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MSL1 plays a central role in assembly of the MSL complex, essential for dosage compensation in *Drosophila*

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In male *Drosophila*, histone H4 acetylated at Lys16 is enriched on the X chromosome, and most X-linked genes are transcribed at a higher rate than in females (thus achieving dosage compensation). Five proteins, collectively called the MSLs, are required for dosage compensation and male viability. Here we show that one of these proteins, MSL1, interacts with three others, MSL2, MSL3 and MOF. The latter is a putative histone acetyl transferase. Overexpression of either the N- or C-terminal domain of MSL1 has dominant-negative effects, i.e. causes male-specific lethality. The lethality due to expression of the N-terminal domain is reduced if *msl2* is co-overexpressed. MSL2 co-purifies over a FLAG affinity column with the tagged region of MSL1, and both MSL3 and MOF co-purify with the FLAG-tagged MSL1 C-terminal domain. Furthermore, the MSL1 C-terminal domain binds specifically to a GST–MOF fusion protein and co-immunoprecipitates with HA-tagged MSL3. The MSL1 C-terminal domain shows similarity to a region of mouse CBP, a transcription co-activator. We conclude that a main role of MSL1 is to serve as the backbone for assembly of the MSL complex.

Keywords: CBP/dosage compensation/FLAG affinity gel/male-specific lethal/MSL complex

Introduction

Drosophila melanogaster dosage compensate (i.e. equalize X-linked gene products) by doubling the amount of gene expression from the one male X chromosome to equal that from the two female X chromosomes (reviewed by Bashaw and Baker, 1996; Lucchesi, 1998). Males homozygous for loss-of-function mutations in the *male-specific lethal1* (*msl1*), *male-specific lethal2* (*msl2*), *male-specific lethal3* (*msl3*), *maleless* (*mle*) or *males absent on the first* (*mof*) genes die due to a failure to dosage compensate. Antibody-binding studies have shown that the MSL1, MSL2, MSL3, MLE and MOF proteins, collectively called the MSLs, co-localize to hundreds of sites along the length of the male X chromosome (Lucchesi, 1998). Acetylation of histone H4 at Lys16 is associated preferentially with the male X chromosome and is dependent on the binding of the MSLs (Turner *et al*., 1992; Bone *et al*., 1994). MOF shows homology to the ESA1 and Tip60 histone acetyl transferases (Hilfiker *et al*., 1997; Smith *et al*., 1998). Histone acetylation would decrease the affinity of histone for DNA (Struhl, 1998) and could destabilize higher order chromatin structure by weakening the interaction between adjacent nucleosomes (Luger *et al*., 1997). Furthermore, several transcription co-activators have been shown to have histone acetyl transferase activity (Brownell and Allis, 1996; Struhl, 1998). This suggests that altering chromatin structure via histone acetylation is a key element of the mechanism by which the MSLs increase X-linked gene expression in males. Of the other MSLs, MLE codes for an RNA–DNA helicase (Lee *et al*., 1997), MSL2 is a RING finger protein (Bashaw and Baker, 1995; Kelley *et al*., 1995; Zhou *et al*., 1995) and MSL3 is a chromodomain protein (Koonin *et al*., 1995). MSL1, however, has no recognizable domains but does have regions that are rich in acidic amino acids (Palmer *et al*., 1993). The MSL complex is not unique in containing both an RNA helicase and histone acetyl transferase. The CREB-binding protein (CBP) transcription co-activator is a histone acetyl transferase (Bannister and Kouzarides, 1996) and binds to RNA helicase A (Nakajima *et al*., 1997). Coupling a helicase with a histone acetyl transferase could be advantageous as histone acetylation could destabilize the chromatin structure and thus facilitate passage of the helicase along the chromosome.

There are several lines of evidence suggesting that the MSLs form a complex. First, the localization of any one of the MSLs to the male X chromosome requires all five msl^+ activities (Palmer *et al*., 1994; Gorman *et al*., 1995; Kelley *et al*., 1995; Gu *et al*., 1998). Furthermore, the stability of the MSL1 protein is strongly dependent on the presence of MSL2 (Chang and Kuroda, 1998). Similarly, MSL3 stability is dependent upon MSL1 and MSL2 (Gorman *et al*., 1995). MSL2 and MSL3 interact with MSL1 in a yeast two-hybrid system (Copps *et al*., 1998). MSL1, MSL2 and MSL3 co-immunoprecipitate and chromatographically co-fractionate from *Drosophila* SL2 cell extract (Copps *et al*., 1998). MLE, however, appears not to be tightly associated with the other MSLs.

In addition to the MSLs, the X-linked non-coding *roX1* (RNA on the X chromosome) and *roX2* genes may have a role in dosage compensation. The male-specific accumulation of roX1 and roX2 RNAs is dependent upon the MSLs, and roX1 RNA 'paints' the male X chromosome in a manner strikingly similar to the 'painting' of the mammalian inactive X chromosome by *Xist* RNA (Amrein and Axel, 1997; Meller *et al*., 1997). Since an RNA component is essential for association of MLE with the X chromosome (Richter *et al*., 1996), this suggests that a

possible role for the roX RNAs is to stabilize the interaction of MLE with the other MSLs (Meller *et al*., 1997; Chang and Kuroda, 1998).

The binding of MSL1 and MSL2 to several 'high affinity' sites on the X chromosome does not require MLE, MOF or MSL3 (Lyman *et al*., 1997). Furthermore, the binding of these three proteins to the male X chromosome is absolutely dependent on MSL1 and MSL2 (Gu *et al*., 1998). Together, these results suggest that the MSLs may bind sequentially to the X. In the first step, the MSL1 and MSL2 complex binds to high affinity sites on the X. Secondly, MSL1 and MSL2 then recruit MSL3, MLE and MOF to the X. The MSL complex does not associate with the female X chromosomes because MSL2 protein is absent from females (Bashaw and Baker, 1995; Kelley *et al*., 1995; Zhou *et al*., 1995).

With the long-term goal of improving our understanding of the mechanism of dosage compensation, we have sought to identify the domains of MSL1 that are important for function *in vivo*. We find that overexpression of two regions of MSL1 causes male-specific lethality, i.e. they behave as dominant-negative mutant forms of MSL1. We present genetic and biochemical evidence that one region interacts with MSL2, the other with both MSL3 and MOF. Our results suggest a central role for MSL1 in assembly of the MSL complex.

Results

Overexpression of regions of MSL1 causes male-specific lethality

We hypothesized that overexpression of a truncated version of MSL1 could cause male-specific lethality if it bound to one or more of the MSLs and reduced the concentration of this MSL available to bind to endogenous MSL1 below a critical threshold required for dosage compensation (and thus male viability). Transgenic flies homozygous for an *msl1* expression construct (Figure 1) were raised at 30°C and heat shocked daily for 1 h at 37°C to maximize MSL1 protein synthesis. Expression of MSL1 and its truncated derivatives was controlled with the *hsp70* heat-shock promoter, which has significantly higher constitutive activity at 30°C compared with 22°C (O'Brien and Lis, 1991). We found that there was 100% male lethality in transformant lines carrying either the *FΔ84*, *FΔ84ΔC**, *FN*, *C* or *FC* constructs (Table I). There was also a smaller but nevertheless significant decrease in male viability in lines expressing either the FMSL1, Δ84 or Δ84ΔC* proteins. Transformant lines expressing either full-length MSL1 or the MID region of MSL1 under these conditions produced approximately equal numbers of males and females (Table I). These results suggest that there are at least two regions of MSL1, one near the N-terminus and the other at the C-terminus, defined by FN and C, respectively, which could be important for assembly of the MSL complex *in vivo*. Interestingly, although the FLAG-tagged FΔ84 and FΔ84ΔC* proteins are essentially identical to the Δ84 and Δ84ΔC* proteins, respectively, overexpression of the former has a much more severe effect on male viability (Table I). Similarly, overexpression of FLAG-tagged MSL1 (FMSL1) but not MSL1 resulted in a small but significant decrease in male viability.

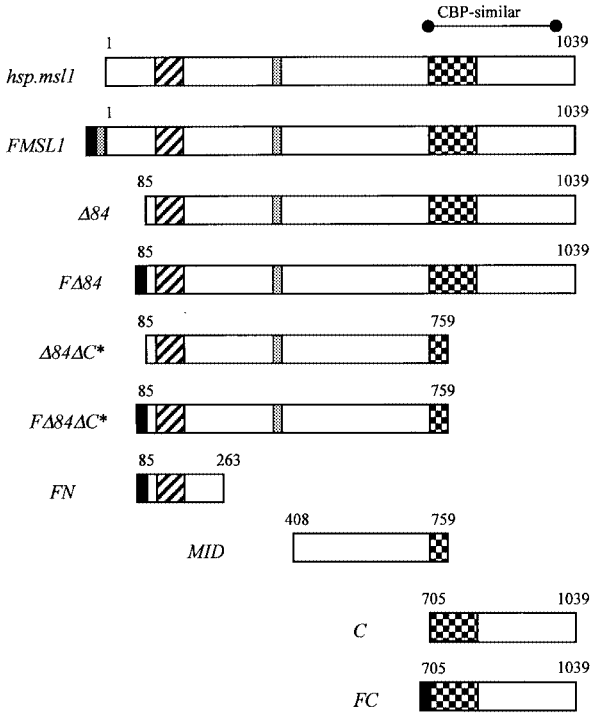

Fig. 1. *msl1* constructs used in this study. The numbers at the beginning and end of each construct indicate the region of MSL1 that is expressed. Full-length MSL1 (1039 amino acids) is shown at the top. The proteins encoded by *FΔ84*, *FΔ84ΔC** and *FC* are identical to those encoded by *Δ84*, *Δ84ΔC** and *C*, respectively, except that the former encode a FLAG tag (DYKDDDDK, shaded black) at their N-termini. The FMSL1 protein is identical to MSL1 except that it has a FLAG peptide at the N-terminus and a linker peptide (RPQKTT, shaded with dots) between the FLAG and the start of MSL1. Features of MSL1 that are highlighted are a predicted amphipathic α-helix (cross-hatched, amino acids 96–172), a highly acidic stretch (shaded grey, amino acids 368–391) and a region where half the amino acids are either S, T or P (chequered, amino acids 708–801). Also indicated is a region (amino acids 712–988) that shows similarity to amino acids 863–1117 of mouse CBP (see Results). The expression of all MSL1 derivatives in transgenic flies was controlled by the *hsp70* promoter.

Table I. Overexpression of FLAG-tagged derivatives of MSL1 causes male-specific lethality

Construct[a]	Males	Females	Male/female ratio[b]
hsp·msl1	319	289	1.1
FMSL1	377	565	0.67
Δ84	296	633	0.46
FΔ84	0	272	0
*Δ84ΔC**	416	1041	0.4
*FΔ84ΔC**	0	374	0
FN	0	189	0
MID	601	559	1.08
C	0	425	0
FC	0	666	0

[a]All lines were homozygous for the construct. Vials were heat shocked daily for 1 h at 37°C. For simplicity, data are shown for just one line although at least two and usually three lines were examined for each construct, all of which gave a similar result.
[b]Significant reduction in male viability ($p < 0.01$, χ^2 test) for all lines except *hsp·msl1* and *MID*.

Viability of FΔ84 but not C males is reduced if heterozygous for msl2

If the dominant-negative mutant forms of MSL1 are binding to a particular MSL, then lowering the concentra-

Table II. Viability of *FΔ84* but not *C* males is reduced if heterozygous for *msl2*

Construct[a]	*msl*	*msl*/+			+/+		
		Males	Females	M/F ratio	Males	Females	M/F ratio
FΔ84[b]	*msl1*	100	301	0.33	147	390	0.38
	msl2	42	495	0.08[c]	105	481	0.22
	mle	141	498	0.28	151	521	0.29
	msl3	158	406	0.39	166	393	0.42
C[d]	*mle*	65	189	0.34	40	157	0.26
	msl2	45	140	0.32	43	131	0.33
	msl3	41	180	0.22	19	170	0.12

[a]*FΔ84* Crosses were raised at 22°C and shocked daily at 37°C for 1 h. *C* crosses were raised at 30°C and shocked daily at 37°C for 30 min.
[b]Full genotype of *FΔ84* crosses (female×male): *msl1*, w^{1118} P[*FΔ84* w^+]34×*msl1*$^{\gamma 269}$ *bw/CyO*; *msl2*, w^{1118} P[*FΔ84* w^+]34×*msl2*$^{\gamma 136}$ *cn bw/CyO*; *mle*, w^{1118} P[*FΔ84* w^+]34×*mle*$^{\gamma 286}$ *bw/CyO*; and *msl3*, w^{1118} P[*FΔ84* w^+]34×*mle*^{3} *red/TM3, Sb Ser.*
[c]Viability significantly reduced ($p < 0.01$, χ^2 test) relative to +/+ siblings.
[d]Full genotype of *C* crosses (female×male): *msl2*, *y w*; P[*C* w^+]25×*msl2*$^{\gamma 136}$ *cn bw/CyO*; *mle*, *y w*; P[*C* w^+]25×*mle*$^{\gamma 286}$ *bw/CyO*; and *msl3*, *y w*; P[*C* w^+]25×*mle*^{3} *red/TM3, Sb Ser.*

tion of that MSL could reduce male viability further, since less of the MSL is available to bind to full-length MSL1. The concentration of a particular MSL was reduced by 50% by crossing either an *FΔ84* or *C* line with a null mutation for an *msl* (Table II). This experiment could not be carried out easily with *mof* since it is X-linked (Hilfiker *et al*., 1997). In order to detect if any of the *msl* mutations can enhance the male-lethal effects of either *FΔ84* or *C*, conditions were used that result in only a modest reduction in male viability. Thus, the offspring of the crosses carry only one copy of either the *FΔ84* or *C* transgenes (homozygous flies were used in Table I), and were raised under milder incubation conditions (either 22°C incubation or 30 min heat shock) than used previously (Table I). This resulted in lower expression of FΔ84 or C proteins. The viability of males carrying the *FΔ84* construct was reduced significantly ($p < 0.01$, χ^2 test) if the males were heterozygous for *msl2* (Table II). There was no significant difference in the relative viability of heterozygous *msl1*, *mle* or *msl3* males compared with their respective wild-type siblings (Table II). These results indicate that in *FΔ84* males the concentration of MSL2 available for dosage compensation is limiting. This is perhaps not surprising since MSL2 is the male-specific MSL (Zhou *et al*., 1995). However, there was no significant difference in the relative viability of heterozygous *msl2*, *mle* or *msl3 C* males compared with their respective wild-type siblings (Table II). While this assay was not informative about which MSL could be interacting with the C region, it would seem unlikely that it is MSL2 given the results with the *FΔ84* construct.

Co-expression of MSL2 rescues males from the lethal effects of dominant-negative mutant forms of MSL1 (ΔMSL1)

If FΔ84 and MSL2 do interact, then co-expression of both proteins should improve male viability compared with expression of FΔ84 alone. An *FΔ84* line was crossed with a transformant line carrying an *hsp·msl2* construct (*msl2* expression driven by the *hsp70* promoter). The offspring of the cross were raised at 30°C and heat shocked daily for 1 h at 37°C. The viability of males that carried both constructs was significantly improved compared with males that carried only the *FΔ84* construct (Table III). Rescue of *FΔ84* males was not complete, probably because the FΔ84 protein contains the C-terminal domain of MSL1 that associates with other MSLs (see below).

To identify the region of MSL1 that interacts with MSL2, transformants carrying either the *FΔ84ΔC** or *FN* constructs were also crossed with *hsp·msl2*. Co-expression of *msl2* significantly improves the viability of both *FΔ84ΔC** and *FN* males (Table III). These results suggest that the domain of MSL1 that interacts with MSL2 maps to the region expressed by the *FN* construct. In comparison, the viability of *C* males was not improved by co-expression of MSL2, MLE, MSL3 or MOF (data not shown).

Dominant-negative mutant forms of MSL1 interact with MSL2, MSL3 and MOF

Immunoprecipitation assays show that MSL1 and MSL2 are part of a complex (Kelley *et al*., 1995), and yeast two-hybrid experiments suggest that MSL1 and MSL2 interact directly in *Drosophila* (Copps *et al*., 1998). We used FLAG affinity chromatography to determine if any of the dominant-negative truncated versions of MSL1 associate with MSL2. To maximize the likelihood of detecting an association between any of the MSL1 forms and MSL2 in a crude fly extract, we co-overexpressed MSL2 with each FLAG-tagged form of MSL1. *FMSL1*, *FΔ84*, *FΔ84ΔC**, *FN* and *FC* transformant lines were each crossed with *hsp·msl2* and the offspring given a single heat shock to induce MSL1 and MSL2 protein synthesis prior to homogenization. Crude cell lysate was applied to an anti-FLAG affinity gel. After repeated washing of the column, bound protein was eluted with an excess of free FLAG peptide. All of the FLAG-tagged versions of MSL1 bound specifically to the anti-FLAG affinity gel (Figure 2A). There was no significant retention of MSL2 alone on the affinity gel (Figure 2B). However, MSL2 did bind to the affinity gel if it was co-expressed with FMSL1, FΔ84, FΔ84ΔC* or FN, but not FC (Figure 2B). We conclude that the dominant-negative effect of the FN region of MSL1 (amino acids 85–263) is due to association with MSL2. This is consistent with yeast two-hybrid experiments which have shown that part of the FN region of MSL1 (amino acids 85–186) associates with MSL2 (Copps *et al*., 1998).

Similar affinity chromatography experiments were performed to determine if MOF, MSL3 or MLE associate

Table III. Co-expression of MSL2 rescues males from the lethal effects of dominant-negative mutant forms of MSL1 (Δ*MSL1*)

Construct[a]	Δ*MSL1*			Δ*MSL1* + *msl2*		
	Males	Females	M/F ratio	Males	Females	M/F ratio
FΔ84[b]	86	354	0.24	268	474	0.57[c]
*FΔ84ΔC**	172	336	0.51	403	415	0.97[c]
FN	23	110	0.21	129	157	0.82[c]

[a]Crosses were raised at 30°C and heat shocked daily at 37°C for 1 h. Offspring contained either one copy of the Δ*MSL1* construct or one copy of the Δ*MSL1* construct plus one copy of *hsp·msl2*. Thus, expression of the *msl1* constructs and *msl2* was controlled by the *hsp70* promoter. Under these incubation conditions, expression of *msl2* has no effect on female viability (data not shown).
[b]Full genotype of crosses (female×male): *FΔ84*, w^{1118}; P[*FΔ84* w^+]33×P[*hsp·msl2*]13/*TM3, Sb e*; *FΔ84ΔC**, w^{1118}; P[*FΔ84ΔC** w^+]13×P[*hsp·msl2*]13/*TM3, Sb e*; *FN* alone, *y w* × *y w*; P[*FN* w^+]13; and *FN* + *msl2*, *y w* P[*hsp·msl2*]14×*y w*; P[*FN* w^+]13.
[c]Significant improvement in male viability ($p < 0.01$, χ^2 test) compared with males that carry only the *msl1* construct.

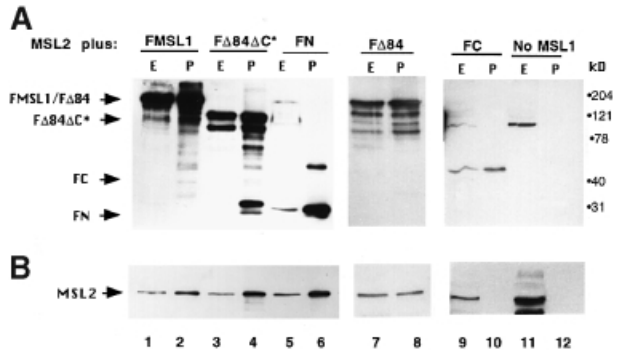

Fig. 2. FLAG affinity chromatography of FLAG-tagged MSL1–MSL2 complexes. Protein extracts were prepared from transformant flies that co-overexpressed MSL2 and either FLAG-MSL1 (lanes 1 and 2), FΔ84ΔC* (lanes 3 and 4), FN (lanes 5 and 6), FΔ84 (lanes 7 and 8) or FC (lanes 9 and 10). Protein extract was also prepared from a line that overexpressed MSL2 alone (lanes 11 and 12). Aliquots of either unpurified extract (E; lanes 1, 3, 5, 7, 9 and 11) or FLAG affinity-purified protein (P; lanes 2, 4, 6, 8, 10 and 12) were separated by SDS–PAGE and transferred to nitrocellulose membranes. The amount loaded on the extract lanes corresponds to ~8% of the material applied to the FLAG affinity gel. Western blots were incubated with either anti-MSL1 (**A**) or anti-MSL2 (**B**) primary antibodies.

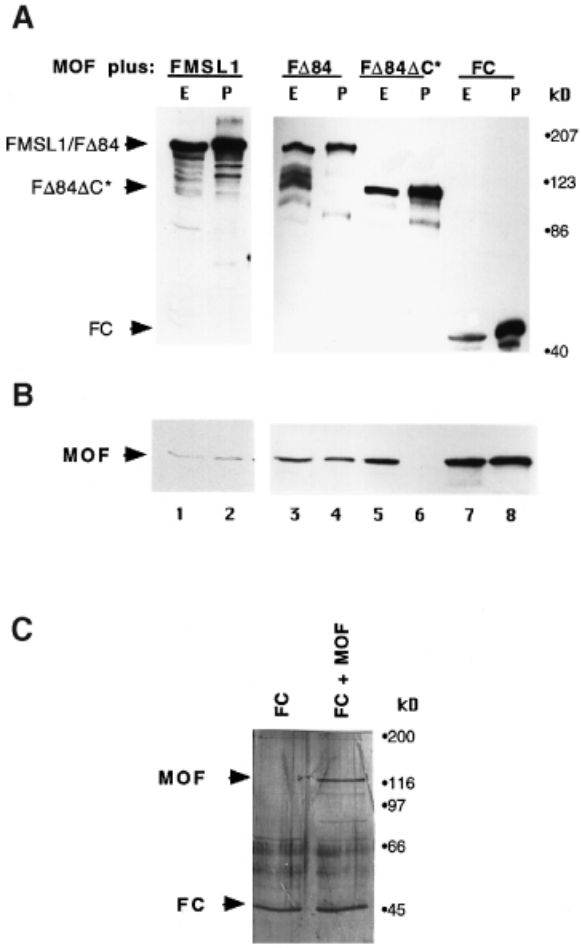

Fig. 3. FLAG affinity chromatography of FLAG-tagged MSL1–MOF complexes. Protein extracts were prepared from transformant flies that co-overexpressed MOF and either FLAG-MSL1 (lanes 1 and 2), FΔ84 (lanes 3 and 4), FΔ84ΔC* (lanes 5 and 6) or FC (lanes 7 and 8). Western blots containing either unpurified extract (E; lanes 1, 3, 5 and 7) or FLAG affinity-purified protein (P; lanes 2, 4, 6 and 8) were incubated with either anti-MSL1 (**A**) or anti-MOF (**B**) primary antibodies as described in the legend to Figure 2. (**C**) Silver stain of affinity-purified proteins

with any of the dominant-negative mutant forms of MSL1. *FMSL1*, *FΔ84*, *FΔ84ΔC** and *FC* transformant lines were each crossed with either *hsp·mof* or *hsp·msl3* and the offspring heat shocked prior to homogenization to induce MSL1 and either MOF or MSL3 synthesis. The crude fly extracts were applied to FLAG affinity columns and bound material eluted with excess FLAG peptide. All of the FLAG-tagged forms of MSL1 were retained on the affinity gels (Figures 3A and 4A). Both MOF (Figure 3B) and MSL3 (Figure 4B) co-purified with FMSL1, FΔ84 and FC, but not FΔ84ΔC*. This suggests that both MOF and MSL3 interact with the C-terminal domain of MSL1 and that these associations are responsible for the dominant-negative effects of this region of MSL1. In contrast, MLE did not co-purify with either the FΔ84 or FC proteins (Figure 5). This is consistent with immunoprecipitation and chromatographic purification experiments that have shown that MLE is only weakly associated with the MSL complex (Copps *et al*., 1998). Immunofluorescence studies have shown that mle^+ function is required for the localization of MOF and MSL3 to the X chromosome (Gu *et al*., 1998). One interpretation of this result is that MLE interacts directly with either MSL3 or MOF. To test this prediction, we co-overexpressed FC, MSL3, MOF and MLE and partially purified the protein complex over FLAG affinity columns. FC (Figure 5A, lanes 7 and 8), MSL3 and MOF (data not shown), but not MLE (Figure 5B, lanes 7 and 8), were retained on the affinity gel. We conclude that either MLE does not interact with

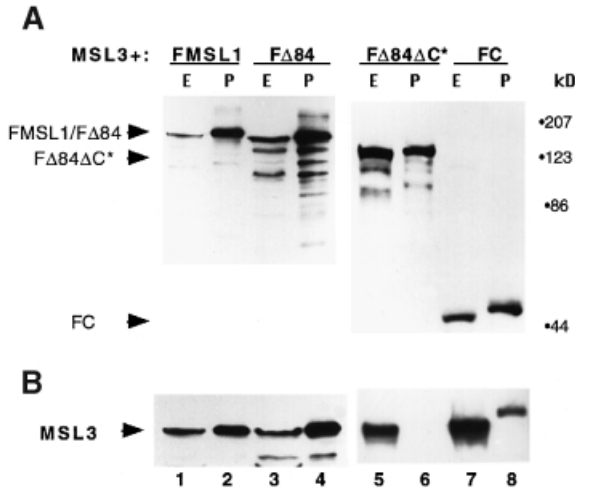

Fig. 4. FLAG affinity chromatography of FLAG-tagged MSL1–MSL3 complexes. Protein extracts were prepared from transformant flies that co-overexpressed MSL3 and either FLAG MSL1 (lanes 1 and 2), FΔ84 (lanes 3 and 4), FΔ84ΔC* (lanes 5 and 6) or FC (lanes 7 and 8). Western blots containing either unpurified extract (E; lanes 1, 3, 5 and 7) or FLAG affinity-purified protein (P; lanes 2, 4, 6 and 8) were incubated with either anti-MSL1 (**A**) or anti-MSL3 (**B**) primary antibodies as described in the legend to Figure 2.

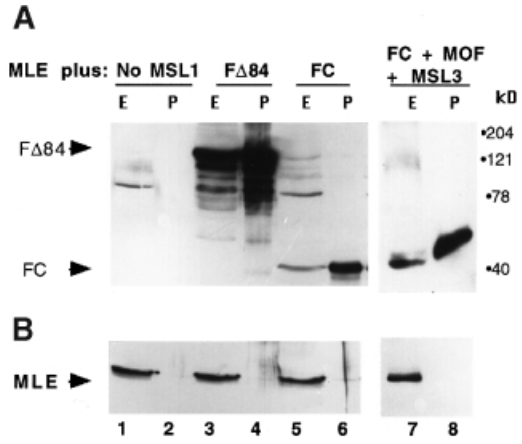

Fig. 5. FLAG affinity chromatography of FLAG-tagged MSL1 co-expressed with MLE. Protein extracts were prepared from transformant flies that co-overexpressed MLE and either FΔ84 (lanes 3 and 4), FC (lanes 5 and 6) or FC, MSL3 and MOF (lanes 7 and 8). Protein extract was also prepared from a line that overexpressed MLE alone (lanes 1 and 2). Western blots containing either unpurified extract (E; lanes 1, 3, 5 and 7) or FLAG affinity-purified protein (P; lanes 2, 4, 6 and 8) were incubated with either anti-MSL1 (**A**) or anti-MLE (**B**) primary antibodies as described in the legend to Figure 2.

either MSL3 or MOF, or any interaction could not be detected by using this approach.

To confirm the interaction between the C-terminal domain of MSL1 and MOF by an alternative method, we prepared GST–MOF fusion protein in *Escherichia coli*. The fusion protein was mixed with crude fly extracts from transformant lines expressing either MSL1 or one of the truncated versions of MSL1. As a control, we also incubated GST with each of the fly extracts. The protein mixes were then applied to glutathione–Sepharose and bound protein eluted with excess glutathione. MSL1 co-purified with GST–MOF but not with GST (Figure 6). This result confirms that MSL1 and MOF interact. The Δ84 (which includes the C-terminal domain) and FC

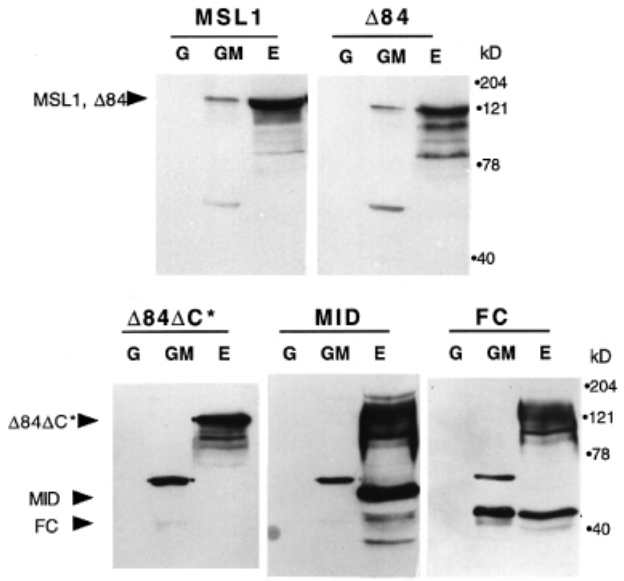

Fig. 6. Glutathione affinity chromatography of GST–MOF–MSL1 complexes. Fly extracts (E) from transformant lines that had been heat shocked to induce protein synthesis were incubated with glutathione–Sepharose beads containing either bound GST (G) or GST–MOF (GM). Bound proteins were eluted with glutathione. An aliquot of each sample was fractionated by SDS–PAGE, transferred to a nitrocellulose membrane, then incubated with anti-MSL1 antibody. The amount loaded on the input lanes corresponds to ~7% of the material applied to the glutathione affinity beads. The band at 68 kDa seen in all panels is an *E.coli* protein that co-purifies with GST–MOF and reacts with the anti-MSL1 antibody. Elution of GST and GST–MOF was confirmed by probing identical membranes with anti-GST antibody (Sigma) (data not shown).

proteins also co-purify specifically with GST–MOF over the glutathione affinity column (Figure 6). However, the Δ84ΔC* and MID proteins do not co-purify with GST–MOF (Figure 6). These results confirm that it is the C-terminal domain of MSL1 that interacts with MOF.

To confirm the interaction between MSL3 and the C-terminal domain of MSL1, we generated transformant lines that express a haemagglutinin (HA) epitope-tagged form of MSL3 following a heat shock. The *hsp·HAmsl3* lines were crossed with *hsp·msl1*, *FΔ84ΔC** or *FC* lines and the offspring heat shocked prior to homogenization to induce MSL1 and HA·MSL3 synthesis. The crude fly extracts were incubated with high affinity HA antibody and immune complexes precipitated using protein G–agarose. HA-tagged MSL3 was precipitated efficiently by the HA antibody (Figure 7A). MSL1 and FC but not FΔ84ΔC* co-precipitated with HA·MSL3 (Figure 7B). These results confirm that the C-terminal domain of MSL1 associates with MSL3.

Since all of the above affinity purifications of MSL1–MSL complexes were from crude fly extracts, it is possible that the interactions between MSL1 and the other MSLs are not direct. To test this possibility, we performed *in vitro* translations with MSL1 C-terminal domain, HA·MSL3 and HA·MOF RNA templates. The [^{35}S]methionine-labelled proteins were mixed and immunoprecipitated with anti-HA antibody (Figure 8). We found that C co-immunoprecipitated with HA·MSL3 but not HA·MOF (Figure 8). These experiments show that the C-terminal domain interacts directly with MSL3 but that the interaction with MOF requires either another factor present in fly extracts

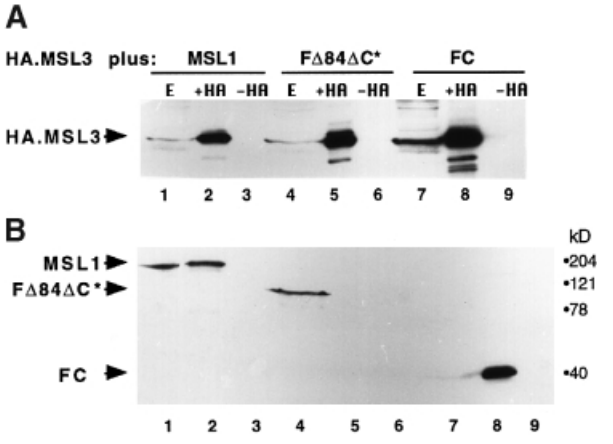

Fig. 7. MSL1 co-immunoprecipitates with HA-tagged MSL3. Protein extracts were prepared from transformant flies that co-overexpressed HA·MSL3 and either MSL1 (lanes 1, 2 and 3), FΔ84ΔC* (lanes 4, 5 and 6) or FC (lanes 7, 8 and 9). Aliquots of either unpurified extracts (E; lanes 1, 4 and 7), protein purified by incubation with protein G beads plus anti-HA antibody (+HA; lanes 2, 5 and 8) or protein precipitated by protein G beads alone (–HA; lanes 3, 6 and 9) were separated by SDS–PAGE and transferred to nitrocellulose membranes. The amount loaded on the extract lanes corresponds to ~4% of the material incubated with protein G beads. Western blots were incubated with either anti-HA (**A**) or anti-MSL1 (**B**) primary antibodies.

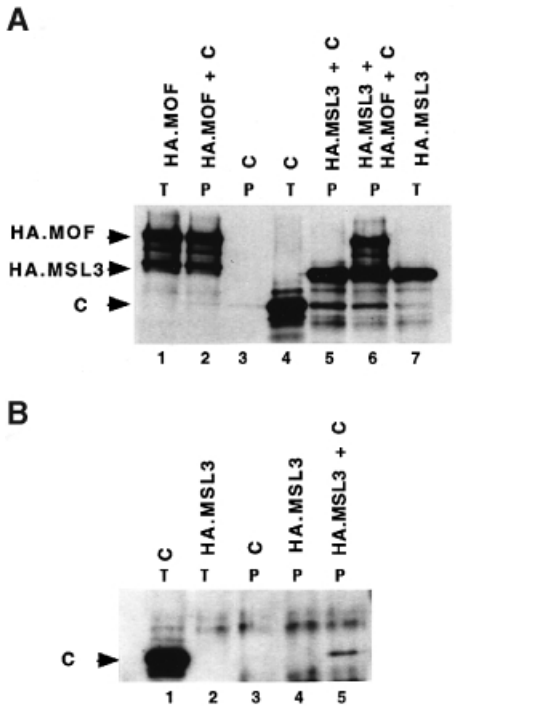

Fig. 8. *In vitro* translated MSL1 C-terminal domain co-immunoprecipitates with *in vitro* translated HA·MSL3 but not with HA·MOF. *In vitro* translation reactions were carried out with RNA templates for HA·MOF (lane 1), MSL1 C-terminal domain (A, lane 4; B, lane 1) or HA·MSL3 (A, lane 7; B, lane 2) in the presence of [^{35}S]methionine. C was mixed with either HA·MOF (A, lane 2) HA·MSL3 (A, lane 5; B, lane 5) or both (A, lane 6), and immunoprecipitated with anti-HA antibody and protein G beads. Proteins were separated by SDS–PAGE and either exposed to X-ray film (**A**) or transferred to a nitrocellulose membrane and incubated with anti-MSL1 antibody (**B**). The amount loaded on the translated protein lanes (T) corresponds to ~5–10% of the protein that was mixed with anti-HA antibody and protein G beads (P). C co-immunoprecipitated with HA·MSL3 (A and B, lane 5) but not with HA·MOF (A, lane 2).

or post-translational modification of MSL1 or MOF. We favour the latter since a silver stain of FLAG affinity-purified FC–MOF complex separated by SDS–PAGE shows only two main bands corresponding to the sizes expected for FC and MOF (Figure 3).

The C-terminal domain of MSL1 contains a region that is high in Ser, Thr and Pro. Not surprisingly, a FASTA homology search of the protein database with the C-terminal domain amino acid sequence identified a number of proteins with similarity restricted to the Ser-, Thr- and Pro-rich region. However, both mouse and human CBP showed similarity across essentially the entire C-terminal domain (24% identity, 58% similarity to amino acids 863–1117 of mouse CBP; 23% identity and 58% similarity to amino acids 861–1116 of human CBP). A comparison of mouse CBP with *Drosophila* CBP (Akimaru *et al*., 1997) showed that, with the exception of the start of the bromodomain, the MSL1-similar region of mouse CBP is not well conserved in *Drosophila* CBP (28% identity to amino acids 1356–1690 of *Drosophila* CBP, 18 gaps in the alignment). Thus, it is perhaps not surprising that the C-terminal domain of MSL1 shows little similarity to *Drosophila* CBP (12% identity, 40% similarity over 276 amino acids).

FMSL1, but not FΔ84, binds to the male X chromosome

The FΔ84 version of MSL1, which is missing only the first 84 amino acids, binds to MSL2, MOF and MSL3 (Figures 2–4) yet causes male-specific lethality when overexpressed. This suggests that the first 84 amino acids are important for function *in vivo*. To determine if this region is required for binding to the X chromosome, polytene chromosomes from the FMSL1 and FΔ84 lines were stained with anti-FLAG and anti-MSL2 antibodies. Heat treatment of transformant lines carrying an *hsp·msl1* construct results in a transient association of MSL1 with all of the chromosomes (Chang and Kuroda, 1998). Similarly, both FMSL1 and FΔ84 bind transiently to all of the chromosomes following a heat shock (M.Scott, unpublished data). However, 24 h after a heat shock, FMSL1 is associated preferentially with the male X chromosome (Figure 9). We could not detect any preferential binding of FΔ84 to the X chromosome (Figure 9). Thus, one possible explanation for why overexpression of FΔ84 causes male-specific lethality is that the first 84 amino acids of MSL1 are required for binding to the male X chromosome.

We have suggested that the dominant-negative effects of overexpression of the FΔ84 and FC regions of MSL1 are due to association with another MSL, thus preventing formation of sufficient MSL complex for dosage compensation. According to this model, expression of either region should result in loss of the MSL complex from the X chromosome. Following a single heat shock to induce expression of FΔ84, MSL2 was still bound to the male X chromosome (Figure 9). However, this is a sensitive assay that can detect low levels of *msl2* expression (Kelley *et al*., 1997). We then tested if expression of FC interfered with the association of MOF with the male X chromosome. Anti-MOF antibody bound to many sites on the male X chromosome in non-heat-shocked controls (Figure 10). However, by 6–9 h after a single heat shock

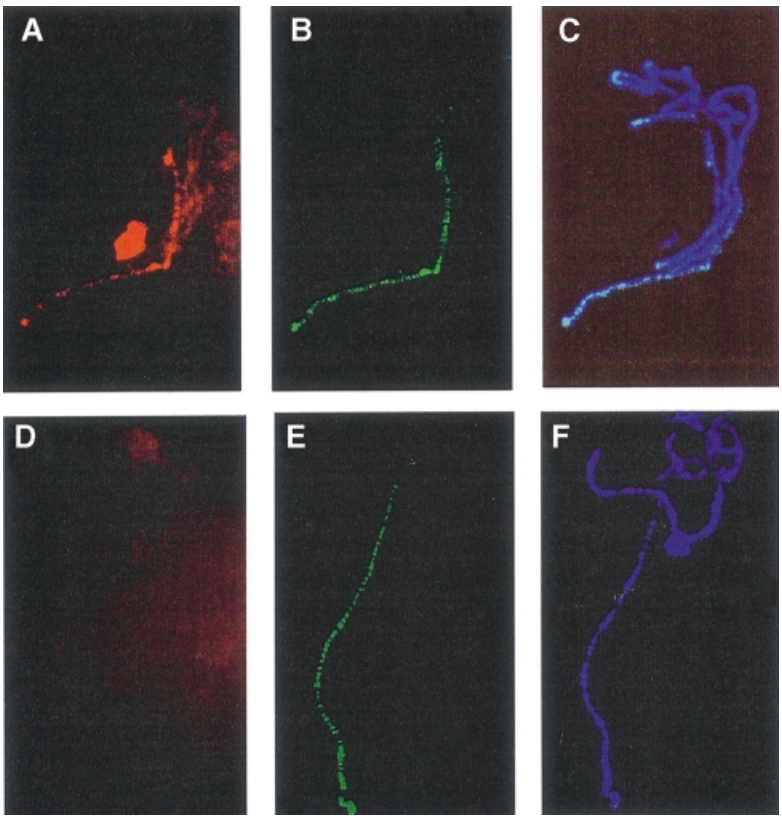

Fig. 9. FMSL1 but not FΔ84 binds to the male X chromosome. Male salivary gland nuclei from *FMSL1* (**A**–**C**) and *FΔ84* (**D**–**F**) transformant lines were stained with anti-FLAG antibody (A and D, red) and anti-MSL2 antibody (B and E, green). The same nuclei were counterstained with DAPI to visualize all of the chromosome arms (C and F, blue). FMSL1 but not FΔ84 bound preferentially to the X chromosome. MSL2 bound to many sites along the X chromosome in nuclei from both lines. The nuclei were isolated from larvae that were given a 1 h heat shock at 37°C then left to recover at 25°C for 24 h. Similar results were obtained from larvae that were given a shorter heat shock (20 min) and were allowed to recover for 8 h (M.J.Scott, unpublished data).

to induce FC expression, binding of MOF to the male X chromosome was not detectable (Figure 10). These results are consistent with our hypothesis that overexpression of FC kills males due to significant loss of MSL complex from the male X chromosome.

Discussion

Assembly of the MSL complex

In general, the amino acid sequences of the MSLs suggested regions or domains within the proteins that could be important for function *in vivo* (Hilfiker *et al*., 1997). Indeed, this has been confirmed by mapping loss-of-function mutations to the domain, such as the helicase domain of MLE (Lee *et al*., 1997), the putative acetylase domain of MOF (Hilfiker *et al*., 1997) and the RING finger region of MSL2 (Zhou *et al*., 1995; Copps *et al.*, 1998). The amino acid sequence of MSL1 was the least informative, containing no recognizable domains, although regions rich in acidic amino acids (Palmer *et al.*, 1993) and possible PEST sequences were identified (Palmer *et al.*, 1993; Chang and Kuroda, 1998). To identify regions within MSL1 that are important for function *in vivo*, we have determined which regions have dominant-negative effects when overexpressed. We find that two regions of MSL1, one near the N-terminus and the other at the C-terminus, are likely to be important for assembly of the MSL complex *in vivo* as overexpression of either region caused male-specific lethality. Genetic evidence, decreased male viability of *msl2* heterozygotes and increased male viability by co-overexpression of MSL2 suggested that the region of MSL1 at the N-terminus was interacting with MSL2. This was confirmed by co-purification of MSL2 with FLAG-tagged versions of MSL1 over FLAG affinity columns. Similarly, the C-terminal region of MSL1 was shown by FLAG affinity chromatography, glutathione affinity chromatography and immunoprecipitation experiments to interact with both MOF and MSL3. Furthermore, expression of the C-terminal domain resulted in significant loss of MOF from the male X chromosome.

The FN region of MSL1 that binds to MSL2 was chosen originally for expression in flies because we predicted, using the method of Woolfson and Alber (1995), that almost half of FN (amino acids 96–172) would form a two-stranded, α-helical, coiled-coil structure (Figure 11). Coiled-coil structures are comprised of a heptad repeat $(abcdefg)_n$ where hydrophobic residues occupy positions a and d on the same side of the α-helix (Cohen and Parry, 1990). The coiled-coil motif of GCN4 mediates dimerization (O'Shea *et al*., 1991). If a similar structure mediates the formation of the MSL1–MSL2 heterodimer, then part of the region of MSL2 that interacts with MSL1 should form a coiled-coil structure. The region of MSL2 that interacts with MSL1 contains a RING finger domain (Copps *et al*., 1998). We predict that the region immediately preceding the RING finger could form a coiled-coil

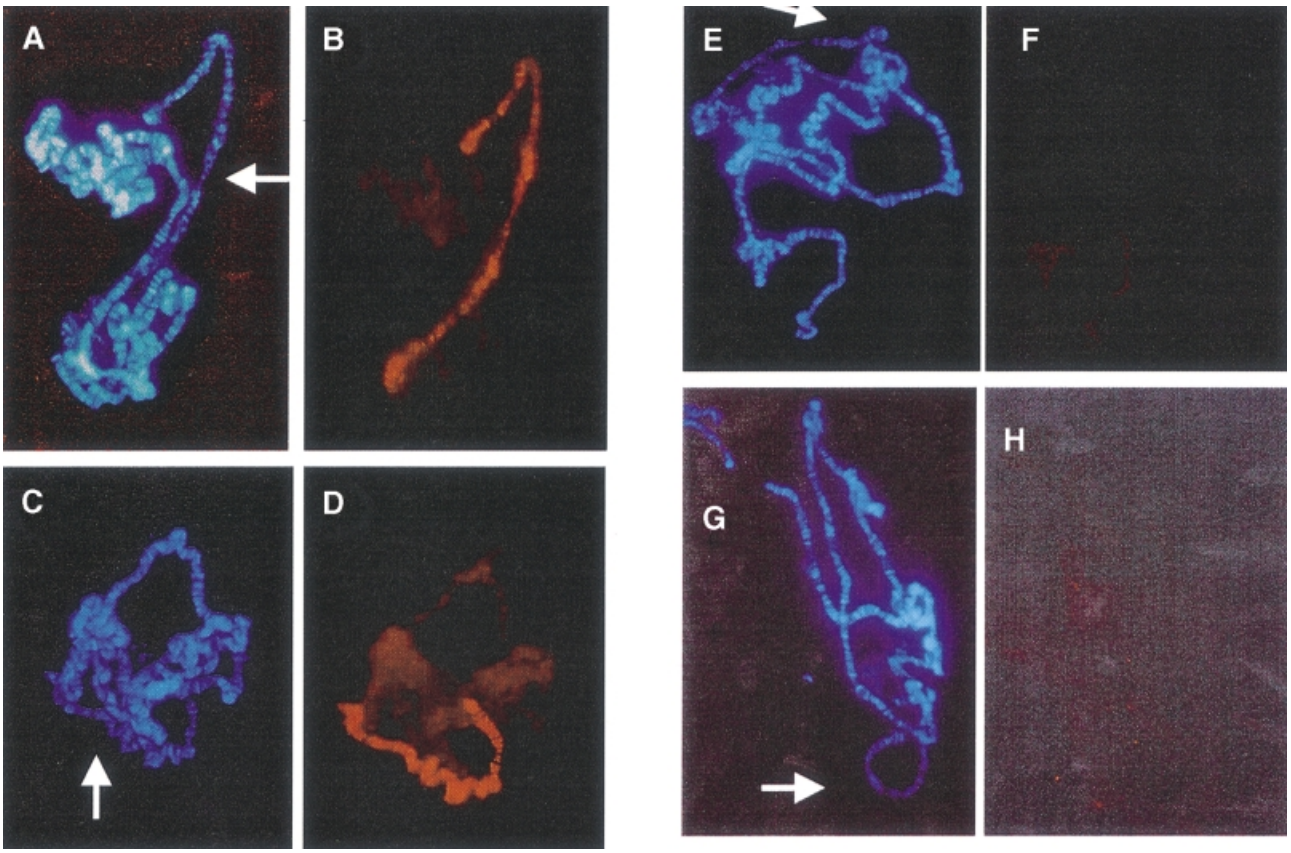

Fig. 10. Expression of the C-terminal domain of MSL1 disrupts binding of MOF to the male X chromosome. Male salivary gland nuclei from a non-transformant line (*y w*) (**A** and **B**) and an *FC* transformant line (**C**–**H**) were stained with DAPI (A, C, E and F) and anti-MOF antibody (B, D, F and G, red). Nuclei were isolated from larvae that were either not heat shocked (A–D) or given a 1 h shock at 37°C then left to recover at 25°C for either 6 h (E and F) or 9 h (G and H). The X chromosome is indicated by an arrow. MOF bound to hundreds of sites on the male X chromosome in non-heat-shocked controls but binding was not detectable by 6–9 h after induction of FC expression.

structure (Figure 11). It is particularly significant that several of the mutations that disrupt the interaction with MSL1 in yeast (Copps *et al*., 1998) introduce amino acid changes that either significantly disrupt the α-helix (leucine to proline) or introduce a charged amino acid into the predicted hydrophobic face of the α-helix (Figure 11). The RING domain is found in a number of proteins including the V(D)J recombination-activating protein RAG1 (Bellon *et al*., 1997). The crystal structure of the RAG1 dimerization domain, which includes the RING finger, reveals that dimerization is stabilized by interaction between α-helices that form a hydrophobic core (Bellon *et al*., 1997). The RING finger is thought to 'form the structural scaffold upon which the dimer interface is formed' (Bellon *et al*., 1997). It is tempting to speculate, by analogy with RAG1, that the association of MSL1 and MSL2 involves the interaction of amphipathic α-helices that depend on the RING finger domain. This could best be addressed by determining the crystal structure of the MSL1–MSL2 complex. Such studies are currently underway in our laboratory.

We have shown that the *in vitro* translated MSL1 C-terminal domain co-immunoprecipitates with *in vitro* translated HA·MSL3 but not HA·MOF. Thus, C interacts directly with MSL3 but the interaction with MOF requires either another factor present in fly extracts or post-translational modification of MSL1 or MOF. While we cannot rule out the possibility of a nucleic acid component of the FC–MOF complex, we favour the latter since a silver stain of FLAG affinity-purified FC–MOF complex separated by SDS–PAGE shows only two main bands corresponding to the sizes expected for FC and MOF (Figure 3). The C-terminal domain of MSL1 is rich in serine and threonine residues, and contains several potential phosphorylation sites (Palmer *et al*., 1993) and a predicted PEST sequence (Chang and Kuroda, 1998). PEST sequences have been suggested to contribute to the instability of the MSL1 protein (Chang and Kuroda, 1998). However, the role of these sequences in MSL1 has not been determined. Indeed, an alternative function for the PEST sequences is suggested by the observations that the PEST domains of PU.1 and IκBβ are required for their respective interactions with Pip (Perkel and Atchison, 1998) and c-Rel (Chu *et al*., 1996). In both cases, phosphorylation of a serine residue within the PEST sequence was required for the respective protein–protein interactions. The recent finding that a serine/threonine kinase is associated preferentially with the male X chromosome (Jin *et al*., 1999) raises the possibility that MSL1 or another MSL is phosphorylated by this enzyme.

In the sequential model for assembly of the MSL complex, the first step involves the binding of the MSL1–MSL2 complex to several 'high affinity' sites on the male X chromosome (Lyman *et al*., 1997; Gu *et al*., 1998). Since the localization of both MOF and MSL3 to the X chromosome requires mle^{+} function, this suggests that the association of MOF and MSL3 with the MSL1–MSL2 complex is MLE dependent (Gu *et al*., 1998). MLE could

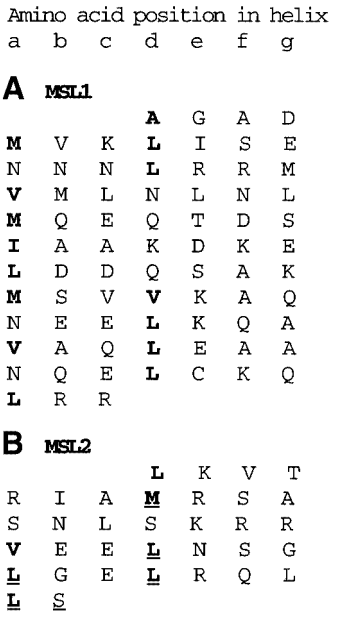

Fig. 11. Predicted coiled-coil, α-helical regions of MSL1 and MSL2. Amino acids 96–172 of MSL1 (**A**) and 7–40 of MSL2 (**B**) are shown following the $(abcdefg)_n$ nomenclature for coiled-coil structures where a and d are usually hydrophobic amino acids. The first zinc-binding site of the MSL2 RING finger begins at Cys41, i.e. immediately following the region shown. Hydrophobic amino acids at positions a and d are shown in bold. Those amino acids in MSL2 that were shown to be important for the interaction with MSL1 in yeast (Copps *et al*., 1998) are underlined. The MSL/MSL2 disruption mutations would either introduce a proline into the predicted α-helix (L28P, L32P or L35P) or introduce a charged amino acid into the predicted hydrophobic face of the helix (M14K or L39R), all of which would be predicted to destabilize a heterodimer. Analysis of favourable interactions between charged amino acids in positions e and g (E106MSL1–K8MSL2, D132MSL1–R24MSL2 and E134MSL1–R36MSL2) suggests that the predicted MSL2 α-helix would dimerize preferentially with amino acids 103–143 of MSL1.

either bind directly to MOF and/or MSL3 (Gu *et al*., 1998), or somehow stabilize the MSL complex together with roX RNA (Chang and Kuroda, 1998). In support of the latter model, we have shown that MOF and MSL3 bind directly to the C-terminal domain of MSL1. Furthermore, MLE did not co-purify with an FC–MOF–MSL3 complex over an affinity column. However, our affinity chromatography experiments were designed to maximize the likelihood of detecting protein–protein association and are not quantitative. It is possible that MOF and MSL3 may have a higher affinity for the C-terminal domain of MSL1 than full-length MSL1. This could explain why the Δ84 protein, which includes the C-terminal domain and binds to GST–MOF (Figure 6), is less effective at killing males than the C-terminal domain alone (Table I). Thus, one possible mechanism is that *in vivo* the C-terminal domain of MSL1 is not freely available to bind to MOF and/or MSL3, and that the binding of MLE to the MSL1–MSL2 complex causes a conformational change in MSL1 such that the C-terminal domain becomes more accessible. This could be addressed by accurately measuring the binding affinities of purified MSL1, Δ84 and C proteins for MOF and MSL3.

Previous searches of the protein sequence database with the complete MSL1 sequence failed to identify any significant similarities (Palmer *et al*., 1993). However, when we carried out a search with just the C-terminal domain sequence, we found some similarity to a 254 amino acid region of mouse CBP. Although the similarity is not high, given that the similarity extends across almost the entire C-terminal domain of MSL1, and that both CBP and the MSL1 C-terminal domain bind to histone acetyl transferases (or putative histone acetyl transferasess), we think it may be significant. If this similarity reflects a conserved function, then it would be predicted that the MSL1-similar region of CBP, which has no known function, would associate with either an MOF-like histone acetyl transferase or an MSL3-like protein in mammalian cells.

It is not known how the MSL complex binds to the male X chromosome. None of the MSLs contain a recognizable DNA-binding motif (Lucchesi, 1998). We have shown that the FΔ84 version of MSL1 binds to MSL2, MSL3 and MOF but does not bind preferentially to the male X chromosome. This suggests that the male lethality which results from overexpression of FΔ84 is due to this protein being able to bind to three MSLs, but being unable to bind to the X chromosome because the first 84 amino acids of MSL1 are required for recognition of the X chromosome. Alternatively, the lack of binding of FΔ84 to the male X chromosome could be because the beginning of MSL1 is required for assembly of the MSL complex *in vivo*. However, if so, then it would be expected that FΔ84 would have bound to the 'high affinity' sites since FΔ84 does bind to MSL2. Assuming that MSL1 and MSL2 are the only components of the high affinity complex (Lyman *et al*., 1997), it would then appear more likely that the first 84 amino acids of MSL1 are required for X chromosome binding rather than complex formation. However, there are several lines of evidence which suggest that the roX RNAs are part of the MSL complex (Meller *et al*., 1997; Franke and Baker, 1999), which raises the possibility that one or both of the roX RNAs could be part of the high affinity complex. Thus it will be of interest to determine if the MSL complex containing the FΔ84 protein binds to roX RNA with a lower affinity than the complex containing full-length MSL1. The inclusion of a FLAG tag at the N-terminus of FΔ84 and, to a much lesser extent, FMSL1, resulted in proteins that were more effective at causing male lethality when overexpressed compared with their respective non-tagged versions of MSL1. One explanation for this result is that the FLAG peptide somehow interferes with the normal function of the N-terminal region of MSL1. Clearly, further experiments are required to clarify the function of the N-terminal domain of MSL1.

Perhaps the most remarkable feature of dosage compensation is that the vast majority of genes on the X chromosome respond equally to the MSL complex, seemingly independently of local chromatin environments. The approach we have taken of generating dominant-negative mutant forms of an MSL coupled with affinity chromatography will complement antibody-binding studies to further our understanding of the mechanism of dosage compensation in *Drosophila*.

Materials and methods

Recombinant DNA

All recombinant DNA manipulations were carried out by using standard procedures (Ausubel *et al*., 1997) unless otherwise specified. To construct

plasmids for expression of regions of MSL1 in *Drosophila*, the general strategy was to insert the appropriate DNA fragment of the *msl1* gene together with a synthetic linker into the P transformation vector pCaSpeR-hs (Thummel and Pirrotta, 1991). In designing the linkers, the sequence upstream of the translation start codon was designed to match the *Drosophila* consensus (Cavener, 1987) and the preferred codons (Ashburner, 1987) were chosen for each of the amino acids in the FLAG peptide. To make the *hsp·msl1*, *Δ84*, *FΔ84*, *MID*, *C* and *FC* constructs, the 3.3 kb *Nco*I (partial)–*Xba*I, 3.0 kb *Nar*I + *Xba*I (*Δ84* and *FΔ84*), 1.2 kb *Sac*I (partial)–*Sph*I and 1.2 kb *Sac*I–*Xba*I (*C* and *FC*) fragments from the *msl1* gene (Palmer *et al*., 1993) were used, respectively. The linkers were made by annealing the following oligonucleotides: for *hsp·msl1*, 5′-GGCGCAAAAAACCAC-3′ and 5′-CATGGTGGTTTTTTGC-3′; for *Δ84*, 5′-AATTAAACATGGCTCCTCCTTCCTCCGGCGGAACCGTGTGTGCCGG-3′ and 5′-CGCCGGCACACACGGTTCCGCCGGAGGAAGGAGGAGCCATGTTT-3′; for *FΔ84*, 5′-AATTAAACATGGACTACAAGGACGACGATGACAAGGCTCCTCCTTCCTCCGGCGGAACCGTGTGTGCCGG-3′ and 5′-CGCCGGCACACACGGTTCCGCCGGAGGAAGGAGGAGCCTTGTCATCGTCGTCCTTGTAGTCCATGTTT-3′; for *MID* and *C*, 5′-AATTACCATGGAGCT-3′ and 5′-CCATGGTT-3′; and for *FC*, 5′-AATTCACCATGGACTACAAGGACGACGATGACAAGGAGCT-3′ and 5′-CCTTGTCATCGTCGTCCTTGTAGTCCATGGTG-3′. The *FLAG·MSL1* construct was made by insertion of a linker into the unique *Not*I site of the *hsp·msl1* construct. The linker was made by annealing the oligonucleotides 5′-GGCCGCAAACCACCATGGACTACAAGGACGACGATGACAAGA-3′ and 5′-GGCCTCTTGTCATCGTCGTCCTTGTAGTCCATGGTGGTTTGC-3′. The protein encoded by the *FLAG·MSL1* construct includes six additional amino acids, RPQKTT, between the FLAG peptide and the start of MSL1. The *Δ84ΔC** and *FΔ84ΔC** constructs were prepared from *Δ84* and *FΔ84*, respectively, by digestion with *Sph*I + *Stu*I, subsequent treatment with T4 DNA polymerase (to remove the 3′ overhang), and ligation. The truncated MSL1 proteins encoded by these constructs include four additional amino acids, PNSS, at the C terminus. The *FN* construct was prepared by ligation of *Eco*RI + *Stu*I-cut *FΔ84* with the ends made blunt by treatment with mung bean nuclease. The truncated MSL1 protein encoded by this construct includes an additional alanine at the C-terminus. Similarly to construct *hsp·mof* and *hsp·msl3*, appropriate fragments containing the respective coding regions (Gorman *et al*., 1995; Hilfiker *et al*., 1997) were inserted into pCaSpeR-hs. The *HA·msl3* construct was made by insertion of a linker into the unique *Eco*RI site of *hsp·msl3*. The linker was made by annealing the oligonucleotides 5′-AATTCACAATGTACCCCTACGATGTGCCCGATTACGCCGA-3′ and 5′-AATTTCGGCGTAATCGGGCACATCGTAGGGGTACATTGTG-3′. The cloning junctions of all constructs were confirmed by DNA sequencing. DNA and protein sequences were analysed using the Wisconsin Package Version 9.1 [Genetics Computer Group (GCG), Madison, WI].

Drosophila stocks

Flies were raised on standard cornmeal–yeast–sugar–agar medium with methyl paraben. Crosses were performed at 25°C unless otherwise indicated. Heat shocks of crosses or transformant lines were performed by submersing all but the top 1 cm of a vial (2.5 cm diameter by 9 cm height with the bottom 3 cm filled with culture medium) in a 37°C waterbath. The $msl1^{\gamma 269}$ and $msl2^{\gamma 136}$ alleles are described by Palmer *et al.* (1993) and Zhou *et al.* (1995). All stocks not specifically mentioned are described in Lindsley and Zimm (1992). Recombinant lines carrying *hsp·mof* and *FC* or *hsp·mle* and *hsp·msl3* were derived by selecting for dark-eyed offspring from the appropriate crosses.

Germline transformation

MSL1 constructs were co-injected into either w^{1118} or *y w* embryos with the Δ2,3 helper plasmid (Laski *et al*., 1986) using standard procedures (Rubin and Spradling, 1982). Single F_1 progeny displaying a non-white eye colour were backcrossed to either w^{1118} or *y w*, then bred to homozygosity. Linkage of P [w^+] was determined by following w^+ segregation in the appropriate crosses.

Affinity chromatography and immunoprecipitation

Adult flies were heat shocked at 37°C for 1 h then incubated at 25°C for an additional 4 h. The flies were then quick-frozen in liquid nitrogen and used immediately or stored at –70°C. Crude whole-cell extracts were prepared by Dounce homogenization of ~100 flies in 2 ml of cell lysis buffer [50 mM Tris–HCl pH 8.5, 300 mM NaCl, 1% NP-40 and 1× complete protease inhibitors (Boehringer Mannheim)]. The extracts were incubated on ice for 30 min then spun at 10 000 *g* for 10 min at 4°C to remove particulate matter and unlysed nuclei. A 1 ml aliquot of the supernatant (containing ~5–10 mg of protein) was taken, diluted with 4 ml of phosphate-buffered saline (PBS; 140 mM NaCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, pH 7.3), then mixed with 300 µl of dilute anti-FLAG M2 beads (bed volume ~60 µl) (BabCo). The mixture was incubated by gentle inversion for 1 h at 4°C then transferred to a 10 ml disposable column (Bio-Rad) and the column allowed to drain. The column was washed five times with 2 ml of TBS (50 mM Tris–HCl, 150 mM NaCl pH 7.4) per wash. Bound protein was eluted with two 500 µl washes of TBS each containing 200 µg of FLAG peptide (BabCo). The eluant was concentrated ~10-fold using a Nanosep microconcentrator (Pall Filtron). All FLAG affinity experiments were performed at least twice from separate starting material and yielded consistent results.

For glutathione affinity chromatography, the diluted crude fly extract was mixed with 200 µl of glutathione–Sepharose 4B (Pharmacia) that had previously been incubated with ~10–20 µg of partially purified GST–MOF protein (prepared from a strain of *Escherichia coli* according to the standard manufacturers' protocols). The mixture was incubated by gentle inversion for 1 h at 4°C then transferred to a 10 ml disposable column (Bio-Rad) and the column allowed to drain. The column was washed five times with 2 ml of PBS (pH 7.3) per wash. Bound protein was eluted with two 500 µl washes of elution buffer (50 mM Tris–HCl, 10 mM glutathione pH 8.0). The eluant was concentrated ~10-fold using a microconcentrator.

For anti-HA immunoprecipitation, 1 ml of the crude fly extract was incubated with 50 µl of protein G–agarose beads (Sigma) at 4°C for 40 min with shaking, then spun at 2000 *g* for 2 min at 4°C. A 1 µg aliquot of high affinity anti-HA antibody (Boehringer Mannheim) was added to the supernatant then incubated at 4°C for 1 h with shaking. A further 50 µl of protein G beads were added, incubated at 4°C for 1 h with shaking then spun at 2000 *g* for 2 min at 4°C. The beads were washed three times each with 1 ml of TBS, then resuspended in 40 µl of 1.5 times sample buffer.

Western blots

A 20 µl aliquot of sample was mixed with sample buffer, boiled for at least 5 min then loaded onto a 7.5% SDS–polyacrylamide gel (Ausubel *et al*., 1997). Proteins were transferred in Tris–glycine buffer (pH 8.3), containing 25 mM Tris, 192 mM glycine and 20% methanol, for 16 h at 20 mA. Membranes were incubated with primary antibody for 1 h at room temperature then detected using the ECL system (Amersham).

In vitro transcription and translation

DNA templates for *in vitro* transcription were prepared by PCR. The sequence of the forward primer was 5′-CACTATCTACTACATCTACTTAATACGACTCACTATAGGGAACCAGCAACCAAGTAAATC-3′ and that of the reverse was 5′-$(T)_{30}$ATCGAAACATTCTTATCAGTCTC-3′. The forward primer includes the promoter for T7 polymerase and the reverse is designed such that a poly(A) tail is added to the RNA. The primers were complementary to the sequences flanking the polylinker of the pCaSpeR-hs vector, so that any of the plasmids described above could be used as templates in a PCR reaction. The amplification reactions were in a 50 µl volume containing 300 pg of DNA template, 200 µM dNTP, 20 pmol of each forward and reverse primer and 1 µl of eLONGase (Life Technologies) in buffer supplied by the manufacturer. The cycling conditions were seven cycles of 94°C for 30 s, 41°C for 30 s and 72°C for 2 min followed by 25 cycles of 94°C for 30 s, 51°C for 30 s and 72°C for 2 min. Since only one predominant product was obtained from each reaction, the primers were removed using the CONCERT PCR purification system (Life Technologies) and the DNA concentrated by ethanol precipitation and resuspended at a concentration of 200 ng/µl. *In vitro* transcription and translation reactions were performed with the TNT T7-coupled transcription–translation system (Promega) according to the manufacturer's instructions. Each 50 µl transcription–translation reaction contained 2 µl of DNA template and 20 µCi of [^{35}S]methionine (Amersham). Immunoprecipitation of *in vitro* translated proteins with the anti-HA antibody was performed essentially as described above except that prior to addition of protein G beads, 2–4 µl of translation reaction(s) were diluted in 450 µl of TBS containing 0.1% Tween-20 and incubated at 4°C for 1 h.

Polytene chromosome squashes

Polytene chromosome spreads were stained with antibodies using the procedure essentially as described by Lyman *et al*. (1997). Mouse anti-FLAG antibody (Babco) was pre-sorbed against *Drosophila* embryos at a concentration of 0.5 mg/ml for 16 h at 4°C and incubated with sample

at a concentration of 25 µg/ml. Rabbit anti-MSL2 and anti-MOF were used at a dilution of 1:50. Anti-FLAG and anti-MSL2 primary antibodies were incubated simultaneously. Secondary incubation was with Texas red-conjugated anti-mouse, fluorescein isothiocyanate (FITC)-conjugated anti-rabbit or rhodamine-conjugated anti-rabbit as described (Lyman *et al*., 1997). DNA was counterstained with 4′,6-diamidino-2-phenylindole (DAPI).

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