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Targeting the chromatin-remodeling MSL complex of *Drosophila* to its sites of action on the X chromosome requires both acetyl transferase and ATPase activities

Weigang Gu, Xierong Wei, Antonio Pannuti and John C.Lucchesi[1]

Department of Biology, Emory University, Atlanta, GA 30322, USA

[1]Corresponding author
e-mail: lucchesi@biology.emory.edu

Dosage compensation in *Drosophila* is mediated by a multiprotein, RNA-containing complex that associates with the X chromosome at multiple sites. We have investigated the role that the enzymatic activities of two complex components, the histone acetyltransferase activity of MOF and the ATPase activity of MLE, may have in the targeting and association of the complex with the X chromosome. Here we report that MLE and MOF activities are necessary for complexes to access the various X chromosome sites. The role that histone H4 acetylation plays in this process is supported by our observations that MOF overexpression leads to the ectopic association of the complex with autosomal sites.

Keywords: ATP-dependent helicase/chromatin remodeling/dosage compensation/*Drosophila*/histone acetylation

Introduction

Dosage compensation is a regulatory process that ensures that males and females have equal amounts of X chromosome gene products. In *Drosophila*, where it is achieved by a doubling of X-linked gene transcription in males relative to females, dosage compensation involves a complex of gene products of at least five known genes (for review see Lucchesi, 1998): *maleless* (*mle*), *male-specific lethal 1*, *2* and *3* (*msl1*, *msl2*, *msl3*) and *males absent on the first* (*mof*). The complex is preferentially associated with numerous sites on the X chromosome in somatic cells of males but not of females and its presence on the X chromosome is correlated with a significant increase of a specific histone isoform: histone H4 acetylated at Lys16 (for review see Turner, 1998). In addition, two untranslated RNAs, RNA on the X1 and 2 (*roX1* and *roX2*), are found only in males or in transgenic females that have been induced to form an MSL complex (for review see Stuckenholz *et al*., 1999). The *roX* RNAs associate with the X chromosome with a distribution that mirrors that of the MSLs, and one of them (*roX2*) is present in a purified MSL complex isolated from cultured cells (Smith *et al*., 2000). Recently, a kinase (JIL-1) has been found to be enriched on the X chromosome of male salivary gland nuclei (Jin *et al*., 1999).

Although the ubiquitous association of each of the five MSLs with the X chromosome, seen in wild-type males, depends on the presence and availability of the other gene products, loss-of-function mutations of *mle* and *msl3* (Palmer *et al*., 1994; Bashaw and Baker, 1995) or *mof* (Gu *et al*., 1998) allow the residual binding of MSL1 and MSL2 to ~30–40 sites. Approximately 30 of these sites have been mapped (Lyman *et al*., 1997) and are thought to be chromatin entry points for the complex. The order of assembly at these sites was shown to be MSL1/MSL2, MLE, MOF and lastly MSL3 (Gu *et al*., 1998). Kelley *et al*. (1999) have reported that the *roX1* and *roX2* genes themselves are located within two entry sites where the formation of complexes containing *roX* RNA occurs.

Here we report that, in order to become stably associated with the numerous other sites along the X chromosome where it is normally found, the MSL complex requires the histone acetyltransferase activity of MOF as well as the ATPase activity of MLE. If either of these activities is impaired, complexes containing the known MSLs are formed but are unable to access X-chromosome chromatin beyond the entry sites. Finally, we report that overexpression of MOF leads to the acetylation of numerous autosomal sites and to the autosomal association of the MSL complex. This study represents the first demonstration that the enzymatic activities of a chromatin remodeling complex are required for its targeting within the genome.

Results

The effect of the mof1 mutation on the distribution of the complex is not due to protein instability

We have shown previously that in the presence of the mof^1 mutation, MSL complexes are targeted only to the entry sites (Gu *et al*., 1998; Figure 1A). Note that, because the salivary glands of dying mutant males are unfavorable for cytological studies, these observations were made on female larvae carrying an $msl2^+$ transgene (H83M2) where the ectopic expression of MSL2 leads to the formation of the MSL complex (Kelley *et al*., 1995). The mof^1 mutation, which is a glycine to glutamic acid replacement at the most highly conserved residue of the acetyl coenzyme A (acetyl CoA)-binding domain (G691E; Hilfiker *et al*., 1997), results in the loss of the histone acetyltransferase activity for both recombinant MOF (Akhtar *et al*., 2000) and the MSL complex (Smith *et al*., 2000). Its effect on the distribution of the complex could be due to loss of histone acetyltransferase activity or to a decrease in the level of MOF^1 protein due to instability. To determine whether the latter is the case, protein extracts from the heads of adult flies were resolved by SDS–PAGE and exposed to antibodies against MOF. The amount of protein loaded was monitored by Coomassie Blue staining. As shown in Figure 1B, the levels of MOF in wild-type

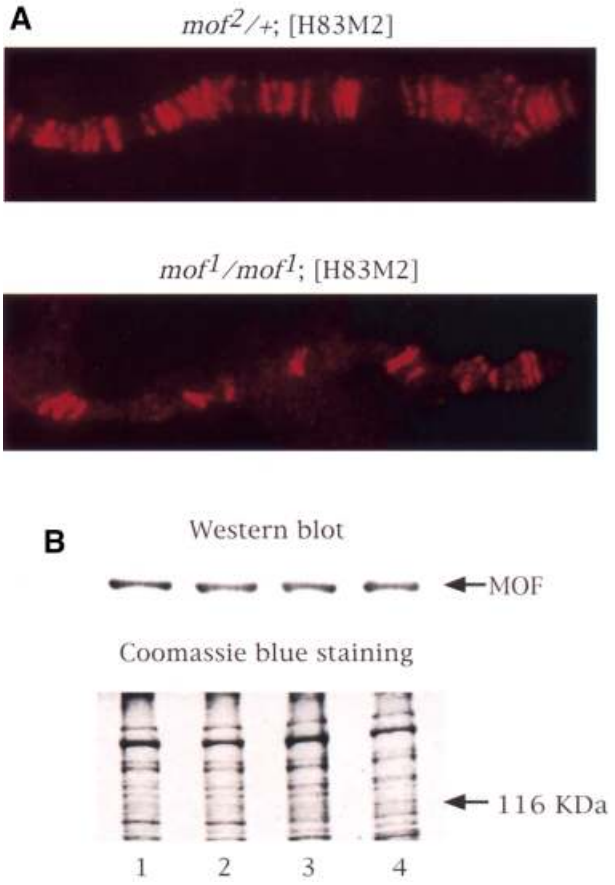


Fig. 1. MSL complexes are targeted only to the entry sites on the X chromosome in the presence of *mof*[1]. (**A**) Polytene chromosomes from control *mof*[2]/+; [H83M2] and *mof*[1]/*mof*[1]; [H83M2] female larvae were immunolabeled with antiserum against MOF. The distribution of the MOF protein is the same as that observed for all of the other MSLs (Gu *et al*., 1998). (**B**) MOF is expressed at a similar level in wild-type males (lane 1), wild-type females (lane 2), *mof*[1]/*mof*[1]; [H83M2] females (lane 3) and *mof*[2]/+; [H83M2] females (lane 4). Protein extracts prepared from adult fly heads were resolved by 7.5% SDS–PAGE. The proteins were transferred to nitrocellulose membrane and incubated with anti-MOF serum. A parallel gel was stained with Coomassie Brilliant Blue R-250 to monitor the amount of protein loaded.

males and females, and in [H83M2] transgenic females homozygous for *mof*[1] or carrying a *mof*[+] wild-type allele over a null allele, were very similar.

In the presence of the mof[1] *mutation, complexes are formed but cannot access the X chromosome beyond the entry sites*

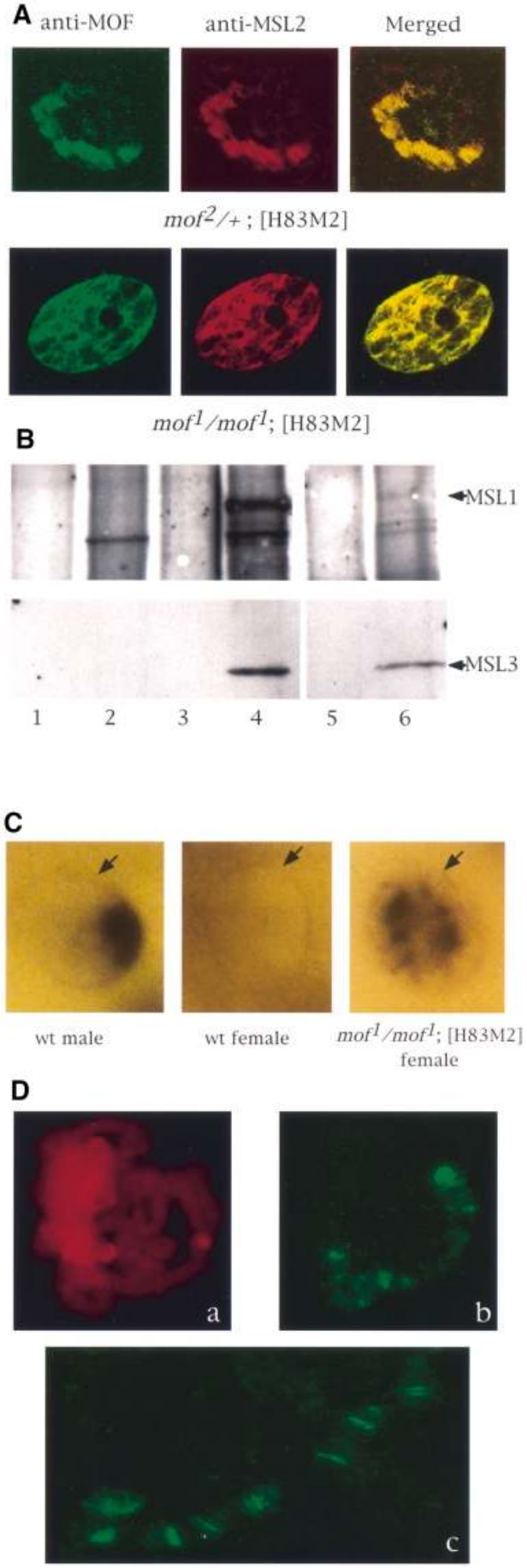
To determine whether the MOF[1] protein that is not found along the X chromosome is free or is present in association with any of the other MSLs, we stained whole salivary glands with antibodies against MSL2 and MOF (Figure 2A). In *mof*[2]/+; [H83M2] control females, these two proteins are found only on the X chromosomes, as expected (the *mof*[2] allele encodes a truncated protein; Gu *et al*., 1998). In *mof*[1]/*mof*[1]; [H83M2] females, there were some bands with strong staining for MOF and MSL2 due to the presence of MSL complexes at the entry sites, as shown previously on polytene chromosome spreads (Figure 1A). Besides these strong bands on the X chromosome, MSL2 and MOF were also co-localized elsewhere in the nuclei. Since there is no evidence of the association of these complex components with autosomal chromatin in polytene chromosome spreads of *mof*[1] males (Hilfiker *et al*., 1997) or in *mof*[1]/*mof*[1]; [H83M2] females such as the one used in Figure 1A (data not shown), our observations suggest the presence of free, untargeted MSL complexes in the nucleoplasm. Co-immunoprecipitation of MSL1 and MSL3 presented in Figure 2B established that such complexes do exist.

Since transcribed *roX* RNAs do not accumulate in the absence of the MSL complex (Meller *et al*., 2000), we reasoned that the presence of these RNAs would confirm the presence of the MSL complex in the nucleoplasm of *mof*[1] mutant individuals. To study the distribution of *roX1* RNA, salivary glands from wild-type males and females, and from *mof*[1]/*mof*[1]; [H83M2] females were hybridized with *roX1* DNA probes. As expected, *roX1* RNA is present in wild-type male but not female nuclei (Figure 2C). *roX1* RNA was also observed in *mof*[1]/*mof*[1]; [H83M2] females. However, in contrast to the wild-type male pattern, in which *roX1* is localized only to the X chromosome, in *mof*[1] mutant individuals *roX1* RNA was dispersed throughout the nuclei. In order to distinguish whether this RNA is associated with all the chromosomes or is present in the nucleoplasm, we performed *in situ* hybridization on polytene chromosome spreads. As shown in Figure 2D, *roX1* RNA was present only at the X chromosome entry sites. Thus, the dispersed pattern of *roX1* RNA in the whole nuclei indicates its presence in the nucleoplasm. Since *roX1* RNA requires the MSL complex for stabilization, this result confirmed that assembled MSL complexes unable to be targeted to the X chromosome are present.

We performed similar experiments on Schneider 2 (S2) cells transfected with *mof*[1] cDNA under the control of the *Mtn* promoter. After induction with $CuSO_4$, in most transfected cells, MSL1 is dispersed in interphase nuclei, although occasionally, because of the variability in the degree of transfection, the level of MOF[1] is not sufficient to compete fully with endogenous MOF and staining of the X chromosome by active complex can be seen. Transfected cells clearly overexpressing MOF[1] had very significantly reduced levels of histone H4 acetylated at Lys16 (H4Ac16) (Figure 3). The absence of complex on the autosomes in salivary gland preparations of *mof*[1]/*mof*[1]; [H83M2] female larvae strongly suggests that in these S2 cells the complex is not associated with autosomal chromatin; rather, it is present in the nucleoplasm. Since propidium iodide stains both DNA and RNA, it is not possible to determine the distribution of the MSL complex on the basis of MSL1 staining in interphase nuclei. For this reason, we searched for mitotic figures in our preparations and were able to establish that, during cell division, the level of MSL1 appears to be substantially lower, perhaps due to disassembly and degradation.

Overexpression of MOF causes association of the MSL complex at ectopic sites

The results reported above demonstrate that the histone acetyltransferase activity of MOF plays an important role in spreading the complex from the entry sites to other sites on the X chromosome. The acetylation of histone H4 at



Lys16, for which MOF is responsible (Smith *et al*., 2000), may render the X-chromosome chromatin accessible to MSL complexes. If this is the case, an induced ectopic acetylation of histone H4 at Lys16 could lead to the association of the MSLs at some autosomal sites. To investigate this possibility, we used S2 cells transfected with *mof* cDNA and selected for stable integration of the construct. Cells overexpressing MOF had a very high level of the H4Ac16 isoform in comparison with mock-transfected S2 cells, where H4Ac16 was only observed on the X chromosome (Figure 4A and C). When MOF-overexpressing cells were stained for MSL1 and MSL3, these two proteins were co-localized on the X chromosomes as well as at many other sites in the nuclei (Figure 4D). To determine whether these complexes are present free in the nucleoplasm or are bound to autosomes, we stained cells with MSL1 antiserum and counterstained them with propidium iodide to identify nuclei with morphologically distinct chromosomes. In cells whose nuclei had entered mitosis, MSL1 was not only present on the X chromosome, but was also clearly associated with the autosomes; little if any complex appeared free in the nucleoplasm (Figure 4E and F). Although there is an X chromosome entry site mapped at 5C where *mof* is located, the autosomal binding in induced cells is unlikely to be due to the creation of ectopic entry sites by the integration of the *mof* cDNA construct at multiple autosomal locations, because (i) this type of autosomal association is absent in transfected but non-induced cells, where the MSL complex is limited to the X chromosome (data not shown) and (ii) in salivary gland preparations of larvae carrying a *mof* cDNA transgene known to rescue the mof^1 mutation at an autosomal location there is no evidence of complex association with the transgene (data not shown).

Loss of MLE function prevents the spreading of the complex by affecting its stability and/or function

The MLE protein is required for assembly of the MSL complex, as demonstrated by the observation that only

Fig. 2. In the absence of MOF activity, MSL complexes are found in the nucleoplasm. (**A**) Whole salivary glands from mof^1/mof^1; [H83M2] and $mof^2/+$; [H83M2] larvae were stained for MSL2 and MOF by indirect immunofluorescent labeling. In the nuclei of larvae expressing mof^+, the two antigens are co-localized along the paired X chromosomes. In mof^1 homozygotes, the MSL complex appears in the interchromosomal spaces. Note that it must also be present at the entry sites on the X chromosomes although these cannot be resolved in this material. (**B**) Co-immunoprecipitation of MSL1 and MSL3. Protein extracts prepared from wild-type female (lanes 1 and 2), wild-type male (lanes 3 and 4) and mof^1/mof^1; [H83M2] female (lanes 5 and 6) flies were immunoprecipitated with pre-immune (lanes 1, 3 and 5) or MSL1 antisera (lanes 2, 4 and 6). The precipitate was analyzed by western blotting using MSL1 and MSL3 antisera. (**C**) Localization of *roX1* RNA, determined by *in situ* hybridization in whole salivary glands. The RNA is localized to a limited area corresponding to the X chromosome in male nuclei and is absent in the nuclei of wild-type females. In mof^1/mof^1; [H83M2] females, *roX1* RNA is dispersed in a pattern that mimics that of the MSLs. The arrows mark the nuclear envelope. (**D**) *In situ* hybridization of *roX1* RNA on the polytene chromosomes of mof^1/mof^1; [H83M2] female larvae. (a) Propidium iodide staining of a nucleus; (b) *roX1* is present at the entry sites on the X chromosome in the same nucleus; (c) a stretched region of X chromosome from another nucleus.

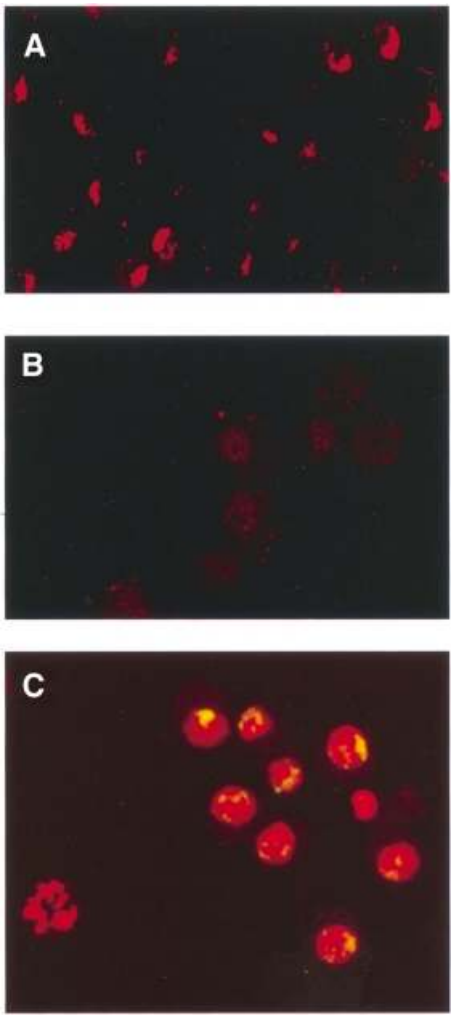


Fig. 3. Overexpression of MOF[1] in S2 cells causes a reduction in the level of H4Ac16 present on the X chromosomes and relocation of MSL complexes. Histone H4Ac16 in S2 cells transfected with the vector (**A**) or with *mof*[1] cDNA (**B**) and induced with $CuSO_4$. (**C**) Merged image of induced S2-*mof*[1] cells stained for MSL1 (green) and counterstained for nucleic acids with propidium iodide (red). The yellow color indicates the presence of the MSL1 protein.

MSL1/MSL2 can be found at the entry sites in its absence (Palmer *et al*., 1994; Gu *et al*., 1998). Using a point mutation that abolishes the helicase activity of MLE (*mle*^{GET}), Lee *et al*. (1997) observed that the mutant protein was able to bind to a reduced number of sites on the X chromosome, as well as to a number of ectopic sites on the autosomes, in mutant males. MSL1 was found to co-localize with MLE^{GET} at a number of the X chromosome sites, the so-called entry sites. As the authors suggested, the MLE^{GET} binding pattern may have been influenced by the moribund state of the mutant male larvae examined. We wished, therefore, to ascertain the role of the *mle*^{GET} mutation in transgenic females and, thereby, to be able to compare its effect with that of the *mof*[1] mutation. We stained polytene chromosomes of *mle*[1]/*mle*[1];*mle*^{GET}/[H83M2] females for the presence of MSL1 and MSL3 (the *mle*[1]mutant allele has a stop codon that truncates the protein after the first 125 amino acids, so that the only MLE present in these females is the mutant MLE^{GET} protein). We observed that the MSL1 and MSL3 proteins (Figure 5) as well as MOF (data not shown) are co-localized at the entry sites. Once again, we used the presence of *roX* RNA as an indicator of MSL complexes to determine whether free, untargeted complexes are present in these nuclei. Surprisingly, no *roX1* RNA was detected in whole salivary gland nuclei by *in situ* hybridization with *roX1* DNA probes (Figure 6A). Since this result is different from those obtained with [H83M2] transgenic females homozygous for the *mof*[1] loss-of-function mutation (Figure 2C), we confirmed the absence of *roX1* RNA signal by RT–PCR using *roX1*-specific primers (Figure 6B). Furthermore, no *roX2* RNA was detected either by *in situ* hybridization in whole salivary glands (Figure 6A) or by RT–PCR (Figure 6B). Thus, it appears that in the absence of the ATP-dependent function of MLE, MSL complexes can be assembled but, once assembled, these complexes no longer contain *roX* RNA.

To determine whether fully or partially formed complexes lacking *roX* RNA exist free in the nucleoplasm of *mle*[1]/*mle*[1];*mle*^{GET}/[H83M2] females, we stained whole salivary gland nuclei for MSL1 and MSL3 by indirect immunofluorescent labeling (Figure 7A). We also performed co-immunoprecipitation of MSL1 and MSL3 from whole mutant female extracts (Figure 7B). The results of these experiments provided evidence for the existence of MSL complexes in the nucleoplasm of *mle*^{GET} mutant larvae.

Discussion

It has long been known that eukaryotic transcription is regulated at initiation by a large group of factors that associate with RNA polymerase II, target it to promoter regions with spatial and temporal specificity, and allow it to clear the promoter. Recently, another level of transcriptional regulation, involving specialized multiprotein aggregates that interact with chromatin components to control the rate of transcription, has come to light. At present, these aggregates can be grouped into two broad categories: (i) complexes that use the energy of ATP hydrolysis to alter nucleosomal conformation (SWI/SNF in yeast and in mammals; RSC, Sfh1p in yeast; NURF, CHRAC and ACF in *Drosophila*); and (ii) complexes that target specific histone acetyltransferases to their site of action and alter chromatin conformation via the acetylation of histones (GCN/ADA, NuA4 and SAGA in yeast). All of these complexes interact with nucleosomal proteins (i.e. histones); they may interact with components of the initiation complex, although they do not activate silent genes, and they enhance the level of transcription of large groups of activated genes (for review see Grant and Berger, 1999; Muchardt and Yaniv, 1999). The dosage compensation complex (or MSL complex) of *Drosophila* is particularly interesting because it includes both an ATP-dependent helicase (MLE) and a histone acetyltransferase (MOF).

Meller *et al*. (2000) have shown that the MLE protein is required very early in the process of assembly of the MSL complex at the sites of *roX* RNA synthesis. MLE is a member of the DEAH/DEAD RNA helicase family (Kuroda *et al*., 1991; Lee and Hurwitz, 1993; for review see Eisen and Lucchesi, 1998). The recombinant protein

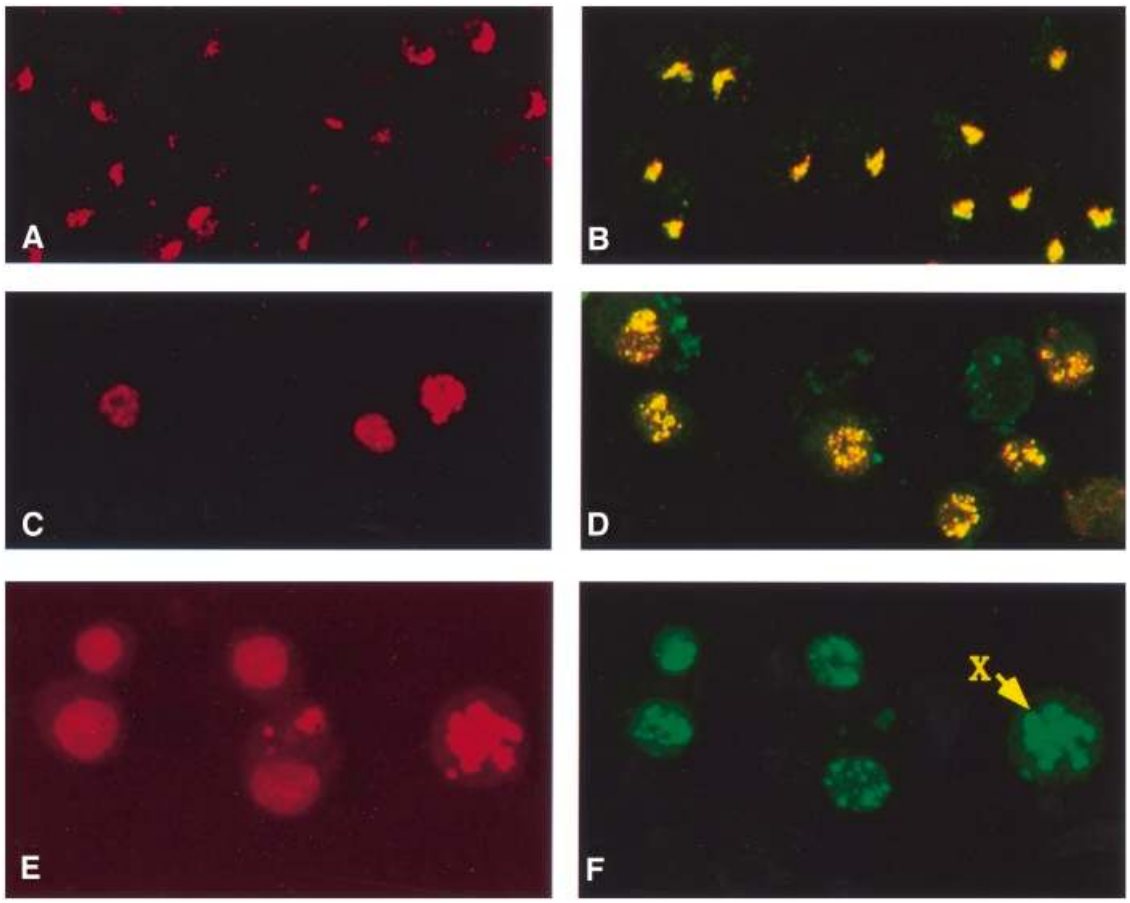


Fig. 4. MSL complexes are targeted to autosomal sites in S2 cells overexpressing MOF. (**A**) Histone H4Ac16 staining is restricted to the X chromosome in cells transfected with the vector induced with $CuSO_4$. (**B**) Co-localization to the X chromosome is also seen in the merged image of immunofluorescent labeling for MSL1 (red) and MSL3 (green) in similar cells. (**C**) In contrast, H4Ac16 is widespread throughout the nuclei in cells transfected with *mof* cDNA and induced with $CuSO_4$. (**D**) Merged images of immunofluorescent labeling for MSL1 (red) and MSL3 (green) in similar cells. The complex distribution generally follows the distribution of H4Ac16. (**E**) Induced S2-*mof* cells were stained for nucleic acids with propidium iodide. (**F**) The same cells were immunolabeled with anti-MSL1 serum. The distribution of MSL1 and, by inference, of the MSL complex is clearly autosomal. The arrow points to a chromosome that is more intensely stained than the others and is presumably the X chromosome.

has been shown to have nucleic acid binding and DNA/RNA helicase activity, and the MLE/*roX* RNA interaction may be similar to that demonstrated for putative DEAD-box helicases during ribosome biogenesis in yeast (Daugeron and Linder, 1998; de la Cruz *et al*., 1998, 1999; Kressler *et al*., 1998). A point mutation generated by *in vitro* mutagenesis in the ATP-binding domain (mle^{GET}) abolishes the helicase activity *in vitro* while the nucleic acid-binding affinity is unaffected (Lee *et al*., 1997). The mle^{GET} gene product cannot rescue the lethality of the *mle*-null genotype, indicating that its helicase activity is required for dosage compensation (Lee *et al*., 1997). Our results in transgenic females that express only the MLE^{GET} protein show that the RNA helicase activity of MLE may not be required for the assembly of MSL complexes. This activity, though, appears to be essential for the stability of the complexes with respect to their ability to retain their *roX* RNA component. *roX* RNA is essential for the assembly of the MSL complex, but once that is accomplished it can be lost and the other complex components are presumably held together by protein–protein interactions. This is consistent with the observation of Akhtar *et al*. (2000) that MSL complexes immunoprecipitated from RNase-treated S2 cell extracts remain assembled in the absence of demonstrable *roX2* RNA. Our results suggest that *roX* RNA-less complexes can access the entry sites but are unable to spread to their sites of action along the X chromosome.

MOF is a member of the MYST family of histone acetyltransferases (Borrow *et al*., 1996; Reifsnyder *et al*., 1996; Hilfiker *et al*., 1997; Neal *et al*., 2000). *In vitro* assays with full-length proteins or truncated fragments have shown that the members of this family acetylate histones H2A and H3, but show a strong preference for H4. The specific residues acetylated on histone H4 have been determined for yeast Esa1p (as a recombinant protein or as part of the NuA4 complex), human Tip60 (as a recombinant protein) and *Tetrahymena* p80. All three of these MYST family members acetylate lysines 5, 8, 12 and 16 (Kimura and Horikoshi, 1998; Smith *et al*., 1998; Ohba *et al*., 1999). In contrast, MOF—both as a recombinant protein (Akhtar and Becker, 2000) and as a member of the MSL complex (Smith *et al*., 2000)—shows a clear, marked preference for Lys16. Since database monitoring has revealed that several MYST family acetyl transferases other than MOF exist in *Drosophila*, it is likely that some

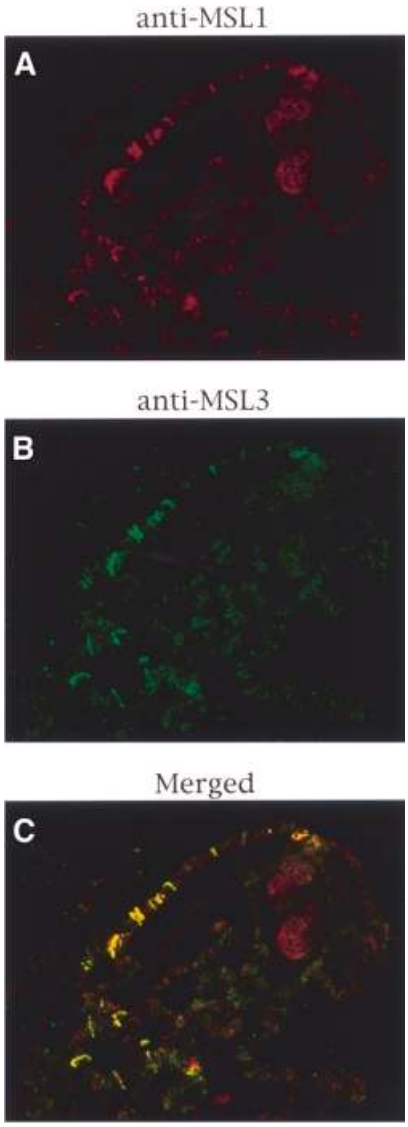


Fig. 5. Effect of the *mle*^{GET} mutation on the localization of the MSL complex. Polytene chromosomes from *mle*^{1}/*mle*^{1}; [*mle*-GET]/[H83M2] larvae were stained for MSL1 (**A**) or MSL3 (**B**). As seen in the merged image (**C**), these two MSLs are co-localized at the entry sites.

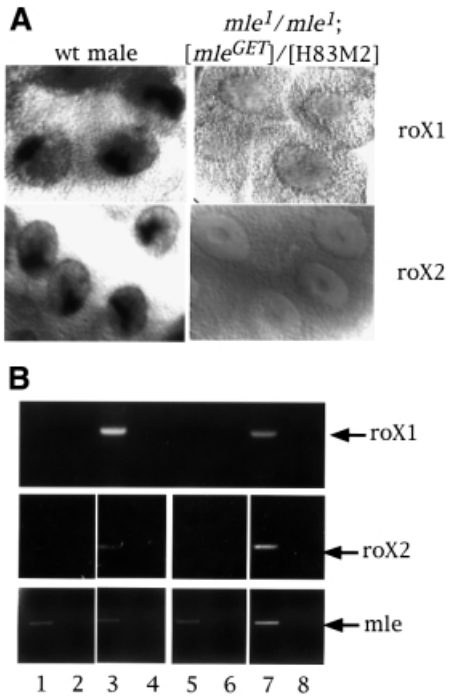


Fig. 6. *roX* RNA is absent in females that produce only the MLE^{GET} mutant protein. (**A**) Localization of *roX1* and *roX2* RNA by *in situ* hybridization in whole salivary glands from a wild-type male and a *mle*^{1}/*mle*^{1}; [*mle*-GET]/[H83M2] female larva. (**B**) RT–PCR for *roX* RNA. Total RNA from adult flies of wild-type females (lanes 1 and 2), wild-type males (lanes 3 and 4), *mle*^{1}/*mle*^{1}; [*mle*-GET]/[H83M2] females (lanes 5 and 6) and *mle*^{1}/+; [*mle*-GET]/[H83M2] females (lanes 7 and 8) were reverse transcribed (lanes 1, 3, 5 and 7) or not (lanes 2, 4, 6 and 8) and then amplified for *roX1* or *roX2* RNA by PCR. The lower panel shows the amplification of MLE RNA by RT–PCR in the same samples.

level of acetylation of histone H4 at Lys16 occurs throughout the chromatin of both males and females. The enrichment of the monoacetylated H4Ac16 form along the X chromosome in males, though, is due to the specific targeting of MOF to this chromosome by the MSL complex. In females, MOF is present but is completely dispensable (Hilfiker *et al*., 1997; Gu *et al*., 1998), leading to the conclusion that it has no function in this sex or that its function is assumed by some other MYST family member. We wish to point out that available data do not allow us to establish whether the specific acetylation mediated by the MSL complex is responsible *per se* for the doubling in transcription of X-linked genes or whether it renders the X chromosome more accessible to other factors, such as the JIL-1 kinase (Jin *et al*., 1999), which are the actual effectors of the enhancement. We also do not know whether components of the complex itself are acetylated by MOF.

The normal association of the MSL complex at hundreds of sites along the X chromosome appears be a process with at least three major steps (Figure 8A). The first is the formation of functional complexes at the two entry sites where the *roX* RNAs are transcribed (Kelley *et al*., 1999). It should be noted that although the MSL1 and MSL2 proteins are able to access the X chromosome at the entry sites and to recruit MLE, further complex assembly can only occur in the presence of the *roX* RNAs. This contention is supported by the observation that, in the absence of the two *roX* genes, no complex is seen to form in embryonic stages where it is normally evident (Franke and Baker, 1999). A caveat is that removal of *roX2* was accomplished by using a deletion of such size that other *roX*-like genes or other unidentified components of the complex or genes whose product is required for complex stability, closely linked to *roX2*, may have been deleted as well. In any event, since the *roX* RNAs are unstable unless they are associated with the complex (Meller *et al*., 2000), the process of assembly can proceed only at the entry sites containing the *roX* genes. Once complexes are formed, they access the X chromosome through all of the entry sites, presumably via the affinity of their MSL1/MSL2 components for these sites. Finally, the complexes spread from the entry sites to the many other sites along the X chromosome where they are normally found. This last step requires the histone acetyltransferase activity of MOF. We suggest that the spreading process involves the

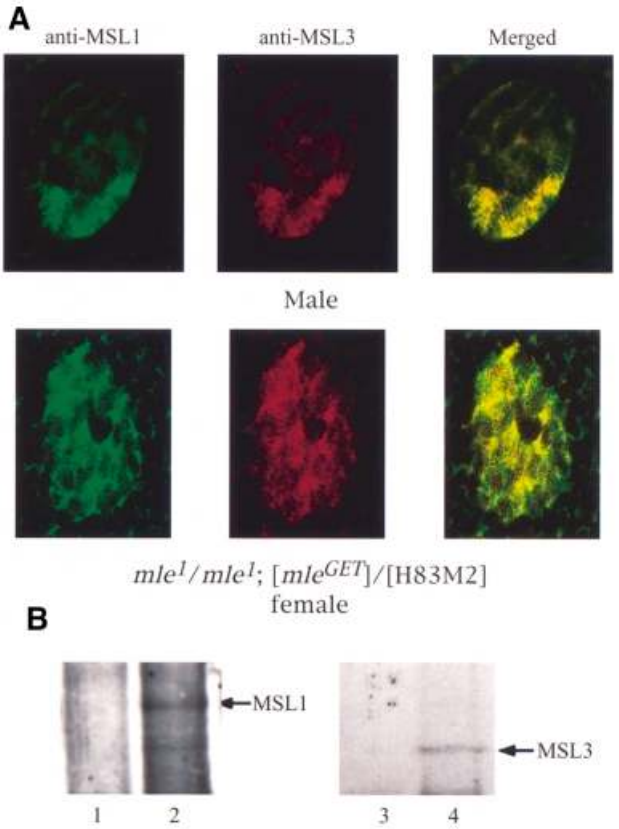


Fig. 7. In the absence of MLE ATPase activity, MSL complexes lacking *roX* RNA are found in the nucleoplasm. (**A**) Whole salivary glands from *mle*^{1}/*mle*^{1}; [*mle*-GET] and wild-type male larvae were stained for MSL1 and MSL3 by indirect immunofluorescent labeling. In the nuclei of male larvae expressing only *mle*^{GET}, the two antigens are co-localized along the X chromosome. In the nuclei of female larvae from *mle*^{1}/*mle*^{1}; [*mle*-GET], although some MSL1 (green) and MSL3 (red) proteins appear to be independent of each other, a substantial amount of these proteins are associated (yellow, in the merged image), leading to the conclusion that the MSL complex exists in the interchromosomal spaces. Note that, as stated in the legend of Figure 5, the complex must also be present at the entry sites on the X chromosomes, although these cannot be resolved in this material. (**B**) Co-immunoprecipitation of MSL1 and MSL3. Protein extracts prepared from *mle*^{1}/*mle*^{1}; [*mle*-GET] female flies were immunoprecipitated with pre-immune (lanes 1 and 3) or MSL1 antisera (lanes 2 and 4). The precipitate was analyzed by western blotting using MSL1 and MSL3 antisera. Note that the lanes presented in this figure were obtained from the gel illustrated in Figure 2, which includes the wild-type male and female controls.

acetylation of neighboring nucleosomes, thereby altering the conformation of adjacent chromatin and rendering it more accessible to the entry of additional MSL complexes. The latter may require the presence of acetylated histone H4 tails in order to stabilize their chromatin association. This conclusion is consistent with our observation that, in S2 cells overexpressing MOF, the resulting abnormal ectopic acetylation of histone H4 at Lys16 leads to the association of the MSL complex along autosomal chromatin. This may mirror the normal situation *in vivo* where complexes, initially attracted to the entry sites, acetylate histone H4 at Lys16 and thereby make adjacent chromatin regions accessible to more complexes. The affinity of the MSL complex for histone H4 tails implied in our model is reminiscent of a similar role played by histone tails in the spreading of complexes containing SIR2, 3 and 4 during the silencing of mating type loci and telomeric heterochromatin formation in yeast (Hecht *et al*., 1996; Stone and Pillus, 1998). Although critical to the spreading process, the role played by the ATPase function of MLE, either directly or in conjunction with roX RNA, is not sufficiently understood to be incorporated in the model.

We believe that the process just described can provide the following explanations for the gaps in MSL binding that occur along the X chromosome, or at ectopic autosomal sites where the complex has been caused to form at the site of a *roX* transgene (Kelley *et al*., 1999). It is possible that the spread of H4 acetylation and complex association may be stopped by some insulator or some as yet uncharacterized boundary elements. This would not necessarily require that the entry sites be entirely responsible for the pattern seen along the X chromosome. As illustrated in Figure 8B, the interphase chromosome is believed to consist of a series of rosettes formed by loops of the chromatid fiber anchored to a central core by dispersed regions that have affinity for one another (Pirrotta, 1997, 1998; Ostashevsky, 1998; Solovjeva *et al*., 1998; Munkel *et al*., 1999). In such an arrangement, a cluster of complexes that have been stopped by some boundary element could acetylate the nucleosomes on a neighboring loop, initiating a spreading process on the other side of a gap.

The above considerations raise a number of questions that remain to be resolved. Is the pattern of complex association on the X chromosome tissue specific? Is it dependent on a tissue-specific distribution of the entry sites (other than those containing the *roX* loci, which must remain invariant in all tissues)? Is the tissue-specific distribution established when the complex first forms in early embryogenesis (Rastelli *et al*., 1995; Franke *et al*., 1996; McDowell *et al*., 1996) and is the pattern perpetuated through the mitotic divisions that give rise to a particular tissue (Lavender *et al*., 1994)? To answer these questions will require a thorough melding of cytological and biochemical approaches.

Materials and methods

Fly stocks

Flies were reared on standard cornmeal–sugar–yeast–agar medium containing propionic acid and methylparaben as mold inhibitors. The [w^{+} H83M2] transgene is a P-element construct that contains the *msl2* open reading frame under *hsp83* promoter control. The insert lacks the SXL-binding sites present in the 5′ and 3′ UTR of *msl2* and, therefore, can give rise to the MSL2 protein in transgenic females (Kelley *et al*., 1995). The [w^{+} *mle*-GET] transgene is similar but the *mle* open reading frame contains a lysine → glutamic acid substitution in the ATP-binding motif GKT (Lee *et al*., 1997). Females of the *w cv mof*^{1}/*w cv mof*^{1}; [w^{+} H83M2] *CyO* genotype were generated by crossing *w cv mof*^{1}/*w cv mof*^{1} virgin females to *w cv mof*^{1}/*Y*; 18H1 *Bc*/[w^{+} H83M2] *CyO* males. Control *y w mof*^{2}/+; [w^{+} H83M2] *CyO* females were produced by crossing *y w*/*y w* virgin females to *y w mof*^{2}/*Y*; 18 H1 *Bc*/[w^{+} H83M2] *CyO* males. In these stocks, 18H1 designates the presence of an insert that covers the *mof* mutation. Larvae were recognized by the lack of black cells and adults by the presence of curly wings. To produce females expressing only the mle^{get} mutant protein, *w*; *pr mle*^{1}/*w*; *pr mle*^{1}; *msl3* [w^{+} H83M2]/*msl3* [w^{+} H83M2] females were crossed to *w*; *pr mle*^{1}/*CyO*; [w^{+} *mle*-GET] males. The resulting *w*; *pr mle*^{1}/*w*; *pr mle*^{1}; [w^{+} *mle*-GET]/*msl3* [w^{+} H83M2] and *w*; *pr mle*^{1}/*CyO*; [w^{+} *mle*-GET]/*msl3* [w^{+} H83M2] larvae were distinguished by the absence or presence of the rearranged *CyO*

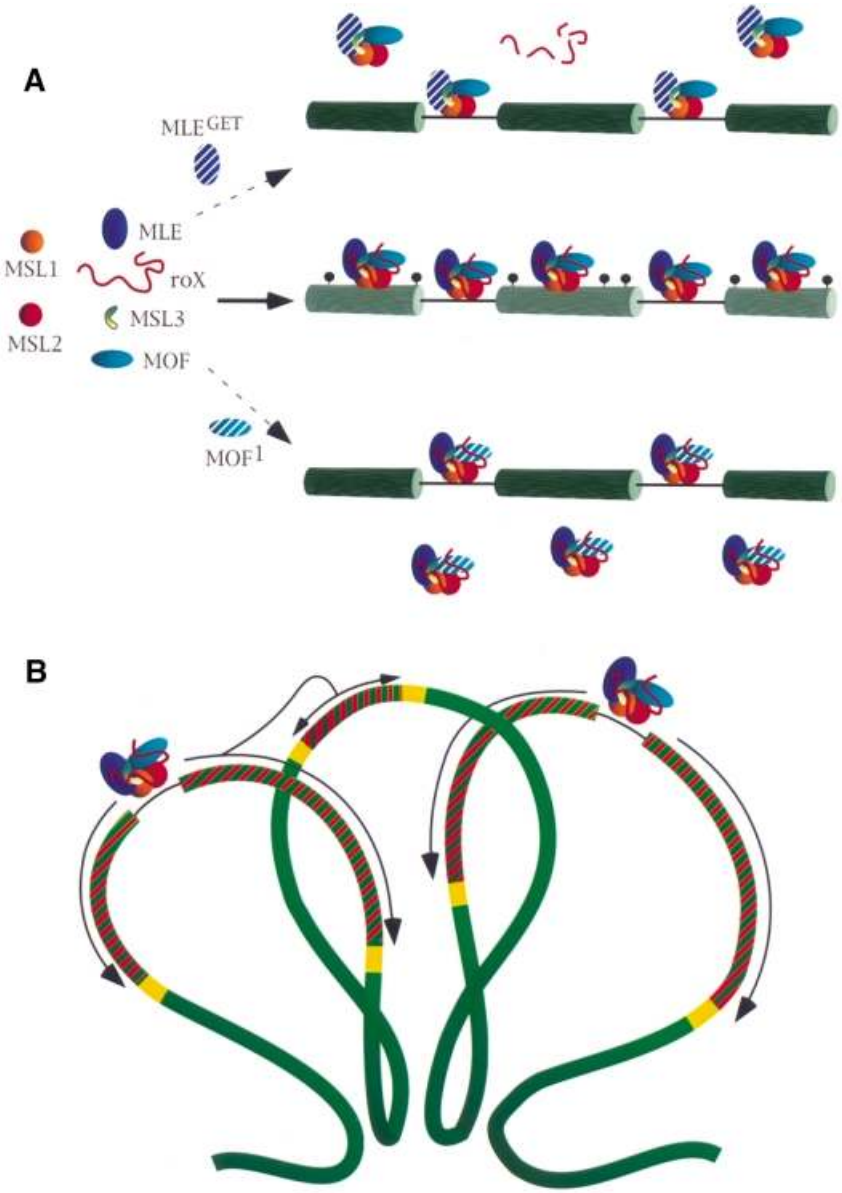


Fig. 8. A model for the association and spreading of the MSL complex along the X chromosome. (**A**) In wild type, the MSL complex assembles at the *roX*-bearing entry sites, moves to other sites and acetylates histone H4 of neighboring nucleosomes at Lys16 (indicated as black circles). In the absence of the ATPase activity of MLE (MLE^{GET}), the association of the complex with *roX* RNA is unstable. Free complexes devoid of *roX* RNA and unable to access the X chromosome are present in the nucleoplasm. If the acetylase function of MOF is impaired by mutation (MOF^1), complexes are formed; although they can access other entry sites, they cannot spread along the X chromosome and are found, unbound, in the nucleoplasm. (**B**) Wild-type MSL complexes present at the entry sites spread along the X chromosome by acetylating nucleosomes. Spreading of the complexes and concomitant acetylation (indicated by the striped regions) may be interrupted by some type of boundary elements (yellow boxes). The looping of the chromatid fiber into rosettes may enable spreading of the complex from a region where acetylation initiated at an entry site to a region where no entry sites are present (from the left to the central loop in the diagram).

chromosome in polytene chromosome spreads of salivary glands. A full description of the mutants listed and of the *CyO* balancer chromosome can be found in FlyBase (http://flybase.bio.indiana.edu).

In situ hybridization of roX RNA

The insert from pZero-2/*roX1* DNA, which contains a 1.9 kb *roX1* DNA fragment amplified from genomic DNA using primers GTTACGTTC-GGAGTGGAAAATGG and GTTTCTTCTGGGTGTAGCTTCTTGG, was used as the probe. The probe for *roX2* RNA was a 1.1 kb *roX2* DNA fragment amplified from genomic DNA using primers CTCCGA-TTGCCTTGCACTCG and AAGTGTCAGTTCTGGTCACCCTGG. Probe was random-prime labeled with digoxigenin using Klenow DNA polymerase (Roche Molecular Biochemicals). *In situ* hybridization of whole salivary glands was performed according to Meller *et al*. (1997). *In situ* hybridization of polytene chromosomes was performed using digoxigenin-labeled antisense *roX1* RNA, which was transcribed *in vitro* with SP6 RNA polymerase (Roche Molecular Biochemicals) using pZero-2/*roX1* cut with *Eco*RV as the template. The hybridization was performed according to Kelly *et al*. (1999) except that the signal was detected using the TSA Fluorescein System (NEN Life Science Products).

Antisera

Anti-MSL antibodies were raised against various fragments of MSL proteins fused to glutathione *S*-transferase (GST) as follows: rabbit anti-MSL1 (amino acids 423–1029), guinea pig anti-MSL2 (amino acids 78–529), guinea pig anti-MSL3 (full-length) and rabbit anti-MOF (amino acids 748–827). Secondary antisera were donkey or goat anti-rabbit IgG

conjugated with Cy5 or anti-guinea pig IgG conjugated with fluorescein isothiocyanate (FITC; Jackson Immunoresearch Laboratories). For immunofluorescent staining, primary antisera were diluted at 1:200 and secondary antisera at 1:250 in phosphate-buffered saline (PBS; 10 mM NaH_2PO_4 pH 7.2, 130 mM NaCl), 1.0% bovine serum albumin (BSA) and 0.2% Tween-20.

Immunofluorescent staining

Polytene chromosome spreads were prepared as previously reported (Gu *et al*., 1998). Chromosome preparations were washed three times with PBS and blocked for 1 h with PBS, 1% BSA, 0.2% Tween-20. Antibody was added and the slides were incubated at 4°C overnight. The slides were then washed with PBS and blocked once again with PBS, 1% BSA, 0.2% Tween-20 and 2% normal goat or donkey serum. Fluorochrome-conjugated secondary antibody was added and the slides maintained at room temperature for 2 h or at 4°C overnight. After extensive washing with PBS, the slides were mounted with Slow-Fade mounting medium (Molecular Probe) and observed with a Bio-Rad confocal microscope.

Whole salivary glands were fixed and permeabilized according to Bone *et al*. (1994). Immunofluorescence procedures were the same as described above for polytene chromosomes.

Wild-type or transfected S2 cells were seeded on the slides the night before staining. Slides were washed briefly with PBS and fixed in 4% formaldehyde in PBS for 15 min at room temperature. After washing three times with PBS, cells were permeabilized for 5 min at –20°C with pre-cooled acetone. Cells were then washed with PBS and processed for antibody staining as described for polytene chromosomes.

Western blot analysis for MOF

Heads from 20–30 adult flies were homogenized in 100 µl of sample buffer (0.1 M Tris pH 6.8, 4% SDS, 0.2% β-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue) and heated at 100°C for 3 min. After centrifugation, 2.5–10 µl of supernatant were loaded on a 7.5% SDS–polyacrylamide gel. The gel was either stained with Coomassie Blue R-250 to monitor the amount of protein loaded or transferred to a nitrocellulose membrane which was exposed to anti-MOF serum.

Immunoprecipitation

About 100–150 flies were ground in liquid nitrogen and then homogenized 15 times in 1 ml of lysis buffer [50 mM Tris–HCl pH 8.8, 300 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 µg/ml leupeptin, 2 µg/ml antipain, 2 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The homogenate was incubated for 40 min on ice after the addition of 1 ml of lysis buffer. The protein extract was then separated from the debris after centrifugation for 20 min at 4°C. The protein extract was cleared by incubation with 20 µl/ml of protein A-coupled agarose beads and then incubated at 4°C overnight with 4 µl of MSL1 antiserum or 6 µl of MSL1 pre-immune antiserum bound to 10 µl of protein A-coupled agarose beads. After washing the beads five times with the buffer (20 mM HEPES pH 7.2, 10% glycerol, 300 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.2% Tween-20, 1 mM PMSF), immunoprecipitated proteins were eluted from the agarose beads with 60 µl of sample buffer and 20 µl were resolved on a 7.5% SDS–polyacrylamide gel. The gel was transferred to nitrocellulose membrane, which was probed with MSL1 and MSL3 antiserum and then incubated with the corresponding secondary antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories). The secondary antibody was detected using ECL western blotting detection reagents (Amersham–Pharmacia Biotech).

Transfection of S2 cells

The coding regions for MOF and MOF^1 were amplified from genomic DNA prepared from wild-type or *w cv mof*^{1} females, using primers caaagatatcctcgagTCTGAAGCGGAGCTGGAA and aaaccattcctaggtcgacATGCGATGATAGCAGAACGG (nucleotides in lower case were added as linkers for the purpose of cloning). The amplified DNA was cut with *Xho*I and *Nhe*I, and the vector pMt/HA (a derivative of pMK322) was cut with *Xho*I and *Spe*I. The restriction fragments were ligated to generate pMt-MOF and pMt-MOF1, which will express MOF and MOF^1, respectively, after induction with $CuSO_4$. S2 cells were transfected as described (Di Nocera and Dawid, 1983). Briefly, 10–15 µg of DNA in 250 µl of 250 mM $CaCl_2$ were added dropwise to 250 µl of 2× HEPES-buffered saline while mixing. The solution was added to S2 cells after 40 min at room temperature. After 24 h, cells were washed with medium and allowed to grow for another 24 h before selection with 200 µg/ml hygromycin. Lines of stably transfected cells were established after continuous selection for ~2 weeks.

RT–PCR

Total RNA was prepared from 60–80 adult flies using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA was eluted in 50 µl of H_2O and then treated with RNase-free DNase I (Promega). After purification, ~100 ng of total RNA were reverse transcribed for 1 h at 37°C using Senscript reverse transcriptase (Qiagen) with primer RevoX1 (GGTCACCCTATCAGTAGCAGTACCACAC) (*roX1*), primer r2CPr3 (GAGCGAGATGACAATAGAGAGG) (*roX2*) or primer mleP3r (ACAGAGTGTGAAGCAGAAGC) (*mle*). Amplification of cDNA was performed in a 50 µl reaction with one-tenth of the reverse transcription reaction, 100 µM dNTPs, 1 U of Perfect-Match PCR-Enhancer (Stratagene) and 10 pmol of each forward and reverse primer. The cycling conditions were 94°C for 5 min followed by 30 cycles (*roX1*) or 25 cycles (*roX2* and *mle*) of 94°C for 1 min, 52°C for 1 min, 72°C for 45 s. The primers were as follows. *roX1*: RevoX1 (see above) and DiroX1 (CATCGTGCAACAATCCCAAAG); *roX2*: r2CPr3(see above) and r2CPd2 (GCCATCGAAAGGGTAAATTGG); *mle*: mleP3r (see above) and mleP3d (CTACTCGGTGCGATTGAG).

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