Association and spreading of the *Drosophila* dosage compensation complex from a discrete *roX1* chromatin entry site

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In *Drosophila*, dosage compensation is controlled by the male-specific lethal (MSL) complex consisting of MSL proteins and *roX* RNAs. The MSL complex is specifically localized on the male X chromosome to increase its expression ~2-fold. We recently proposed a model for the targeted assembly of the MSL complex, in which initial binding occurs at ~35 dispersed chromatin entry sites, followed by spreading *in cis* into flanking regions. Here, we analyze one of the chromatin entry sites, the *roX1* gene, to determine which sequences are sufficient to recruit the MSL complex. We found association and spreading of the MSL complex from *roX1* transgenes in the absence of detectable *roX1* RNA synthesis from the transgene. We mapped the recruitment activity to a 217 bp *roX1* fragment that shows male-specific DNase hypersensitivity and can be preferentially cross-linked *in vivo* to the MSL complex. When inserted on autosomes, this small *roX1* segment is sufficient to produce an ectopic chromatin entry site that can nucleate binding and spreading of the MSL complex hundreds of kilobases into neighboring regions.

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Introduction

Transcription of eukaryotic genes is highly sensitive to the way DNA is packaged into chromatin. Recent reports from a number of model systems demonstrate that alteration in chromatin conformation is a major strategy for gene regulation (reviewed in Kingston and Narlikar, 1999). One striking observation is that changes to chromatin architecture often spread from an initiation point, sometimes discontinuously. Examples of repressive chromatin spreading include heterochromatic position effect variegation in flies (Wakimoto, 1998), yeast mating-type cassette silencing (Stone and Pillus, 1998; Donze *et al.*, 1999) and X inactivation in mammals (White *et al.*, 1998;

Lyon, 1999). In contrast, male-specific lethal (MSL)-mediated dosage compensation in male *Drosophila* provides an example of spreading active chromatin from initiation sites to affect flanking genes (Kelley *et al.*, 1999). This spreading requires the two known enzymatic activities of the complex, the Maleless (MLE) ATPase and the Males absent on the first (MOF) acetyltransferase (Lee *et al.*, 1997; Gu *et al.*, 2000). Therefore, the study of *cis*-acting sites needed to bind the MSL complex and initiate spreading should be of general relevance to the understanding of chromatin-based gene regulation.

The MSL complex is required for X chromosome dosage compensation in *Drosophila* (Lucchesi, 1998; Kelley and Kuroda, 2000). Dosage compensation occurs primarily by increasing X-linked transcription in males, to make the output of the single male X similar to both female X chromosomes (Mukherjee and Beermann, 1965). A histone H4 isoform specifically acetylated at lysine residue 16 (H4Ac16) is highly concentrated on the male X chromosome and is dependent on MSL complex function, suggesting that hypertranscription of the male X is due, at least in part, to chromatin structure alteration through acetylation of nucleosomes (Turner *et al.*, 1992; Bone *et al.*, 1994; Hilfiker *et al.*, 1997; Akhtar and Becker, 2000; Smith *et al.*, 2000).

The *Drosophila* MSL complex contains at least five proteins and two RNAs (Lucchesi, 1998; Kelley and Kuroda, 2000). Mutation in any protein member of the MSL complex causes male-specific lethality and lack of enrichment of H4Ac16 or the MSL complex itself on the male X (Bone *et al.*, 1994; Hilfiker *et al.*, 1997). The five identified MSL proteins are MLE, a DEXH helicase, MSL1, a novel acidic protein, MSL2, a Ring finger protein, MSL3, a chromodomain protein, and MOF, which has both a chromodomain and the histone H4 acetyltransferase activity. Two non-coding RNAs, RNA on X1 (*roX1*) and *roX2* are also members of the MSL complex (Gu *et al.*, 2000; Meller *et al.*, 2000; Smith *et al.*, 2000). These RNAs are male-specific and paint the male X chromosome (Amrein and Axel, 1997; Meller *et al.*, 1997). *roX1* is not essential for male-viability or dosage compensation, but may be functionally redundant with *roX2* (Franke and Baker, 1999). A simple mutant allele of *roX2* has not been reported. Recently, the JIL-1 protein kinase has been found associated with the MSL complex, although its function in the dosage compensation system is still unknown (Jin *et al.*, 2000).

A complete set of MSL proteins is needed to mediate hypertranscription of the hundreds of active genes along the male X (Kelley and Kuroda, 2000). When any subunit is removed through mutation, the MSL complex is lost from most sites along the X, dosage compensation fails, and males die. However, in some mutant backgrounds, a partial MSL complex remains at ~35 unusual sites

interspersed along the X, which we have called chromatin entry sites (Kelley *et al.*, 1999). These sites have been postulated to be locations of initial MSL recognition of the X or sites of complex assembly. They are most readily observed in *msl3* or *mof* mutants, but a very similar, if weaker, MSL staining pattern can be seen in *mle* mutants (Palmer *et al.*, 1994; Lyman *et al.*, 1997; Gu *et al.*, 1998). When a chromatin entry site is moved to an autosome, it has the remarkable property of recruiting MSL complexes to the site of insertion, where they can subsequently spread *in cis* over flanking autosomal genes (Kelley *et al.*, 1999).

Only two of the estimated 35 chromatin entry sites have been cloned, and they harbor the genes coding for *roX1* and *roX2*, the RNA components of the MSL complex (Kelley *et al.*, 1999). This finding presented a number of questions. Is the process of transcription of *roX* RNA, or the accumulation of this RNA at the site of transcription a hallmark characterizing a functional entry site? And if so, do other chromosome entry sites also encode additional, non-coding RNA species? Do the MSL proteins capture nascent *roX* transcripts as they are being assembled into MSL complexes or do they recognize a specific DNA sequence within the *roX* genes? And if such a DNA element could be found, is its presence limited to *roX* genes or also shared with other entry sites? We have approached these questions by initiating a detailed molecular analysis of the *roX1* chromatin entry site. We find that the MSL proteins bind a small DNA region near the middle of the *roX1* gene that corresponds to a male-specific DNase I hypersensitive site in chromatin of intact nuclei. No similar DNA sequence could be recognized in the *roX2* gene, suggesting the existence of either a rather degenerate DNA target or DNA elements of variable primary sequence but similar function. This short DNA element is sufficient to initiate epigenetic MSL spreading along the flanking chromatin in the absence of active *roX1* transcription, suggesting that other chromatin entry sites need not necessarily synthesize *roX* RNAs. However, we did find that spreading was more consistent in cases where the transgene was transcribed. Furthermore, we found a critical early role for the MLE helicase in recruiting the other MSL proteins to the *roX1* gene similar to that previously shown for *roX2* (Meller *et al.*, 2000). These observations leave open the possibility that RNA components play an early role in MSL binding, assembly or spreading.

Results

roX1 exons are sufficient for recruitment of the MSL complex

In previous studies we found that a 4.9 kb *roX1* genomic fragment, inserted into autosomal chromatin, can function as a chromatin entry site for MSL complex nucleation and spreading (Kelley *et al.*, 1999). However, it was not known whether the spreading activity might be a property of the *roX1* 5'- and 3'-flanking regions, the exons, or the RNA itself. In addition, it was not known whether sequences required for binding the complex were also sufficient for spreading, or whether separate sites were necessary to initiate spreading from a binding site. As a first test, we analyzed a transgene carrying a 3.4 kb *roX1* cDNA fragment driven by the constitutive activity of the Hsp83

Fig. 1. A cDNA fragment of *roX1* recruits the MSL complex. (A) Diagram of the 3.4 kb *roX1*c20 cDNA driven by the Hsp83 promoter and a deletion derivative lacking a 30 bp segment that shares 25/30 identity with *roX2*. An intron near the 5' end is indicated in each construct. The sequence comparison of *roX1* and *roX2* in the 30 bp region deleted in *roX1*Δ30 is shown below. (B) Polytene chromosomes from a [Hsp83-*roX1*]61B male. (C) Polytene chromosomes from a [Hsp83-*roX1*Δ30]21D male. Chromosome squashes were immunostained with rabbit anti-MSL1 (red) and counterstained with 4',6-diamine-2-phenylindole (DAPI) (blue). Both nuclei show a strong site of MSL localization at the insertion site of the respective transgenes (arrowheads). A heavily stained X chromosome is also seen in (B).

promoter (Figure 1A). In transgenic animals, strong MSL1 and MLE signals were observed on the autosomal transgenes only in males, indicating that *roX1* exons are sufficient for recruitment of the MSL complex (Figure 1B). Since *roX1* and *roX2* cDNAs can both attract the MSL complex *in vivo*, we next assessed the importance of a short region of similarity (25/30 bp identity near their 3' ends, Figure 1A; Franke and Baker, 1999) for attraction of the MSL complex to an autosomal transgene. A Hsp83-*roX1*Δ30 transgene lacking the 30 bp region of similarity showed strong binding, indistinguishable from a full-length cDNA (Figure 1C), while a multimer of this sequence in the absence of the rest of the *roX1* cDNA failed to attract the MSL complex (data not shown). Thus, this short region of sequence similarity between *roX1* and *roX2* is a poor candidate for the MSL target.

De novo synthesis of *roX1* RNA is not essential for recruitment and spreading of the MSL complex

One appealing model for MSL binding to *roX* genes postulates that the *roX* RNAs are incorporated into MSL complexes cotranscriptionally. MSL proteins might only recognize nascent *roX* transcripts, not the underlying DNA. This model makes a simple prediction that MSL binding to autosomal *roX* transgenes should be strictly dependent upon active transcription of *roX* RNA. As is shown in Figure 2, this is not the case. Two transgenes were assayed for MSL binding when inserted on

Fig. 2. Localization of MSL complexes to roX1c3.4 transgenes. MSL1 localization in male nuclei, detected by immunostaining with anti-MSL1 antibodies (red). (A) Expressed antisense construct [Hsp83-roX1AS]87A, (B) [roX1c3.4]66C, from which transcription has not been detected, (C) [roX1c3.4]33DE, (D) [roX1c3.4]51A. The [roX1c3.4] transgenes show MSL1 binding at the insertion sites of the transgenes (B–D, arrows), as well as in neighboring regions (C and D, arrowheads). (E) Northern analysis shows fusion RNA expression of *roX1* from flanking sequences in the [roX1c3.4]33DE and [roX1c3.4]51A lines, in which relatively frequent spreading was also observed. All transgenic lines carry a mutant allele (*roX1^{ex6}*) at their endogenous *roX1* locus. The [roX1c3.4]66C, 52DE and 47C lines do not express detectable *roX1* RNA. The wild-type strain (y w; WT) expresses endogenous *roX1* RNA.

autosomes. The first contained a constitutive Hsp83 promoter, analogous to Hsp83-roX1 (Figure 1), but transcribing the antisense strand (Hsp83-roX1AS) (Figure 2A). The second carried the same nearly full-length *roX1* cDNA without an exogenous promoter (roX1c3.4) (Figure 2B–D). Both of these transgenes provided excellent MSL targets *in vivo*.

We expected that the roX1c3.4 transgene would not be transcribed. However, *Drosophila* P-elements have a

preference for integrating into promoter or regulatory regions and in some cases a neighboring promoter might transcribe into a transgene (O’Kane and Gehring, 1987). Therefore, we performed northern analysis on five lines carrying the transgene at different locations to determine whether any read-through transcription of *roX1* sequences occurred. A significant amount of apparent *roX1* fusion RNA was detected in two lines, but the other three lines failed to make detectable amounts of *roX1* RNA (Figure 2E). The two fusion RNAs were male specific, presumably because *roX* RNAs require the male-specific MSL complex for stability (Meller *et al.*, 2000). RNA for this northern analysis was isolated from adults, while all MSL binding assays were performed on larval salivary glands. Therefore, we extended our analysis by testing roX1c3.4 expression directly in salivary glands by RNA *in situ* hybridization. The results of the larval analysis were similar to those in adults; roX1c3.4 lines 33DE and 51A produced *roX1* RNA that could be detected at a low level on the X chromosome, while the other three lines did not. Consistent with this result, the 33DE and 51A fusion RNAs were transcribed in the same orientation as authentic *roX1* RNA (data not shown).

We examined hundreds of nuclei containing the roX1c3.4 transgene at the five different insertion sites to assay their ability to support spreading of the MSL complex (Figure 2C and D; Table I). MSL spreading was observed in each line, but the fraction of nuclei undergoing spreading varied from 2 up to 57%. The two roX1c3.4 lines exhibiting read-through transcription showed the most consistent spreading of MSL complexes into flanking autosomal chromatin (Table I). We conclude that transcription from the *roX1* transgene is not essential for nucleation and spreading of the MSL complex, but it might be a factor that influences the frequency of spreading. Alternatively, the differences in spreading frequency may reflect poorly understood position effects at each insertion site assayed.

The MLE helicase is essential for MSL complex association with the *roX1* gene

MSL1 and MSL2 are thought to form a core complex that binds to chromatin entry sites, while MLE and other components including *roX* RNAs may be added sequentially (Lucchesi, 1998; Gu *et al.*, 2000; Meller *et al.*, 2000). To assess whether all the MSL components are required to bind to the *roX1* exons, we analyzed MSL1 localization on the roX1c3.4 transgene in *msl* mutant animals. Since *msl* mutant males show poor morphology of polytene chromosomes, we used females carrying a Hsp83-MSL2 transgene. Females normally lack MSL2 and can not form MSL complexes, but when MSL2 is constitutively expressed in females, the complex forms and is localized to both X chromosomes (Kelley *et al.*, 1995). The roX1c3.4 transgene recruits the MSL complex in the absence of functional *mof* (Figure 3A, arrowhead) as well as in cells lacking *msl3* (Figure 3B, arrowhead), as expected for an authentic chromatin entry site. However, in *mle* mutant animals, surprisingly, the transgene did not bind detectable partial complexes (Figure 3C). By careful re-examination, we found that the endogenous *roX1* locus at 3F shows two strong MSL binding sites as a tight doublet in the absence of *msl3*, but shows a faint, single

Table I. Analysis of epigenetic spreading of MSL complexes from roX1c3.4 transgenes

Strain	No. of nuclei observed	No. of nuclei with no signal	No. of nuclei with a single band	No. of nuclei with spreading	Spreading frequency (%) ^a
33DE	288	2	215	71	24.7
47C	285	11	266	8	2.8
51A	140	0	60	80	57.1
52DE	295	0	271	24	8.1
66C	183	0	179	4	2.2

^aPercentage of observed nuclei with spreading.

MSL signal in *mle* mutants (arrows, Figure 3B and C). This suggests that there are two distinct MSL binding sites located at 3F, one site within the *roX1* gene that requires MLE, and an additional site, similar to the majority of entry sites, that can attract partial MSL complexes in the absence of MLE. It should be noted that even at entry sites that attract partial complexes independent of MLE, the strength or stability of binding to those sites is weak (Palmer *et al.*, 1994; Lyman *et al.*, 1997). Since the requirement for MLE is also a characteristic of the endogenous *roX2* locus (at 10C) (Meller *et al.*, 2000), it is possible that this is a common feature of entry sites that encode *roX* RNAs.

Functional *msl3* and *mof* are not required for MSL binding to chromatin entry sites including *roX1*, but in their absence the complex fails to associate with the additional hundreds of sites seen in its wild-type pattern on the X chromosome (Palmer *et al.*, 1994; Gu *et al.*, 1998). This has been interpreted as an inability of the complex to spread between chromatin entry sites in the absence of either MSL3 or MOF. To test this hypothesis at the level of an individual entry site, we examined a strain in which MSL1 showed frequent spreading from a *roX1* transgene (roX1c3.4-51A). We found that lack of functional *msl3* or *mof* prevented spreading, as it does on the endogenous X chromosome (Figure 3A and B). Taken together, the MLE helicase protein is essential for the MSL complex to bind to the *roX1* transgene, while the MSL3 chromo-domain protein and MOF histone acetyltransferase are required only for spreading.

Male-specific DNase I hypersensitivity within the *roX* gene

The polytene X chromosome in *Drosophila* males shows relatively diffuse morphology compared with that of the female, suggesting that the dosage compensation machinery controls chromatin architecture. The interaction of regulatory complexes with chromatin frequently leads to perturbations in chromatin structure that are revealed by the hypersensitivity of associated DNA upon mild digestion of intact nuclei with DNase I. Mapping of DNase I hypersensitive sites allows the identification of regulatory elements, such as transcription enhancers (Nitsch *et al.*, 1990). In order to identify chromatin perturbations caused by the MSL complex we treated nuclei from male and female adult flies with increasing amounts of DNase I and monitored the appearance of hypersensitive sites by indirect end labeling. We found two such sites within *roX1* in the chromatin of male, but not in female flies (Figure 4). One of the sites mapped near

Fig. 3. MSL complex binding at *roX1* transgenes requires MLE. Females carrying [Hsp83-MSL2] and [roX1c3.4]51A in addition to being homozygous mutant for *mof* (A), *msl-3* (B) or *mle* (C). Polytene chromosomes were stained with anti-MSL1 antibodies (red) and DAPI (blue). In each of these mutants the MSL complex associates with the X chromosome only at chromatin entry sites. Arrowheads and arrows represent the *roX1* transgene and the endogenous *roX1* locus at 3F, respectively. Top right inset in each panel shows an enlarged image of anti-MSL1 immunostaining of the endogenous *roX1* locus.

the beginning of the gene and may therefore correspond to its active promoter. The second site mapped to a region ~1000–1250 bp into the *roX1* gene.

A 217 bp *roX1* region is sufficient for recruitment and spreading of the MSL complex

To determine whether the male-specific DNase I hypersensitive site in *roX1* corresponds to a recognition site for

the MSL complex, and to survey the *roX1* gene for additional sites, we performed a transgenic deletion analysis. We produced transgenes with the 3.4 kb *roX1* cDNA divided into various overlapping fragments, roX1a (1.1 kb), roX1b (1.1 kb), roX1c (1.4 kb), roX1.R5' (0.9 kb) and roX1.R3' (2.6 kb) (Figure 5A). Only two of these fragments, roX1a and R3', showed strong MSL1 binding in transgenic animals (Figure 5B and C). These two constructs share a 300 bp overlap, coincident with the DNase hypersensitive site mapped in the endogenous *roX1* gene. When most of this region was deleted from an otherwise full-length transgene (roX1ΔDHS), MSL1 binding to the transgene was significantly reduced (two lines) or undetectable (two lines). To determine which part of the roX1ΔDHS construct was required for the weak MSL binding detected in some lines, we subdivided that construct into its 5' and 3' parts. The 5' portion (R5'+100), which is 100 bp longer than construct R5' at its 3' end, showed a faint signal, similar to roX1ΔDHS. The 3' segment (roX1bc), which has the same 5' end as roX1b but extends to the end of the gene, showed no signal

(Figure 5A and data not shown). Thus, the 300 bp overlap between construct roX1a and construct R3', encompassing the male-specific DNase hypersensitive region, accounts for the major MSL recruiting activity of the *roX1* chromatin entry site. A minor activity detected in roX1ΔDHS is derived from the region immediately 5' to the major activity.

To determine whether the 300 bp region required for MSL complex binding and spreading was also sufficient for chromatin entry site activity, we analyzed transgenes containing 302 bp (roX1-L), 217 bp (roX1-S) or nine tandem repeat copies of a 217 bp DHS fragment (roX1-SM). Each of these transgenes showed discrete MSL recruiting activity (Figure 5D–G, data not shown for roX1-L), but failed to produce transcripts on northern blots (data not shown). The construct with nine tandem copies, roX1-SM, showed an especially striking signal of MSL1 staining, even stronger than that of the full-length 3.4 kb construct. In addition, one of three lines of roX1-SM transgenics showed frequent spreading into flanking chromatin (Table II; Figure 5G). These results clearly indicate that a 217 bp *roX1* fragment carries the predicted activities for a chromatin entry site, being sufficient for recruitment and spreading of the MSL complex.

Fig. 4. DNase I hypersensitivity within the *roX1* locus. DNase I cleavages were mapped within an *EcoRI* fragment containing the *roX1* gene, schematized to the left (open box; +1 indicates the approximate 5' end of the gene). Nuclei from female (left) or male (right) adult flies were treated with increasing concentrations of DNase I. DNA was isolated and digested to completion with *EcoRI*. DNase I cleavages were revealed by Southern blotting using a probe adjacent to an *EcoRI* site within the *opt* gene. Arrows highlight male-specific DNase I hypersensitive sites in *roX1* chromatin. M, DNA size markers (bp).

Chromatin immunoprecipitation confirms preferential association of the MSL complex within the *roX1* gene

We performed chromatin immunoprecipitation experiments in order to physically map the association of the MSL complex with the endogenous *roX1* gene (Figure 6). To facilitate *in vivo* cross-linking, these experiments were conducted in SL2 tissue culture cells, where the MSL complex is known to associate with the X chromosome (Copps *et al.*, 1998; Akhtar *et al.*, 2000; Gu *et al.*, 2000). SL2 cell nuclei were cross-linked with formaldehyde and chromatin was sheared and purified. MSL proteins were immunoprecipitated with specific antibodies and the associated DNA was amplified by ligation-mediated PCR (LM-PCR) (Orlando *et al.*, 1997), labeled by random priming and used as a probe to reveal the presence of *roX1* DNA (Figure 6). For this purpose the *roX1* cDNA was cleaved into smaller fragments by restriction endonucleases (Figure 6A), which were analyzed by Southern blotting and probing with DNA amplified from the chromatin immunoprecipitate (Figure 6B). Control reactions lacking specific antibodies were performed in parallel ('mock IP'). Normalization of signal intensities for each fragment revealed a significant enrichment of some *roX1* fragments by immunoprecipitation with antibodies directed against MSL proteins (plotted in

Table II. Analysis of epigenetic spreading of MSL complexes from roX1-SM transgenes

Strain	No. of nuclei observed	No. of nuclei with no signal	No. of nuclei with a single band	No. of nuclei with spreading	Spreading frequency (%) ^a
35EF	184	25	158	1	0.5
57C	115	16	38	61	53.0
86AB	194	2	186	6	3.1

^aPercentage of observed nuclei with spreading.

Fig. 5. Deletion analysis used to map MSL complex-recruiting activity within *roX1* transgenes. (A) Diagram of the *roX1* fragments tested in transgenic assays. (B–G) Male polytene chromosomes from each transgenic strain were immunostained with anti-MSL1 (red) and counterstained by DAPI (blue). The integration site of each transgene is represented by an arrowhead. Two constructs that share a 300 bp overlap and show strong anti-MSL staining were [roX1a]66B (B) and [roX1.R3']82CD (C). A 217 bp fragment [roX1-S]48AB (D and E) and a 9× tandem repeat of roX1-S [roX1-SM]57C, F and G) also showed MSL1 localization to the transgene insertion site. Spreading from these small *roX1* derivatives is seen in (E) and (G) (arrowheads).

Figure 6C). Complexes were not enriched on autosomal control genes coding for topoisomerase II, *Acfl* and *hsp26* (data not shown). A *HhaI*–*HindIII* digestion was most informative, mapping the dominant site of interaction to a 788 bp fragment containing the male-specific DNase hypersensitive site. Fragmenting the *roX1* cDNA with *XmnI* and *RsaI* further supported the conclusion that MSL

complexes interact with a discrete, DNase I hypersensitive site within *roX1* chromatin in male cells.

Discussion

We recently proposed a model for how the MSL complex might distinguish the male X chromosome from the

Fig. 6. Mapping of *in vivo* interactions of MSL proteins within the *roX1* gene by chromatin immunoprecipitation. (A) Restriction map of *roX1* cDNA. (B) Raw data of a representative experiment. Left panel: SYBR gold stained agarose gel containing various fragments derived from the *roX1* cDNA. The cDNA was excised from pBluescript with *XhoI* and *XbaI* and gel purified (lane 8). This fragment was further digested with *XmnI* (lane 1), *RsaI* (lane 2), *Esp3I-ScaI* (lane 3), *Esp3I-BstEII* (lane 4), *BanII-NsiI* (lane 5), *HhaI-HindIII* (lane 6), *EcoRI-HindIII* (lane 7). Center panel: Southern blot probed with amplified DNA isolated by immunoprecipitation of SL-2 chromatin with anti-MSL2 antibody. Right panel: Southern blot using a DNA probe isolated from a mock immunoprecipitation of SL-2 chromatin (lacking a specific antibody). (C) Summary of one representative experiment. Each column shows the normalized hybridization profile obtained for the chromatin immunoprecipitation of the protein indicated above the column. Each row shows the data derived from one *roX1* digest. Row 1, *XmnI* (see lane 1, B); row 2, *RsaI* (see lane 2, B); row 3, *HhaI-HindIII* (see lane 6, B). The y-axes of the diagrams show 1/100th of the normalized PhosphorImager counts. Note the scale changes between columns. DHS, position of the DNase I hypersensitive site as mapped in Figure 4. Asterisks indicate two fragments that could not be separated on the gel. The value indicated assumes that both fragments contribute the same signal, which is not necessarily the case.

autosomes. Chromatin entry sites distributed along the X play a critical role in that model as the nucleation points for spreading of the MSL complex into flanking genes. In this report, we define one chromatin entry site within a 217 bp fragment near the middle of the *roX1* gene. This region binds MSL complexes tightly *in vivo*, as shown by cytological analysis of transgenic animals and by immuno-

precipitation of *roX1* DNA cross-linked to MSL proteins. This MSL binding site overlaps a strong male-specific DNase hypersensitive site within *roX1*, suggesting that the MSL complex either induces a chromatin conformational change or recognizes a pre-existing open architecture that is male specific. Not only does this fragment recruit MSL complex to ectopic autosomal sites, but it also supports

variegated spreading into flanking genes that were never before targets of dosage compensation.

These results resolve a number of questions posed by our earlier model. First, it was unclear whether the MSL proteins were recruited to chromatin entry sites by binding DNA sequences or nascent *roX* transcripts. Here we show that MSL proteins bind autosomal transgenes carrying even small fragments of *roX1* in the absence of detectable transcription *in cis* (Figure 5 and data not shown). The realization that chromatin entry sites need not produce RNA to function means that the other ~35 chromatin entry sites may not necessarily encode additional species of *roX* RNAs. They may function as small DNA targets that recruit mature MSL complexes to initiate local MSL spreading. The presence of only a few distinct *roX* RNAs is consistent with the report of Franke and Baker (1999) that MSL complexes form in the presence of just *roX1* or *roX2* RNA, but not if both are absent.

The idea that the *roX1* and *roX2* genes may be atypical chromatin entry sites, in that they produce RNA components of the MSL complex, is consistent with our results using *mle* mutants. Partial MSL complexes are readily detectable at all ~35 chromatin entry sites when either MSL3 or MOF is removed by mutation. In contrast, when the MLE helicase is mutated, partial MSL complexes can be found weakly bound to most chromatin entry sites. However, the 10C site that contains the *roX2* gene and is the single brightest staining site in a *msl3* mutant, is almost undetectable in a *mle* mutant (Lyman *et al.*, 1997; Meller *et al.*, 2000). We now find that the same is true for *roX1*. This was previously overlooked because the 3F band is a composite of two or more very tightly linked entry sites, one due to *roX1* and at least one other that has not been characterized. When we separated the *roX1* chromatin entry site from its neighbors by moving it to an autosome, we found that MLE was also essential for any partial MSL complex to bind.

These results present a paradox. If production of RNA is not required from the *roX1* chromatin entry site, what would be the function of MLE in entry site recognition? MLE possesses not only RNA–RNA but RNA–DNA and DNA–DNA helicase activities *in vitro*, so it might somehow interact with DNA to stabilize weak interactions of MSL1 and 2 (Lee *et al.*, 1997). Although MLE alone clearly does not have X chromosome specificity (Kuroda *et al.*, 1991; Richter *et al.*, 1996), it might contribute directly to the ability of MSL1 and 2 to associate with DNA. Alternatively, the critical role for MLE may be to incorporate *roX2* RNA into MSL complexes, and *roX2* may be essential to establish an MSL complex conformation necessary to recognize the *roX1* chromatin entry site (Meller *et al.*, 2000).

We propose a model for assembly of MSL complexes at two different classes of chromatin entry sites (Figure 7). MSL1 and 2 promote weak interactions with most chromatin entry sites. However, the two entry sites known to encode RNA components of the MSL complex, *roX1* and *roX2*, additionally require the MLE helicase early, perhaps to package *roX* RNA into growing complexes. Partial complexes containing at least MSL1, MSL2, MLE and perhaps a *roX* RNA molecule bind efficiently to all chromatin entry sites. Following addition of the remaining subunits, enzymatically active complexes

Fig. 7. Model for MSL complex assembly and spreading on the X chromosome. (A) MSL1 and MSL2 show a weak interaction with most chromatin entry sites in the absence of MLE, but *roX1* and *roX2* are particularly poor targets under these conditions. (B) When MLE is present, *roX2* RNA can be incorporated into complexes, potentially altering the conformation to allow strong binding to chromatin entry sites. (C) When complete complexes are present, containing at least five MSL proteins and one or more *roX* RNAs, binding and spreading occur in a banded pattern along the length of the X chromosome.

are able to spread into flanking chromatin to dosage compensate target genes. The discrete assembly steps presented in this model reflect the points where various mutants are arrested. In wild-type males, assembly may proceed in a more concerted fashion, perhaps with *roX* RNAs being incorporated into partial MSL complexes prior to any interactions with the X chromosome.

Materials and methods

Plasmid DNA construction

To construct Hsp83–*roX1* and Hsp83–*roX1AS*, a 3.4 kb *EcoRI* fragment containing *roX1* cDNA clone c20 (Meller *et al.*, 1997; DDBJ/EMBL/

GenBank accession No. AB051842) was subcloned in both orientations into a derivative of pCaSpeR-Hsp83 (Horabin and Schedl, 1993) containing a 470 bp *Pst*I fragment from the *tra-2* gene to provide a 3' poly-A site (W.Mattox, personal communication). The same *roX1* fragment was subcloned into pCaSpeR-3, and designated roX1c3.4. For the R5' and R3' constructs, 0.9 and 2.6 kb *Eco*RI fragments of *roX1* cDNA clone c3 (Meller *et al.*, 1997) were cloned separately into pCaSpeR (Pirrotta *et al.*, 1988).

To construct Hsp83-roX1Δ30, a pair of PCRs were performed, using *roX1* c20 as a template. Primer 13A (5'-GCAAGTAAATGCAAACTATAAAAAGAAAATTGAAATC-3') and M13 reverse primer (5'-CAGGAAACAGCTATGAC-3') were used in one reaction, while primer 7A' (5'-ACCAGCAGTTGATTTGCG-3') and primer 13B (5'-ATAGTTTGCAATTTTACTTGCTTTGGC-3') in the other. The resulting two fragments were denatured, re-annealed and amplified in a second PCR using primer 7A' and the M13 reverse primer. The amplified fragment was digested by *Hind*III and substituted for a 1.2 kb *Hind*III fragment in pBlueScriptII-roX1c20. A 30 bp deletion of the *roX1/roX2* homology region (position 3308–3337) was checked by sequencing, digested by *Eco*RI (3.4 kb) and subcloned into pCaSpeR-H83 in the correct orientation.

To construct roX1a, roX1b, roX1c, R5'+100 and roX1bc, fragments of *roX1* cDNA c20 (positions 1–1121, 1096–2175, 2041–3390, 1–892 and 1096–3390, respectively) were amplified with additional *Nhe*I sites introduced at both ends and cloned into pCR-Blunt II TOPO (Invitrogen), followed by sequencing. Clones with the correct sequence were digested with *Nhe*I and subcloned into the *Xba*I site of pCaSpeR.

For the roX1ΔDHS construct, the pBlueScript-roX1c20 clone was amplified by using primer 4A (5'-TAGTATGCTAGCACAGACTGCAAAAGCAGC-3') and 4B (5'-ATATTCGCTAGCCGTCTTCTCGAAACGCAAG-3'), digested by *Nhe*I and self-ligated. A resulting plasmid had a 350 bp deletion (positions 868–1202) adjacent to an introduced 6 bp *Nhe*I site. The insert was digested by *Eco*RI and cloned into pCaSpeR.

For roX1-L and S constructs, *roX1* fragments from the *roX1* c3 cDNA clone (1021–1237 and 980–1281, respectively) were PCR amplified and cloned into a pUC19 variant *Ava*I site. To make the 9× multimer of roX1-S (roX1-SM), the roX1-S fragment was multimerized by ligation and cloned into a pUC19 variant containing an *Ava*I site. After digestion by *Eco*RI and *Hind*III, those fragments were subcloned into *Eco*RI-*Not*I cut pCaSpeR-3 (Pirrotta *et al.*, 1988) using a *Hind*III-*Not*I adapter.

Transformation

Transgenic flies are produced by P-element-mediated transformation (Spradling and Rubin, 1982) and, if needed, remobilized from original lines by transposase derived from P[ry⁺, Δ2-3]99B (Robertson *et al.*, 1988).

Immunostaining of polytene chromosomes

Transgenic flies were grown at 23°C on standard cornmeal-molasses fly media. Preparation of polytene chromosomes and immunostaining using anti-MSL antibodies were as previously described (Kelley *et al.*, 1999).

Genetic crosses

msl mutant females carrying [Hsp83-MSL2] and a [roX1c3.4] transgene were generated by genetic crosses. To generate *mof* mutant larvae, *w cv mof*¹; [Hsp83-MSL1][Hsp83-MSL2] females were crossed to *w cv mof*¹; [*mof*^{16.8}]18H1/[roX1c3.4]51A males. The [*mof*^{16.8}]18H1 transgene includes 6.88 kb of the *mof* genomic region and is able to rescue *mof*¹ mutant males (Hilfiker *et al.*, 1997). Most female larvae carrying the paternal [*mof*^{16.8}]18H1 transgene died or showed delayed development at late third-instar larval stage, due to ectopic expression of MSL1 and MSL2. *mof* female larvae, *w cv mof*¹; [roX1c3.4]51A/+; [Hsp83-MSL1][Hsp83-MSL2]/+, were also distinguished by partial staining of the X chromosome by anti-MSL1 antibodies, due to the absence of the [*mof*^{16.8}]18H1 transgene. To generate *msl-3* mutant larvae, *y w; msl-3* [Hsp83-MSL2] females were crossed to *y w; [roX1c3.4]51A; msl-3/TM6C, Sb Tb* males. *msl-3* female larvae, *y w; [roX1c3.4]51A/+; msl-3* [Hsp83-MSL2]/*msl-3*, were distinguished by absence of the *Tb* phenotype. To generate *mle* mutant larvae, *y w; pr mle*¹; [Hsp83-MSL2] females were crossed to *y w; pr mle*¹ [roX1c3.4]51A/CyO, *y⁺ bw* males. *y w; pr mle*¹ [roX1c3.4]51A/*pr mle*¹; [Hsp83-MSL2]/+ female larvae were distinguished by the brown color of their mouthhooks.

Northern analysis

RNA extraction was performed using the Trizol™ Reagent (Gibco-BRL) according to the supplier's instruction. Twenty micrograms of total RNA

were applied in each lane for electrophoresis, then transferred to Hybond-N⁺ (Amersham) and hybridized (Church and Gilbert, 1984). For probe preparation, *roX1* cDNA c20 fragments (nt 271–587 or 909–1144) were amplified by PCR and used as templates for random priming.

Mapping of DNase I hypersensitivity

Adult flies were sorted manually according to sex. Two grams of flies were frozen in liquid nitrogen and squashed using mortar and pestle. The powder was suspended in 10 ml of buffer A [60 mM KCl, 15 mM NaCl, 13 mM EDTA, 0.1 mM EGTA, 15 mM Tris pH 7.4, 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM dithiothreitol (DTT)] containing 0.5% NP-40 (buffer ANP). The suspension was homogenized five times in a motor-driven homogenizer (Yamato), dounced 20 times with a tight pestle and filtered through Miracloth (Calbiochem). The filtrate was layered on top of 4 ml of buffer A containing 0.3 M sucrose (buffer AS) and centrifuged for 5 min at 3000 r.p.m. in an HB4 rotor (4°C). The pellet was resuspended in 10 ml of buffer ANP, dounced 10 times with a loose pestle and centrifuged again through 4 ml of buffer AS. The pellet was washed twice in 10 ml of buffer A and once in buffer B (60 mM KCl, 15 mM NaCl, 5 mM MgCl₂, 0.1 mM EGTA, 15 mM Tris pH 7.4, 1 mM DTT, 0.3 M sucrose) and finally resuspended in 2 ml of buffer B. The suspension was split into 12 aliquots for DNase I digestion. The nuclei were digested with 0.2–20.5 U of DNase I diluted in buffer B containing 5 mM CaCl₂ and 10 mM MgCl₂ for 3 min at 25°C, and the reaction stopped by addition of 1/7th volume of 4% SDS, 0.1 M EDTA. DNA was prepared by successive proteinase K and RNase A treatments, phenol-chloroform extraction and ethanol precipitation. Ten micrograms of DNA of each DNase I digest were digested to completion with *Eco*RI and separated on a 0.8% agarose gel. The DNA was blotted and hybridized as described (Church and Gilbert, 1984). The RNA probe was prepared by *in vitro* transcription of a 443 bp restriction fragment abutting an *Eco*RI cleavage site 1053 bp upstream of the *roX1* transcription start site.

Chromatin immunoprecipitation

Chromatin immunoprecipitations were carried out as described previously (Orlando *et al.*, 1997) with modifications. Approximately 2 × 10⁹ *Drosophila* SL2 cells were fixed in 1.1% formaldehyde (Calbiochem) and sonicated to release soluble chromatin with a modal length of 1 kb. Chromatin was purified by CsCl gradient ultracentrifugation in a Beckman SW60Ti rotor at 38 kr.p.m. for 40–44 h. Fractionated gradients were dialysed overnight against 1 l of dialysis buffer (5% glycerol, 10 mM Tris pH 8, 1 mM EDTA, 0.5 mM EGTA) using a 28-well microdialysis system (Life Technologies). Chromatin-containing fractions were identified by reversing the cross-links of 50 μl samples, and pooled. The 0.5 ml aliquots were transferred into RIPA buffer (1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) and 2–5 μg of antibodies were added (2 μl anti-MSL1, 2 μl anti-MSL2, 0.5 μl anti-MSL3, 5 μl anti-MOF or 5 μl pre-immune sera for mock immunoprecipitations) and incubated overnight at 4°C. Immunocomplexes were purified using 40 μl of 50% slurry of protein G (MSL3) or protein A (other antibodies) Sepharose. Complexes were washed five times in 1 ml of RIPA buffer, once in 1 ml of LiCl buffer (250 mM LiCl, 10 mM Tris pH 8.0, 1 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate) and once in 1 ml of 10 mM Tris pH 8, 1 mM EDTA before reversing cross-links. Purified immunoprecipitated DNA was resuspended in 22 μl of H₂O and overhanging ends created by sonication were extended using *Pfu* DNA polymerase. The DNA was then amplified by ligation-mediated PCR (LM-PCR) to produce sufficient amounts for labeling and use as a probe in Southern blotting. The linker for LM-PCR was created by the annealing of two oligonucleotides (AGAAGC-TTGAATTCGAGCAGTCAGp and CTGCTGGAATTCAAGCTTCT), the latter of which was also used for PCR amplification.

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