

RESEARCH COMMUNICATION

Local spreading of MSL complexes from *roX* genes on the *Drosophila* X chromosome

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MSL proteins and noncoding *roX* RNAs form complexes to up-regulate hundreds of genes on the *Drosophila* male X chromosome, and make X-linked gene expression equal in males and females. Altering the ratio of MSL proteins to *roX* RNA dramatically changes X-chromosome morphology. In protein excess, the MSL complex concentrates near sites of *roX* transcription and is depleted elsewhere. These results support a model for distribution of MSL complexes, in which local spreading in *cis* from *roX* genes is balanced with diffusion of soluble complexes in *trans*. When overexpressed, MSL proteins can recognize the X chromosome, modify histones, and partially restore male viability even in the absence of *roX* RNAs. Thus, the protein components can carry out all essential functions of dosage compensation, but *roX* RNAs facilitate the correct targeting of MSL complexes, in part by nucleation of spreading from their sites of synthesis.

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The establishment and maintenance of chromatin organization by histone modification and chromatin-remodeling complexes has been postulated to occur in some cases by initial recognition of a nucleation site, followed by spreading in *cis* into flanking sequences (Lee and Jaenisch 1997; Bannister et al. 2001; Lachner et al. 2001; Nakayama et al. 2001; Ho et al. 2002). In dosage compensation of the *Drosophila* male X chromosome, MSL (male-specific lethal) proteins and *roX* (RNA on X) RNAs form large complexes that modify histone tails and can spread long distances from initiation sites into flanking chromatin (Kelley et al. 1999; Meller et al. 2000; Park et al. 2002). The two known *roX* genes are located on the X chromosome (Amrein and Axel 1997; Meller et al. 1997) and are thought to have dual functions. First, *roX* RNAs are components of the MSL complex (Meller et al. 2000;

Smith et al. 2000). The MSL proteins are unable to bind the X chromosome efficiently without *roX* RNA, resulting in male lethality (Franke and Baker 1999; Meller and Rattner 2002). Second, *roX* transgenes can function as nucleation sites for ectopic targeting and spreading of MSL complexes into flanking autosomes regardless of location (Kelley et al. 1999). We have inferred that similar spreading around the endogenous *roX* genes could contribute to targeting the MSL complex to the correct chromosome.

Ectopic spreading of MSL complexes on autosomes is profoundly influenced by the number of *roX* genes in the nucleus (Park et al. 2002). When both endogenous *roX* genes are deleted so that a single *roX* autosomal transgene is the sole source of *roX* RNA, consistent spreading of the MSL complex occurs for long distances (several megabase pairs) into flanking chromatin. However, when several *roX* genes compete for a limiting amount of MSL proteins, ectopic spreading from autosomal *roX* transgenes is very rare, and instead all MSL complexes diffuse to the X chromosome. A model based on these observations proposes that there are two pools of MSL complexes. If MSL proteins are abundant and rapidly assemble onto growing *roX* transcripts, functional complexes will be completed before release of the nascent *roX* transcript from the DNA template. These complexes are postulated to immediately bind the flanking chromosome regardless of sequence and begin spreading in *cis* (Fig. 1A). When multiple *roX* genes are present, they compete for a finite supply of MSL proteins, thus slowing the assembly of complete complexes. Under these conditions, nascent *roX* RNA will be released from the template with an incomplete set of MSL subunits. After maturation is completed in solution, these complexes are postulated to diffuse through the nucleus until encountering the X chromosome (Fig. 1B). How such soluble MSL complexes recognize and bind the X chromosome is not understood. We initially postulated that, besides the two *roX* genes, there were ~35 additional "chromatin entry sites" that would also initiate MSL spreading (Kelley et al. 1999). However, the nature of these sites remains a mystery.

To date, all evidence for *cis* spreading comes from autosomal *roX* transgenes. Here, we demonstrate that MSL complexes do spread locally from the endogenous *roX* genes on the X chromosome, the natural target of dosage compensation. We find that wild-type males require a balance of MSL proteins and *roX* RNAs to evenly distribute MSL complexes both locally and at a distance along the X chromosome. When we artificially increase the amounts of MSL1 and MSL2, thought to be the limiting proteins (Kelley et al. 1997; Chang and Kuroda 1998; Park et al. 2002), MSL complexes spread predominantly over a local segment of the X chromosome surrounding a *roX* gene. More remote regions bind little MSL complex. This dramatically alters the morphology of polytene X chromosomes. Surprisingly, we found that overexpressing MSL1 and MSL2 partially restored viability to males lacking *roX* RNA. This indicates that the MSL proteins have intrinsic affinity for the X chromosome that is enhanced or stabilized in wild-type males by the *roX* RNAs.

[**Keywords:** *Drosophila* dosage compensation; noncoding *roX* RNAs; MSL proteins]

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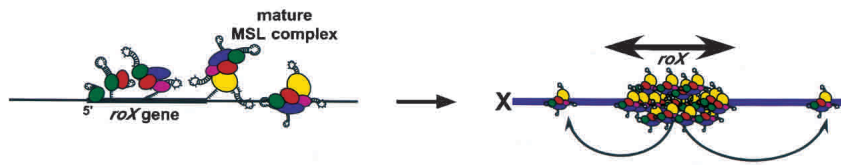
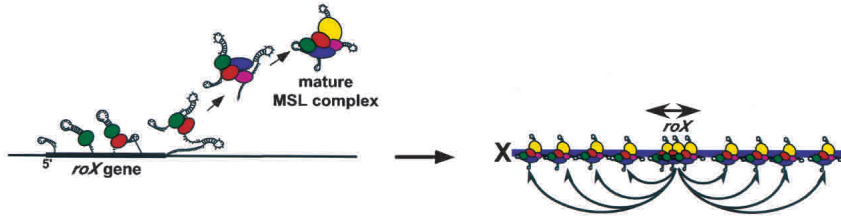
A High ratio of MSL proteins / *roX*RNA (*cis* >> *trans*)**B Normal ratio of MSL proteins / *roX*RNA**

Figure 1. Model for *cis*-versus-*trans* interaction of MSL complexes with the X chromosome. (A) If the local concentration of MSL proteins is high at the site of *roX* transcription, a complete set of MSL proteins will be assembled efficiently on the nascent *roX* RNA, leading to local MSL spreading in *cis*. If the rate of MSL protein assembly is accelerated by overexpressing MSL1 and MSL2, extensive *cis* spreading of MSL complexes will predominate and *trans* interactions will decrease. (B) Conversely, if the local concentration of MSL proteins is low, only partial MSL complexes will be assembled on the nascent *roX* RNA prior to release from the DNA template. Released complexes can finish assembly in the nucleoplasm, but are unlikely to return to the site of *roX* synthesis. In normal conditions, the rate of *roX* RNA transcription and the rate of MSL protein assembly on nascent *roX* RNA are optimized, resulting in balanced *cis* and *trans* interactions of MSL complexes with the X chromosome.

Results and Discussion*Spreading of MSL complexes from roX genes on the X chromosome*

There are two known *roX* genes located on the X chromosome; *roX1* at 3F near the telomere and *roX2* at 10C in the middle of the chromosome (Fig. 2A). The even distribution of MSL complexes in a banded, reproducible pattern in single *roX* mutants, with no preference for the regions surrounding 3F or 10C, does not suggest regional spreading (Meller et al. 1997; Kelley et al. 1999; Meller and Rattner 2002). Our recent finding, that the ratio of MSL proteins to *roX* RNAs affects whether or not MSL complexes spread in *cis* near autosomal *roX* transgenes (Park et al. 2002), prompted us to ask if increasing the levels of MSL proteins would alter balanced targeting of the X chromosome. We used the previously characterized Hsp83 promoter-driven transgenes, [H83M1] (previously known as M1-ECTOPIC) and [H83M2] (Chang and Kuroda 1998) to overexpress the *msl1* and *msl2* genes in *roX1*[−] or *roX2*[−] single mutants. Under these conditions, we found that the *roX* genotype greatly affected X-chromosome morphology. In *roX1*[−] larvae, MSL localization to the X chromosome was concentrated over the middle region of the X chromosome surrounding the *roX2*⁺ gene (Fig. 2B). In contrast, in *roX2*[−] larvae, MSL localization was extensive around the tip of the X chromosome around the *roX1*⁺ gene (Fig. 2C). More distant regions of the X chromosome were deficient for MSL complexes. The MSL-faint regions of the X chromosome displayed a collapsed, narrow morphology reminiscent of that seen in *mle* or *roX1*[−] *roX2*[−] mutants (Belote and Lucchesi 1980; Meller and Rattner 2002). MSL-bright regions surrounding either *roX*⁺ gene showed very diffuse DAPI

staining, reminiscent of chromosome puffing. Although chromosome morphology varied from nucleus to nucleus within the same individual, ~20%–30% of the nuclei showed local alteration of morphology around *roX* genes. These results strongly suggest that spreading really occurs from the *roX1* and *roX2* genes in their native locations, and can be exaggerated by overexpression of MSL proteins. The width of polytene chromosomes is often correlated with transcriptional activity (Offermann 1936; Dobzhansky 1957). The gross alteration in X-chromosome morphology seen in these males suggests that affected regions might be overexpressed and depleted regions underexpressed. This could lead to reduced male viability (Table 1).

To determine whether increased spreading would be evident when both *roX* genes were functional, we overexpressed MSL1 and MSL2 in otherwise wild-type males. These males displayed short, wide X chromosomes that stained strongly with MSL antibodies but showed faint, diffuse staining with DAPI (Fig. 2D).

This morphology suggests overall enhanced MSL binding and function on the X chromosome that could be caused by extensive spreading from both *roX* loci. This is somewhat similar to the phenotype of *iswi* or *nurf301* mutants (Deuring et al. 2000; Badenhorst et al. 2002), raising the strong possibility that it reflects aberrant chromatin organization. Although male viability was decreased by overexpression of MSL1 and MSL2 (Table 1), both male and female flies can tolerate surprisingly large perturbations in MSL localization patterns on the X chromosome (Kelley et al. 1997, 1999).

To test our assumption that the *roX* genes are the precise sites of initiation of MSL spreading, we moved the *roX1*⁺ or *roX2*⁺ genes to new locations along the *roX* X chromosome. In each of 10 sites tested, additional MSL1 and MSL2 protein produced bright MSL staining and diffuse chromosome morphology surrounding the relocated *roX*⁺ gene (Fig. 2E,F; data not shown). Again, more distant regions of the X chromosome had much less MSL staining. Because a strong bias for local MSL spreading was seen at each segment of the X chromosome tested, the sequences surrounding the endogenous *roX* genes are unlikely to contain special spreading elements. Moreover, some *roX* transgenes displayed a strong preference for regional spreading from their site of insertion with only wild-type levels of MSL1 and MSL2 (Fig. 2G). This indicates that our conditions of MSL1 and MSL2 overexpression were not extreme, and that changes in the MSL protein:*roX* RNA ratio or chromatin environment can produce large shifts in the pattern of local MSL spreading.

To determine whether overexpression of MSL1 and MSL2 affected the steady-state levels of *roX* RNAs, we performed Northern analysis (Fig. 2H). Compared with normal males (Fig. 2H, lanes 1,3,5), the levels of *roX* RNAs were increased when MSL1 and MSL2 were over-

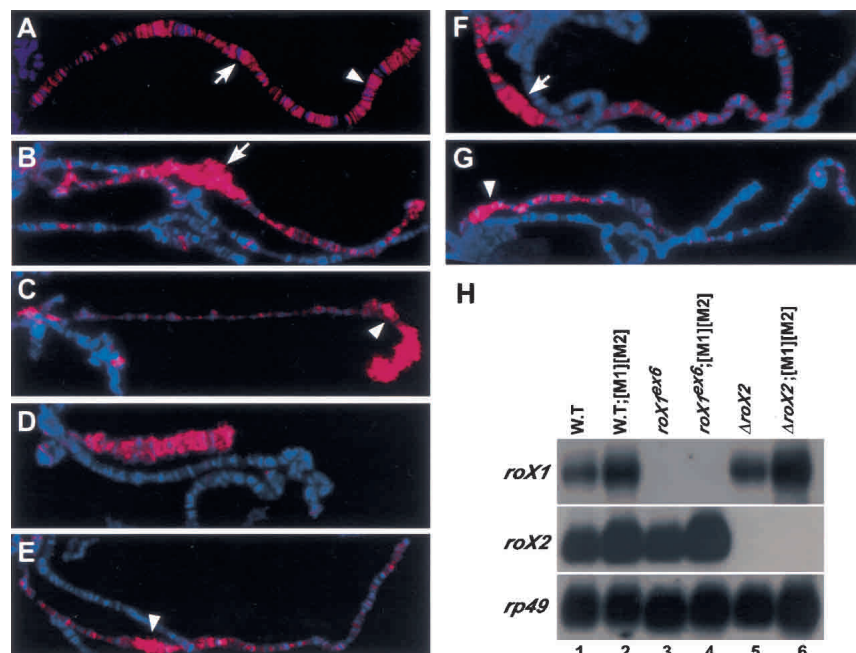


Figure 2. (A–G) Local *cis* spreading of MSL complexes on the X chromosome. Male polytene chromosomes were stained with anti-MSL1 antibodies and visualized with Texas Red-conjugated secondary antibody (red). DNA was stained with DAPI (blue). Arrowheads and arrows indicate the locations of the *roX1* and *roX2* genes, respectively. The genotypes are wild type (A), *roX1*^{ex6}; [M1][M2] (B), *Df(1)roX2*⁵²; [M1][M2] (C), wild type; [M1][M2] (D), *roX1*^{ex6} *Df(1)roX2*⁵² [w⁺ GMroX1-13A]; [M1][M2] (E), *roX1*^{ex6} *Df(1)roX2*⁵² [w⁺ GMroX2-14F]; [M1][M2] (F), *roX1*^{ex6} *Df(1)roX2*⁵² [w⁺ GMroX1-18C] without extra MSL1 and MSL2 (G). [M1][M2] stands for [w⁺ H83M1][w⁺ H83M2]/+ on the third chromosome. Full genotypes of crosses are listed in Materials and Methods. (H) Northern blot showing that the steady-state level of *roX* RNA is increased with overexpression of MSL1 and MSL2. The same membrane was hybridized to an *rp49* probe as a loading control.

expressed (Fig. 2H, lanes 2,4,6). Under these conditions, the local assembly of MSL complexes might be amplified through a positive feedback loop. For example, the transcription of *roX* genes might be increased by the high concentration of MSL complexes, or the accelerated rate of MSL protein assembly with nascent *roX* RNA could lead to increased stabilization of the RNA that might otherwise be degraded. Thus, the higher density of MSL complexes around the *roX* genes (Fig. 2B–G) probably results from two factors. There is an overall increase in MSL complexes, and complexes normally destined for remote parts of the X chromosome instead remain local. This shows that the wild-type male X chromosome is not saturated with MSL complex, but rather that the distribution must be under active control.

Overexpressed MSL1 and MSL2 can partially overcome a lack of *roX* RNAs

Males lacking both *roX1* and *roX2* have only small amounts of MSL proteins bound to a subset of sites on the X chromosome (Meller and Rattner 2002). Depending on poorly understood differences in genetic background, ~95%–99.9% of such males die before adulthood. Surprisingly, a small number of adult males somehow survive without any detectable *roX* RNA (Meller and Rattner 2002; Stuckenholtz 2002). Larval polytene chromosomes from such animals display very poor morphology, with ectopic MSL binding to the chromocenter

and some autosomal sites (Fig. 3A; Meller and Rattner 2002). For comparison, mutants for each of the five known *msl* genes show a complete male lethal phenotype with no escapers. Because wild-type and single *roX* mutant males each had altered X-chromosome morphology when MSL1 and MSL2 were overexpressed, we asked whether *roX1*[−] *roX2*[−] double-mutant X chromosomes would also be affected by these conditions. We found that overexpression of MSL1 and MSL2 improved the morphology of all polytene chromosomes in *roX*[−] males. In addition, the X chromosome displayed much more robust binding of MSL1 (Fig. 3B) and MSL2 (data not shown) than brothers with only wild-type levels of MSL proteins (Fig. 3A). This indicates that the MSL proteins have an intrinsic ability to recognize the X chromosome. At normal MSL levels, this binding is weak but greatly enhanced by *roX* RNA. When MSL proteins are artificially overproduced, however, MSL binding to the X chromosome is at least partially RNA-independent.

Given their greatly improved X-chromosome morphology and MSL localization, we examined *roX1*[−] *roX2*[−]; [H83M1][H83M2]/+ males for viability (Table 1). We crossed *roX* mutant females from three genetically different backgrounds to male flies overexpressing MSL1 and MSL2.

Progeny from all three lines showed enhanced male viability (up to 90-fold improvement) with overexpression of MSL1 and MSL2. This suggests that the protein components of the MSL complex mediate the chromatin changes during dosage compensation. Normally the *roX* RNAs play a crucial role in assembly or targeting of complexes, which can be partially recapitulated by MSL overexpression.

Screening for additional noncoding RNA component(s) of MSL complexes

One qualification of our results is that the *roX1*[−] *roX2*[−] double-mutant stocks might not be truly null for all *roX* RNAs. Although the *roX2* allele is a full deletion, the *roX1*^{ex6} allele removes only the 5' half of the gene, but retains the last ~2.3 kb of the locus (Kelley et al. 1999). If a truncated *roX1* transcript were incorporated into MSL complexes, we would expect it to accumulate to detectable levels. However, using Northern analysis (Fig. 4A) or reverse transcriptase PCR (RT-PCR; Fig. 4B), we were unable to detect any *roX1* RNA in *roX*[−] escaper males carrying the *roX1*^{ex6} allele. Coupled with the observation that the *roX1*^{ex6} allele showed no evidence of MSL attraction and spreading in our polytene chromosome assay (Fig. 2B,E–G), these results suggest that the *roX1*^{ex6} allele is nonfunctional.

We next asked whether additional species of *roX* RNA from unidentified genes might have gone undetected. We

Table 1. Effect of overexpression of MSL proteins on male viability of *roX* mutants

Cross	<i>roX</i> genotype ^a	% viability of progeny overexpressing MSL1 and MSL2			
		Female	Female; [M1][M2]/+	Male	Male; [M1][M2]/+
1	WT	100 ^b (1181) ^c	0.9 (10)	56.5 (667)	31.3 (370)
2	<i>roX1</i> ⁻	100 (1364)	0.4 (6)	63.4 (865)	34.2 (466)
3	<i>roX2</i> ⁻	100 (646)	0.5 (3)	86.5 (559)	75.5 (488)
4	<i>roX1</i> ⁻ <i>roX2</i> ⁻	100 (1286)	11.1 (143) ^d	0.1 (1)	7.2 (93)
5	<i>roX1</i> ⁻ <i>roX2</i> ⁻	100 (1185)	9.3 (110) ^d	0.9 (11)	25.4 (301)
6	<i>roX1</i> ⁻ <i>roX2</i> ⁻	100 (2239)	16.0 (358) ^d	6.6 (147)	44.3 (992)

Full genotypes of crosses:

1. *y w × w/Y; msl2 cn; [M1][M2]/+*
2. *y w roX1^{ex6} × w/Y; msl2 cn; [M1][M2]/+*
3. *w Df(1)roX2⁵²; [w⁺ 4Δ4.3] × w/Y; msl2 cn; [M1][M2]/+*
4. *y w roX1^{ex6} Df(1)roX2⁵² [w⁺ 4Δ4.3] × w/Y; msl2 cn; [M1][M2]/+*
5. *y w roX1^{ex6} Df(1)roX2⁵²; [w⁺ 4Δ4.3] × w/Y; msl2 cn; [M1][M2]/+*
6. *roX1^{ex6} Df(1)roX2⁵²; [w⁺ 4Δ4.3] × w/Y; msl2 cn/+; [M1][M2]/TM3 Sb*

The number of XO males and XXY females is not included.

^aThis indicates the male X genotype; females are heterozygous, as they also carry one wild-type X chromosome.

^bNumbers represent the percent of expected when compared with control females that do not overexpress MSL1 and MSL2.

^cNumbers in parentheses show actual number of progeny.

^dCompared with the survival of wild-type females overexpressing MSL1 and MSL2, these females show enhanced viability, possibly because they have one wild-type and one *roX* mutant X chromosome.

immunoprecipitated MSL complexes from wild-type embryos, recovered RNA, prepared cDNA, performed subtractive hybridization using excess female cDNA to deplete nonspecific contaminants, and cloned the enriched products. Out of 15 randomly picked colonies, we found that the resulting small cDNA library contained ~70% *roX1* and *roX2* subclones (Supplementary Table 1A). To increase the chance of discovering new *roX* RNA(s), colonies containing *roX1* or *roX2* were excluded by colony hybridization with *roX1* and *roX2* probes. Another 31 clones were sequenced, but all represented non-specific abundant RNAs (Supplementary Table 1B). Thus, we found no candidates for additional *roX* species. We conclude that if the MSL complex contains other RNA species, they have very different characteristics from *roX1* and *roX2*. The fact that the MSLs spread from only the *roX2*⁺ locus in a *roX1*⁻ mutant (Fig. 2B), or the *roX1*⁺ locus in a *roX2*⁻ background (Fig. 2C), also strongly suggests that there are no additional RNAs functioning like *roX1* and *roX2*.

Colocalization of MSL proteins to the X chromosome without *roX* RNA

We further investigated how loss of *roX* RNA affected MSL complexes in males overexpressing MSL1 and MSL2. Polytene chromosomes were stained with antibodies to each of the five MSL proteins and histone H4 acetylated at Lys 16. We found that MSL1, MSL2, MSL3, MOF, and H4Ac16 were colocalized to the X chromosome under these conditions (Fig. 3B–D; data not shown), showing substantial rescue of MSL localization to the X chromosome in the *roX*⁻ mutant. The restoration of the precise MSL pattern was incomplete, as MSL proteins were still present at an increased number of autosomal sites and the heterochromatic chromocenter, as is seen in *roX*⁻ mutants with wild-type levels of MSL proteins (Fig. 3A). Surprisingly, localization of the MLE helicase differed significantly from the other MSL pro-

teins. Although MLE binds the X chromosome robustly in wild-type nuclei (Fig. 3E), it shows only very faint staining on the X chromosome in the absence of *roX* RNAs. However, it is still colocalized to some autosomal sites and the chromocenter with the other MSL proteins (Fig. 3F–H). These results demonstrate that stable localization of MLE to the male X chromosome is strongly dependent on *roX* RNAs, and is consistent with previous results showing that association of MLE with the X chromosome is particularly RNase-sensitive (Richter et al. 1996). Earlier work demonstrated the reciprocal result: *roX* RNA cannot bind the X chromosome without MLE helicase (Meller et al. 2000). We do not understand what attracts MLE to the ectopic autosomal sites and chromocenter, but note that MLE has an RNase-sensitive affinity for all chromosomes when overexpressed (Richter et al. 1996).

Given that males cannot live without *mle* function, why do some of these males survive without MLE enrichment on the X chromosome? We tested the possibility that the *mle* requirement also could be overcome by overexpression of MSL1 and MSL2. However, we recovered no *mle* escaper males in crosses in which excess MSL1 and MSL2 were supplied (data not shown). Therefore, although MLE is no longer concentrated on the X chromosome in *roX* mutant males, it still performs an essential function in dosage compensation. Perhaps it acts catalytically, associating only transiently with the other MSL proteins (Copps et al. 1998), or is sufficient when present at substoichiometric levels relative to the other MSL subunits.

If *roX* RNAs are not absolutely essential for MSL targeting, could they play some other role? One possibility is that assembly with the RNAs might stabilize the MSL proteins. To test this possibility, we checked the level of each MSL protein in *roX*⁻ adult male escapers by Western blot (Fig. 4C). The amount of each MSL protein in these males was similar to that of wild-type males. This does not exclude a transient role for *roX* RNAs in MSL

protein integrity but suggests that they instead play a specific role in efficient assembly or X localization of the MSL complex.

Model for MSL targeting to the X chromosome

Earlier observations that the MSL complex could spread in *cis* from an autosomal *roX* transgene lead to speculation that complexes normally spread from the endogenous *roX* loci on the X chromosome to paint the entire chromosome (Kelley et al. 1999). However, the initial characterization of *roX1* clearly demonstrated that soluble MSL complexes could diffuse between chromosomes (Meller et al. 1997; Kelley et al. 1999). More recent work revealed that the ability of the MSL complex to spread from a site of *roX* transcription, or diffuse away, is highly sensitive to the balance between MSL proteins and *roX* transcripts in the nucleus (Park et al. 2002). Here, we demonstrate that the wild-type pattern of MSL complexes along the male X chromosome is the result of a delicate interplay between two targeting strategies. Lo-

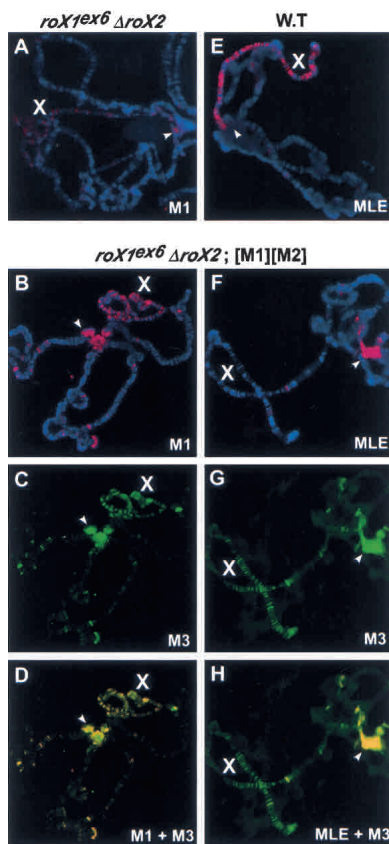


Figure 3. Colocalization of MSL proteins to the X chromosome without *roX* RNA. Male polytene chromosomes were immunostained and visualized by FITC-conjugated secondary antibodies for anti-MSL3 (green) or Texas Red-conjugated secondary antibodies for anti-MSL1 or anti-MSL2 (red). DNA was stained with DAPI (blue). Arrowheads indicate the heterochromatic chromocenter, which shows robust MSL binding in *roX*⁻ mutants. The genotypes of male larvae are *roX1^{ex6} Df(1)roX2⁵²; [w⁺ 4Δ4.3]* (A), *y w roX1^{ex6} Df(1)roX2⁵² [w⁺ 4Δ4.3]; msl2 cn/+; [M1][M2]/+* (B–D,F–H), and *y w* (wild type; E). *roX*⁻ mutants with different genetic backgrounds and escaper frequencies showed similar chromosomal staining (data not shown).

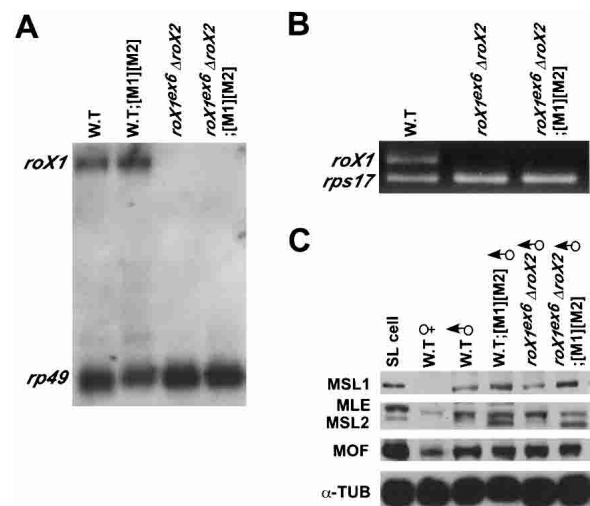


Figure 4. MSL proteins are partially functional without *roX* RNA. To determine whether an incompletely deleted *roX1* RNA retains residual expression, Northern blotting (A) and RT-PCR (B) were performed. (A) A full-length *roX1* genomic fragment was used as a probe to detect any aberrant RNA from the *roX1^{ex6}* gene in males. The full genotype of the *roX* mutant is *roX1^{ex6} Df(1)roX2⁵²; [w⁺ 4Δ4.3]*, which showed the highest escaper frequency. Hybridization to an *rp49* probe is a loading control. (B) cDNA from RNA of adult males from each genotype was amplified by PCR (30 cycles). Primers from sequences retained in *roX1^{ex6}* were used for *roX1* (261 bp) amplification. *rps17* (210 bp) was amplified in the same tube as a positive control. No-RT controls produced no PCR products (data not shown). (C) Western analysis of *roX*⁻ mutants. The total cell extract from one adult fly was loaded in each lane; 80 μg of total SL-2 cell extract was loaded as a positive control. The antibodies used for blotting are shown at left. The full genotype of the *roX* mutant is *y w roX1^{ex6} Df(1)roX2⁵² [w⁺ 4Δ4.3]; msl2 cn/+*, which showed the lowest escaper frequency. *roX*⁻ mutants with different genetic backgrounds and escaper frequencies showed similar results (data not shown). A similar level of MSL3 was also detected with or without *roX* RNA (data not shown). A Western blot for α-tubulin was a loading control.

cal spreading from *roX* loci operates in parallel with a second route where soluble MSL complexes diffuse and reattach to distant segments of the X chromosome. We can alter the proportion of MSL complexes entering each pathway by manipulating the amount of MSL proteins or *roX* RNAs present. We speculate that the underlying mechanism controlling these two outcomes rests on how efficiently MSL subunits can assemble into functional complexes (Fig. 1).

Materials and methods

Fly stocks and genetic crosses

Flies were raised on standard cornmeal-yeast-agar-molasses medium containing propionic acid. To obtain X-chromosome insertions of *roX* transgenes, [*w⁺* GMroX1-69C] and [*w⁺* GMroX2-40A] were remobilized in a *roX*⁻ mutant background [*y w roX1^{ex6} Df(1)roX2⁵² [w⁺ 4Δ4.3]*; Park et al. 2002] using genomic *P* transposase (Robertson et al. 1988). The new transgenic insertion sites were mapped by inverse PCR and sequencing. To overexpress MSL1 and MSL2, *y w* (wild type), *y w roX1^{ex6}* (Kelley et al. 1999), *w Df(1)roX2⁵²; [w⁺ 4Δ4.3]* (Meller and Rattner 2002), *y w roX1^{ex6} Df(1)roX2⁵² [GMroX1-13A][w⁺ 4Δ4.3]* or *y w roX1^{ex6} Df(1)roX2⁵² [GMroX2-14F][w⁺ 4Δ4.3]* females were crossed with *w/Y; msl2, cn/+; [w⁺ H83M1][w⁺ H83M2]/TM6B, Tb* males, and non-*Tb* male third-instar larvae were collected for anti-MSL1 immunostaining of polytene chromosomes. For Northern and Western blots, *y w* (wild type), *y w roX1^{ex6}, w Df(1)roX2⁵²; [w⁺ 4Δ4.3]* or *y w roX1^{ex6} Df(1)roX2⁵² [w⁺ 4Δ4.3]* females were crossed with *w/Y; msl2 cn; [w⁺ H83M1][w⁺ H83M2]/+* males. The

resulting adult male progeny carrying the MSL1 and MSL2 transgenes were selected by eye color. [*w⁺ 4Δ4.3*] supplies essential genes lost in *Df(1)roX2⁵²* (Meller and Rattner 2002).

Immunostaining, Northern, and Western analyses

Immunostaining of polytene chromosomes, Northern, and Westerns were as described previously (Kelley et al. 1999).

RT-PCR

Five micrograms of total RNA from adult males was reverse-transcribed using SuperScriptII reverse transcriptase (Invitrogen) and oligo(dT) primers, as described previously (Meller et al. 2000). Ten percent (2 μL) of the RT reaction was used in a 50-μL PCR amplification by using 0.2 μM *rps17* primers (F: 5'-CGAACCAAGACGGTGAAGAAG-3' and R: 5'-CC TGCAACTTGATGGAGATACC-3') and *roX1* primers (5A: 5'-CCCAGA AGAAACTGCCACTGC-3' and 7B': 5'-AATGTCCCTTTTCGAGCG-3'). PCR was performed as follows: at 94°C for 4 min, 30 cycles (at 94°C for 30 sec, at 55°C for 30 sec, at 72°C for 1 min), and at 72°C for 10 min. RT-PCR products were electrophoresed and stained with EtBr.

Immunoprecipitation and subtractive hybridization of RNA from MSL complexes

Total protein extracts of wild-type embryos (0–24 h old) were prepared by homogenization and sonication as described previously (Smith et al. 2000) in extract buffer [20 mM HEPES at pH 7.6, 70 mM KCl, 2 mM DTT, 0.1% NP-40, 8% glycerol, 1 mM PMSF, 1 U/μL RNasin (Promega), protease inhibitor cocktail; Lanz et al. 1999]. Extracts containing 10 mg of total protein were incubated with 10 μL of rabbit polyclonal α-MLE antibody, and the RNA from that immunoprecipitate was isolated as described previously (Meller et al. 2000). This RNA was used to make tester cDNA, whereas 10 μg of total RNA from adult female flies was used to make driver cDNA for subtraction (Clontech PCR-select cDNA subtraction kit). The resulting MSL-complex-specific cDNA fragments that survived the subtraction were amplified and subcloned for sequencing.

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