Current Biology, Vol. 14, 481–487, March 23, 2004, ©2004 Elsevier Ltd. All rights reserved. DOI 10.1016/j.cub.2004.03.004

Multiple Classes of MSL Binding Sites Target Dosage Compensation to the X Chromosome of *Drosophila*

Hyangyee Oh,[1,4] James R. Bone,[1,5] and Mitzi I. Kuroda[1,2,3,4,*]
[1]Interdepartmental Program in Cell and Molecular Biology
[2]Howard Hughes Medical Institute
[3]Department of Molecular and Cellular Biology
Baylor College of Medicine
One Baylor Plaza
Houston, Texas 77030

Summary

MSL complexes bind hundreds of sites along the single male X chromosome to achieve dosage compensation in *Drosophila*. Previously, we proposed that ~35 "high-affinity" or "chromatin entry" sites (CES) might nucleate spreading of MSL complexes in *cis* to paint the X chromosome. This was based on analysis of the first characterized sites *roX1* and *roX2*. *roX* transgenes attract MSL complex to autosomal locations where it can spread long distances into flanking chromatin. *roX1* and *roX2* also produce noncoding RNA components of the complex. Here we identify a third site from the 18D10 region of the X chromosome. Like *roX* genes, 18D binds full and partial MSL complexes in vivo and encompasses a male-specific DNase I hypersensitive site (DHS). Unlike *roX* genes, the 510 bp 18D site is apparently not transcribed and shows high affinity for MSL complex and spreading only as a multimer. While mapping 18D, we discovered MSL binding to X cosmids that do not carry one of the ~35 high-affinity sites. Based on additional analyses of chromosomal transpositions, we conclude that spreading in *cis* from the *roX* genes or the ~35 originally proposed "entry sites" cannot be the sole mechanism for MSL targeting to the X chromosome.

Results and Discussion

Analysis of High-Affinity MSL Binding Activity within the 18D Region of the X Chromosome

To explore a model in which ~35 high-affinity sites, including *roX1* and *roX2*, initiate spreading of MSL complexes into flanking chromatin [1], we have characterized an additional high-affinity site at 18D10 (Figure 1A). We constructed an overlapping cosmid contig around 18D10, created transgenic lines for each of the cosmids, and tested them for MSL binding at their new sites of insertion. In an *msl3*$^-$ genetic background in which the high-affinity sites are most easily monitored, only 18Dcos5 lines showed a strong MSL signal, comparable to the endogenous 18D10 region on the X chromosome (Figure 1B). However, all three of the 18D cosmids tested were able to recruit MSL complex in wild-type males (Figures 1C–1E). 18Dcos3 and 18Dcos4 do not contain a high-affinity site, but nevertheless MSL complex was recruited to their insertion sites. This result demonstrates that spreading *in cis* from high-affinity sites is not the sole mechanism for attracting MSL complexes to the X chromosome.

To determine whether the high-affinity site in 18Dcos5 has properties similar to *roX* genes, we assayed transgenic lines for MSL spreading. In wild-type, 18Dcos5 transgenes showed stronger MSL binding than 18Dcos3 or 18Dcos4 and infrequently (<5%) showed very limited spreading (usually two bands) (Figure 1C, inset). The spreading frequency at one location (56C) increased up to 80% in *roX1*$^-$ or *roX2*$^-$ backgrounds (data not shown). This behavior is typical of autosomal *roX* transgenes, which show markedly higher spreading frequency when the number of endogenous *roX* genes is decreased [2]. Thus, 18D transgenes may face competition for MSL complexes from endogenous *roX* genes and perhaps other high-affinity sites on the X chromosome.

In the absence of *roX* RNA, MSL proteins bind to several regions on the X chromosome, which may be analogous to the previously mapped high-affinity sites [3]. To see if 18Dcos5 recruits MSL proteins without *roX* RNA, polytene chromosome immunostaining was performed in *roX*-deficient male larvae, which showed consistent MSL protein binding to 18Dcos5 transgenes inserted at cytological positions 56C and 60C (Figures 1F and 1G) but not to 18Dcos3 or 18Dcos4 transgenes (data not shown). This result demonstrates that the MSL binding site located within 18Dcos5 is different from the sites within *roX* genes, which require *roX* RNAs for binding [4].

To narrow down the genes or sequences functioning as a high-affinity site around 18D10, five overlapping subfragments from 18Dcos5 (Figure 2A) were tested for MSL binding in vivo. 18D-5B and 18D-5D showed significant binding and some modest spreading (<1%) in wild-type males (Figures 2B and 2C). However, in the absence of MSL3, only 18D-5B showed MSL binding (Figure 2D), which was significantly weaker than binding to the full-length 18Dcos5 (Figure 1B, arrow). This result indicates that 18D-5B (8.8 Kb) contains a high-affinity MSL binding site. Since 18D-5A did not interact with MSL complex, it seems that the 3′ region of 18D-5B contains the binding activity (Figure 2A). To test this, three more constructs, 18D-5B1 (4.5 Kb), 18D-5B2 (2.6 Kb), and 18D-5B3 (2.1 Kb), containing the 3′ end of 18D-5B (8.8 Kb) were tested for MSL complex binding in transgenic flies (Figure 2A). Although all three fragments still displayed the ability to recruit MSL complexes in wild-type males (Figures 2E–2G), they lost binding to partial MSL complexes lacking MSL3 (data not shown), suggesting the possibility that multiple sites are required for interaction with incomplete MSL complexes. However, the 18D-5B3 (2.1 Kb) subclone still showed modest but rare spreading (<1%) (Figure 2G, inset).

*Correspondence: mkuroda@genetics.med.harvard.edu
[4]Present address: Harvard-Partners Center for Genetics & Genomics, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, Massachusetts 02115.
[5]Present address: Upstate USA, 706 Forest St., Charlottesville, Virginia 22903.

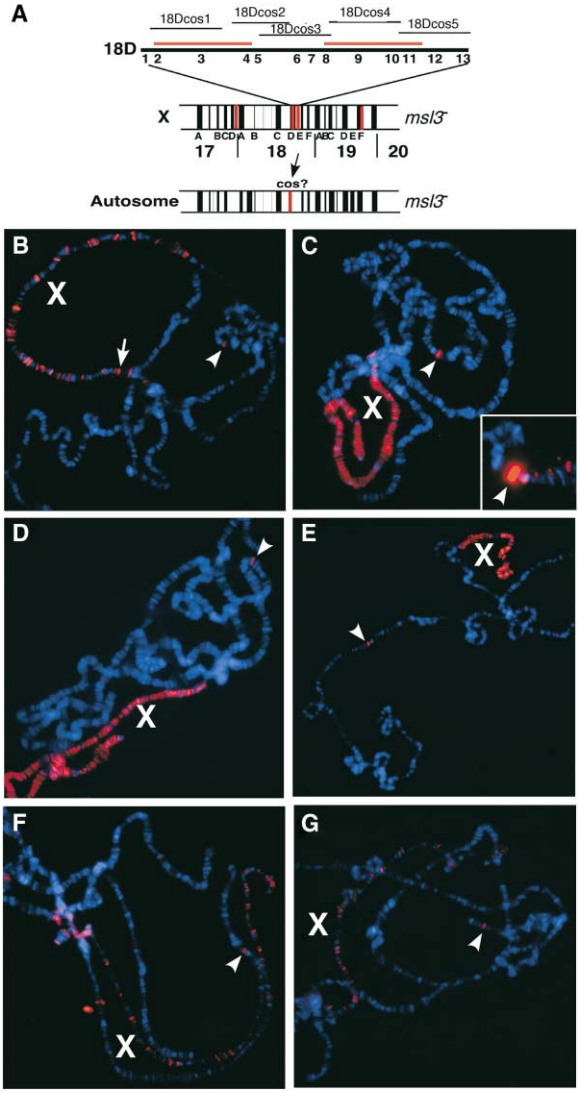

Figure 1. In Vivo Analysis of MSL Complex Binding at the 18D Region

(A) A diagram of the overlapping cosmid map around 18D and a strategy to find binding sites within autosomal transgenes in an *msl3*$^-$ mutant. Red bars indicate the two putative CES regions near 18D.

(B) Polytene chromosomes containing 18Dcos5 at 56C in an *msl3*$^-$ female expressing MSL2 ectopically.

(C) Chromosomes containing 18Dcos5 at 56C in a wild-type male. Bottom right inset in this panel shows an enlarged image of MSL spreading from a different nucleus.

(D and E) Chromosomes containing 18Dcos3 at 49D (D) and 18Dcos4 at 53F (E) in wild-type males.

(F and G) Chromosomes containing 18Dcos5 at 56C (F) and at 60C (G) in *roX*$^-$ males. Chromosomes were stained with anti-MSL1 antibodies (red) and DAPI (blue). Arrowheads designate the transgenes, and an arrow in (B) points to the endogenous 18D10 CES.

Coincidence of a Male-Specific DNase I Hypersensitive Site and In Vivo MSL Binding within 18D10

Previously, a series of short blocks of conserved sequences associated with MSL binding to the *roX1* and *roX2* genes were identified [4]. However, this configuration of consensus sequences was not found at other locations in the genome. The core of the consensus sequence within the *roX* genes, GAGAG and CTCTC, was not present within subclone 18D-5B, confirming that this MSL binding site is distinct. MSL binding sites in *roX* genes are coincident with male-specific DNase I hypersensitive sites (DHS) [4, 5]. Therefore, we assayed 18D-5B for DNase I hypersensitivity and found a male-specific site in the 3′ part of the fragment (Figure 3A), consistent with the location of MSL binding based on our transgenic studies. To confirm that this male-specific DHS is caused by direct MSL complex interaction, we analyzed the region by chromatin immunoprecipitation (ChIP) using anti-MSL2 antibodies and salivary gland tissue. The larvae utilized had low MSL2 expression [6], in which complexes bind only to high-affinity sites (Figure 3B) and also carried an extra copy of 18D10 (18Dcos5 at 56C). To evaluate the ChIP experiment, *roX1* (positive control) and *pka* (negative control) primers were used to measure enrichment of *roX1* in the immunoprecipitated DNA (Figure 3C). To locate MSL binding within 18D, we analyzed subfragments of the 8.8 Kb 18D-5B subclone by Southern blotting with probe prepared from the α-MSL2 IP (Figure 3D). Compared to the control IP, the 3′ end of 18D-5B was enriched in the α-MSL2 immunoprecipitation (Figures 3D and 3F). This was further narrowed down to smaller subfragments (Figure 3E), showing that MSL binding overlaps the male-specific DHS. The binding activity maps to intergenic DNA 3′ of CG12237, whose function is unknown (Figure 3F). These results show that the MSL complex interacts with 18D10 and modifies its chromatin structure as it does in the *roX* genes. However, unlike the *roX* genes, transcription of the 18D MSL binding site was not detected by Northern or RT-PCR using 18D DHS probes and primers (data not shown).

Four Tandem Repeats of a 510 bp Fragment from the 18D10 Region Are Sufficient for Recruitment and Limited Spreading of the MSL Complex

To determine the importance of the male-specific DHS from 18D10 in MSL complex recruitment, we performed a transgenic deletion analysis. We deleted 128 bp (ΔS) or 618 bp (ΔL) of the DHS region from the 18D-5B3 transgene (2.1 Kb). All three ΔL lines and three of the four ΔS lines completely lost the ability to recruit MSL complexes, and the remaining ΔS transgene showed only a very weak signal (Figure 4A). These data demonstrate that the 128 bp region deleted in the ΔS transgene, and perhaps additional elements in the ΔL 618 bp region, contain important *cis*-elements for MSL complex recruitment.

Previously it was shown that ~200 bp of a *roX* DHS is sufficient for recruitment of the MSL complex even in the absence of MSL3 [4, 5]. In addition, when the *roX1* DHS is multimerized, it can show limited spreading into flanking chromatin [5]. To determine whether the 18D10 DHS carries similar activities, we analyzed at least four independent insertions of the following transgenes (Figure 4A): 510 bp (18D10-DHS-L), 271 bp (18D10-DHS-S), four tandem repeats of 510 bp (18D10-DHS-L4mer), or seven tandem repeats of 271 bp (18D10-DHS-S7mer). Unlike the *roX1* DHS, 18D monomers of 510 bp and

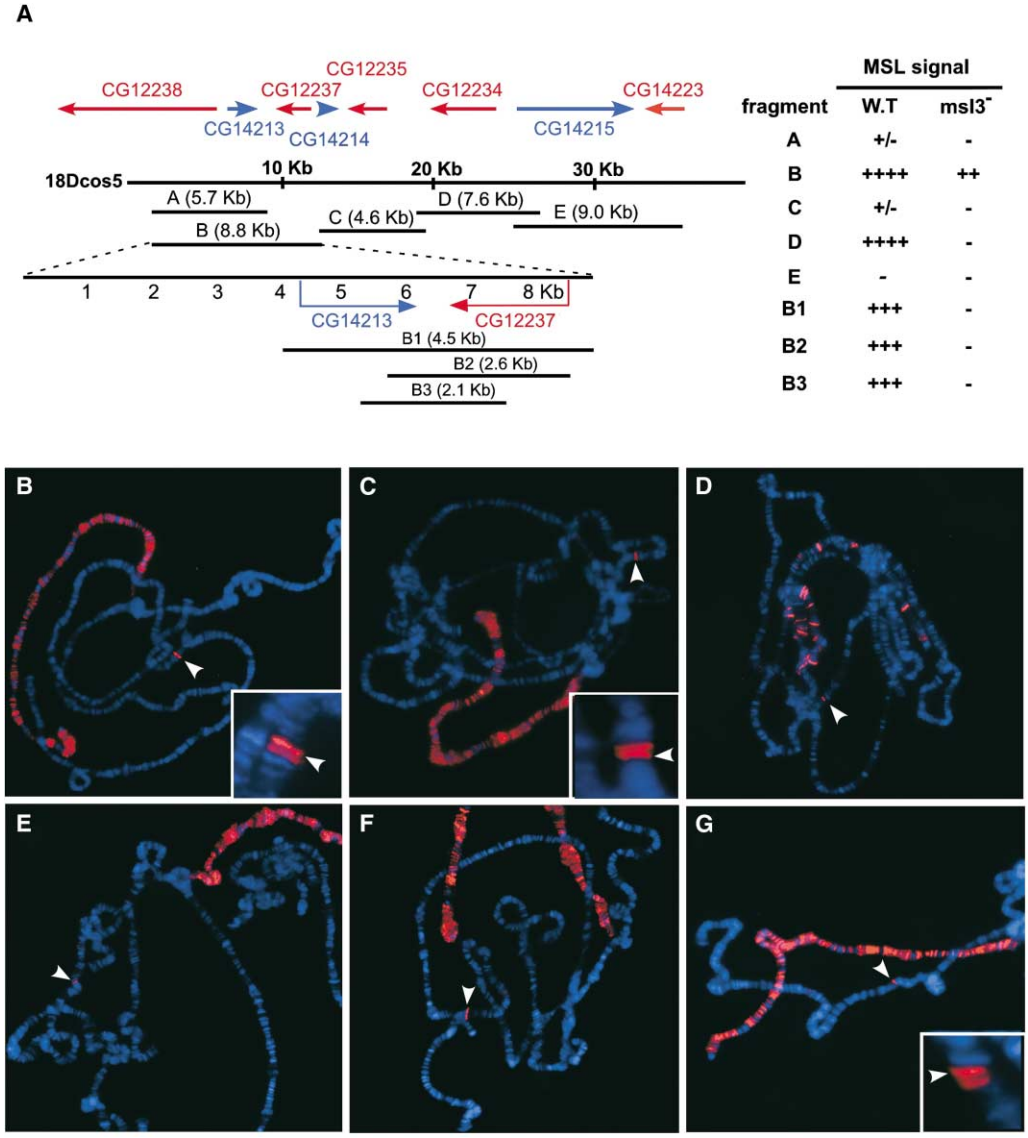

Figure 2. In Vivo Analysis of MSL Complex Binding to Smaller Fragments Derived from 18Dcos5

(A) A diagram of the 18D10 region showing the overlapping map of smaller fragments from 18Dcos5. Transcripts from this region are depicted as arrows (blue and red) to show the direction of transcription. MSL binding to each fragment is summarized in the right side.

(B and C) Polytene chromosomes containing 18D-5B at 82B (B) and 18D-5D at 34D (C) in wild-type males. Bottom right insets show enlarged images of limited MSL spreading.

(D) Chromosomes containing 18D-5B at 82B in an *msl3*$^{-}$ female expressing MSL2 ectopically.

(E–G) Chromosome containing the 18D-5B1 (4.5 Kb) transgene at 90A (E), 18D-5B2 (2.6 Kb) transgene at 93D (F), and 18D-5B3 (2.1 Kb) transgene at 86E (G), which shows MSL spreading from a different nucleus in the bottom right inset. Polytene chromosomes were stained with anti-MSL1 antibodies (red) and DAPI (blue). Arrowheads represent the transgenes.

271 bp were extremely weak for MSL complex binding. Seven tandem repeats of the 271 bp segment also showed very weak MSL complex recruiting activity (Figure 4A). However, the transgene with four tandem copies of 510 bp showed a strong signal for MSL1 staining (Figure 4B) and occasional mild spreading (<5%) (Figure 4B, inset). Even in the absence of MSL3, the 18D10-DHS-L4mer was sufficient to recruit the incomplete MSL complex, with very strong and consistent signals of MSL1 staining (Figure 4C), even stronger than that of 18D-5B (8.8 Kb) in an *msl3*$^{-}$ background. However, unlike 18Dcos5, the 18D10-L4mer did not recruit MSL proteins without *roX* RNA (data not shown). These results indicate that a 510 bp 18D10 fragment carries key sequences for MSL complex targeting. However, despite the strong MSL binding observed, no sequence motifs common to the *roX* genes or otherwise enriched on the X chromosomes [7–11] were detected (data not shown).

Evidence for CES-Independent *cis*-Acting Sequences on the X Chromosome

Given our surprising finding that all 18D cosmids tested were able to recruit wild-type MSL complexes, we ex-

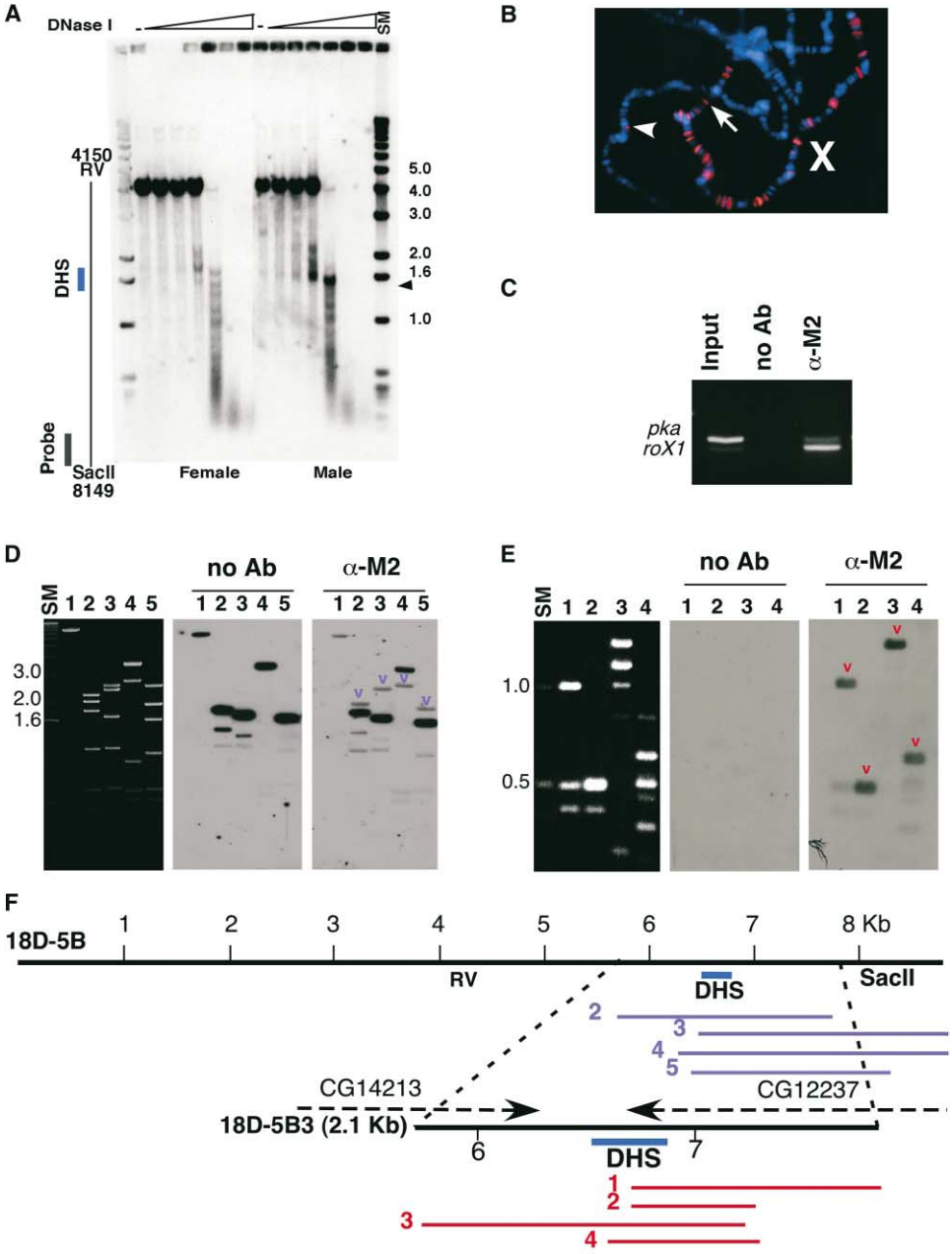

Figure 3. Mapping of In Vivo Interactions of MSL Proteins within the 18D Region

(A) Male-specific DNase I hypersensitivity site (DHS) in the 3′ region of 18D-5B. DNase I cleavages were mapped within an EcoRV/SacII fragment located between CG14213 and CG12237. Living nuclei from female (left) or male (right) adult flies were treated with increasing concentrations of DNase I. DNA was isolated and digested with EcoRV and SacII to produce a 4 Kb genomic fragment. The DHS was identified by Southern blotting and hybridization with a probe adjacent to the SacII site near CG12237. A blue line indicates the DHS. SM, DNA size markers (Kb).

(B) A genetic cross between flies containing 18Dcos5 at 56C and flies containing an *msl2* gene mutated in its SXL binding region produces females showing MSL binding to the high-affinity sites on the X chromosome and 18Dcos5 at 56C (arrowhead). An arrow represents the endogenous 18D10 CES. The same female salivary glands were used to conduct the ChIP assay (see Supplemental Experimental Procedures).

(C) To evaluate the ChIP assay, amplified DNAs from immunoprecipitations with α-MSL2 or no Ab were used as PCR templates using primers from *roX1* (positive control) and *pka* (negative control). An IP lacking chromatin also was performed, which showed no PCR products (data not shown).

(D) The 8.8 Kb 18D-5B was cut with different sets of restriction enzymes in each lane (left). Lane 1, uncut; 2, BamH1/EcoRI; 3, AseI/HindIII; 4, ClaI/HindIII; 5, AccI. Restriction fragments were blotted and hybridized to probes prepared from amplified DNA from control (no Ab) IP (middle). The membrane was stripped and rehybridized with probe immunoprecipitated by α-MSL2 (right). The fragments which appeared only in the α-MSL2 IP are marked with purple arrows. They are 2.1 Kb (lane 2), 2.6 Kb (lane 3), 2.7 Kb (lane 4), and 2.0 Kb (lane 5).

(E) To narrow down the MSL binding sites, the 2.1 Kb fragment (18D-5B3) was cut into smaller pieces with several restriction enzymes (left). Lane 1, NsiI/SphI; 2, NsiI/XhoI; 3, PvuII; 4, SalI/XhoI/SphI. Southern blotting and hybridization were as described above. The fragments which appeared only in the α-MSL2 immunoprecipitation are marked with red arrows. These are 1.0 Kb (lane 1), 0.5 Kb (lane 2), 1.4 Kb (lane 3), and 0.6 Kb (lane 4).

(F) ChIP analyses from (D) and (E) summarized with purple lines and red lines, respectively, which overlapped with the male-specific DHS region (blue line).

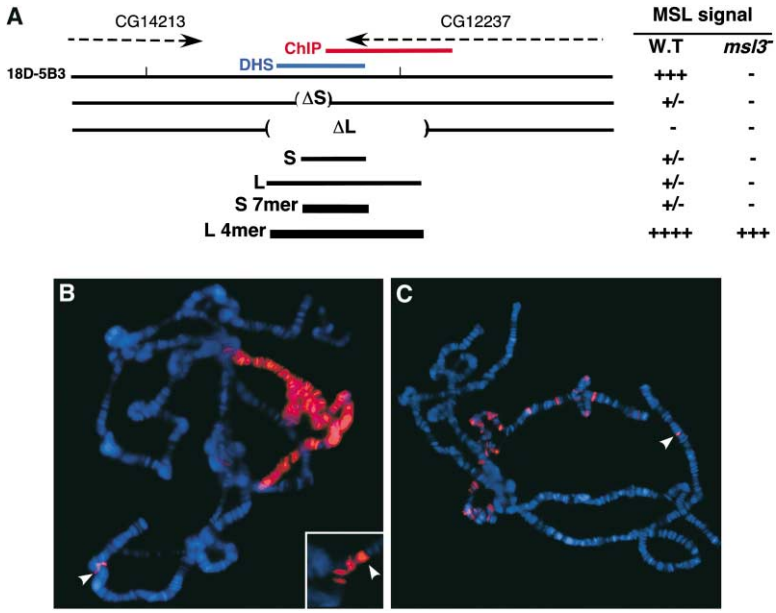

Figure 4. Fine Structure Mapping of the 18D10 CES

(A) A diagram of 18D10 fragments tested in the transgenic assay for MSL binding. ΔS is a deletion of 128 bp (SphI/NsiI) from the 18D-5B3 (2.1 Kb) fragment (BamH1/EcoRI), and ΔL is a deletion of 618 bp (NdeI/ScaI). L and S are 510 bp and 271 bp, respectively. DHS and ChIP assays are depicted as blue and red, respectively. MSL binding of each fragment is summarized in the right side.

(B and C) Polytene chromosomes containing the 18D10-DHS-L4mer transgene at 98B were immunostained with MSL1 antibody (red) and DAPI (blue) in wild-type males (B) and *msl3*$^{-}$ females expressing MSL2 ectopically (C). The bottom right inset in (B) shows an enlarged image of MSL spreading in a second nucleus. Arrowheads indicate the site of transgenes.

tended our analysis to other regions of the X chromosome. Large X to autosome transpositions have been shown to retain both the ability to dosage compensate, as well as the characteristic diffuse appearance of the male X chromosome [12–14]. We analyzed four fly lines containing large X-ray-induced X to autosome transpositions obtained from the *Drosophila* stock center. Their cytological locations and size are summarized in Supplemental Table S1 (see Supplemental Data available with this article online). Males hemizygous for the inserted chromosome segment contain an unpaired portion of the polytene chromosome protruding from the wild-type autosome (Figures 5A–5D, arrows). As observed in line *Tp (1;3) rb*$^{+}$*71g* (Figure 5D), the region of transposed X chromosome appears as wide as the paired autosomes that flank the insertion, suggesting that the transposed section of chromosome adopts a less compact chromatin structure similar to that of the intact male X chromosome. All four X to A transposition stocks that we tested showed MSL binding within the transposed fragments, including two that lack a mapped high-affinity site (Figures 5A and 5B). These data provide further evidence that *cis*-acting sequences are present in large pieces of the X chromosome that enable them to recruit MSL complex regardless of whether they contain a putative chromatin entry site.

In contrast to the X to autosome transposition flies, an autosome to X transposition stock lacked MSL staining of a region of the third chromosome that was transposed to the X. MSL1 protein was detected at sites flanking the break points of the transposition (Figure 5E, vertical arrows), but no staining was observed within the transposed section of the third chromosome even though there is a nearby high-affinity site (4C12-16). These data indicate that linking autosomal sequences to the X chromosome is not sufficient to allow recruitment of the MSL complex, contradicting a key prediction of a simple spreading model.

To test the requirement of entry sites for recruitment of MSL complexes to smaller fragments (<39 Kb), we immunostained transgenic lines harboring X-derived DNA fragments variable in size from 39 Kb to 0.3 Kb (Supplemental Table S2). Interestingly, each cosmid showed strong MSL binding in wild-type, and in at least one case (cos13E) there was even some apparent spreading (<1% of nuclei). These results demonstrate that even without a nearby high-affinity site, some X-derived fragments contain *cis*-acting sequences for MSL complex binding. Our results raise the possibility that spreading in *cis* from the two *roX* genes may not be the major mechanism for MSL binding to the X chromosome.

Our focus in this study was to identify additional putative chromatin entry sites and understand how they attract MSL complexes and whether they, like the *roX* genes, can nucleate MSL spreading. We were successful in isolating the site from cytological location 18D10, and we analyzed its primary sequence, chromatin structure, MSL interaction, and ability to nucleate spreading. We found that the behavior of this site was significantly different from the behavior of *roX* genes in several ways. Our current data can be interpreted in the following framework (Figure 5F). Perhaps there are diverse DNA recognition elements on the X chromosome that have different affinities for MSL complex; high, intermediate, or weak. High-affinity *cis*-elements, such as within the *roX* genes, do not require additional *cis*-elements for recruiting MSL complexes and might be involved in multifold gene activation instead of 2-fold hypertranscription. This interaction might be strengthened by *roX* RNA (Figure 5F, middle). An intermediate-affinity *cis*-element, like the 18D10 site, might require additional intermediate- and/or weak-affinity elements for robust binding and would have the ability to attract partial MSL complexes with a minimal MSL1/MSL2 composition (Figure 5F, right). Third, weak-affinity *cis*-elements might require interaction with several additional weak-affinity *cis*-elements, which might explain occasional autoso-

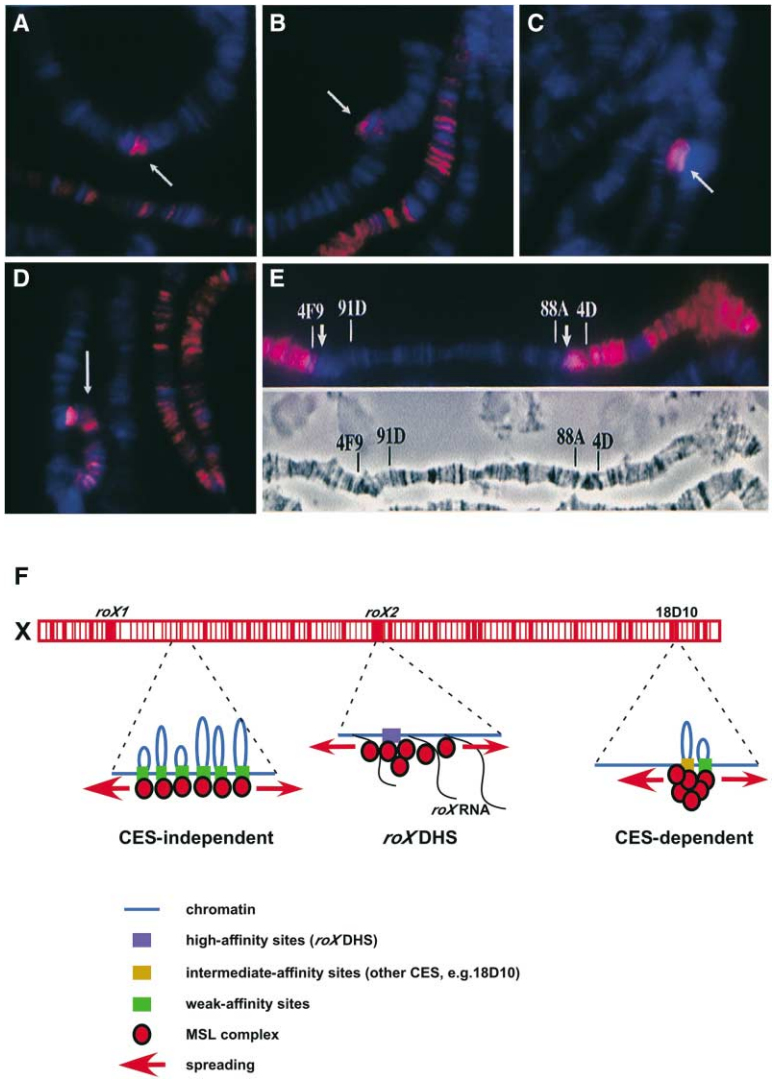

Figure 5. In Vivo Analysis of MSL Binding to Large X Fragments

(A–E) Polytene chromosome squashes from male larvae containing large X to autosome (A–D) or autosome to X (E) transpositions, double stained with DAPI (blue) and antibodies specific for the MSL1 protein (red). (A) *Tp(1;3) sta*, (B) *Tp(1;3) JC153*, (C) *Tp(1;2) r*$^{+}$*75C*, (D) *Tp(1;2) rb*$^{+}$*71g*, and (E) *Tp(3;1) O5*. Arrows indicate the site of transpositions.

(F) A model for MSL binding and spreading on the X chromosome. MSL complex interactions with the X chromosome may require different strategies depending on the *cis*-elements. Without a high-affinity site, several weak-affinity sites cooperate together to recruit the MSL complex, followed by spreading only when complex reaches a threshold concentration (left). A *roX* DHS is sufficient to recruit enough MSL complex for occasional spreading, probably because the local concentration of MSL complex is high due to the interaction between *roX* RNA and MSL proteins (middle). An intermediate-affinity site requires another intermediate- or weak-affinity site for recruiting and spreading of MSL complex (right). These sites show relatively strong affinity for the MSL complex even without *roX* RNA.

mal MSL signals and how X fragments on the autosomes attract wild-type MSL complexes even without a CES (Figure 5F, left).

Supplemental Data

Supplemental data including experimental procedures are available at http://www.current-biology.com/cgi/content/full/14/6/481/DC1.

Acknowledgments

We are grateful to R.L. Kelley and X. Chu for initiating the cosmid library screening, R. Richman for affinity-purified antibodies, and the Bloomington Stock Center for *Drosophila* stocks. We thank Kuroda lab members for critical reading of the manuscript. This work was supported by the Welch Foundation (Q-1359), the National Institutes of Health (GM45744), and the Howard Hughes Medical Institute. M.I.K. is an HHMI Investigator.

Received: December 4, 2003
Revised: January 15, 2004
Accepted: January 29, 2004
Published: March 23, 2004

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