X Chromosome Sites Autonomous Recruit the Dosage Compensation Complex in Drosophila Males

Delphine Fagegaltier, Bruce S. Baker*
Department of Biological Sciences, Stanford University, Stanford, California, United States of America

It has been proposed that dosage compensation in Drosophila males occurs by binding of two core proteins, MSL-1 and MSL-2, to a set of 35–40 X chromosome “entry sites” that serve to nucleate mature complexes, termed compensasomes, which then spread to neighboring sequences to double expression of most X-linked genes. Here we show that any piece of the X chromosome with which compensasomes are associated in wild-type displays a normal pattern of compensosome binding when inserted into an autosome, independently of the presence of an entry site. Furthermore, in chromosomal rearrangements in which a piece of X chromosome is inserted into an autosome, or a piece of autosome is translocated to the X chromosome, we do not observe spreading of compensasomes to regions of autosomes that have been juxtaposed to X chromosomal material. Taken together these results suggest that spreading is not involved in dosage compensation and that nothing distinguishes an entry site from the other X chromosome sites occupied by compensasomes beyond their relative affinities for compensasomes. We propose a new model in which the distribution of compensasomes along the X chromosome is achieved according to the hierarchical affinities of individual binding sites.

Introduction

Most X chromosomal genes are essential or relevant to both sexes. To cope with the difference in the number of copies of these genes in females (XX) and males (XY), organisms have evolved a variety of mechanisms, collectively termed dosage compensation, to equalize the levels of X-linked gene products in the two sexes. In Drosophila males the expression of most of the genes on the single X chromosome is doubled. At least six protein-coding genes, collectively referred to as male specific lethals (msls), are required for dosage compensation (Baker et al. 1994; Marin et al. 2000; Meller 2000): msl-1, msl-2, and msl-3, whose functions remain unknown; maleless (mle), encoding an RNA helicase; males absent on the first (mof), encoding a histone acetyltransferase; and jil-1, encoding a histone kinase. The products of these genes, together with noncoding RNAs encoded by the RNA on the X genes (roX1 and roX2) (Amreen and Axel 1997; Meller et al. 1997; Franke and Baker 1999), are all reproducibly associated with hundreds of locations along the length of the polytenized salivary gland X chromosome in males. MOF has been shown both in vivo and in vitro to acetylate H4Lys16, a specific histone modification also found at sites where compensasomes are associated with the male X (Hilfiker et al. 1997; Smith et al. 2000; Akhtar and Becker 2001). Recently, JIL-1, which phosphorylates H3Ser10, was shown to be enriched at the MSL binding sites in males (Wang et al. 2001). Thus, MSL proteins and roX RNAs are thought to function in a ribonucleoprotein complex (compensasome) to mediate dosage compensation by altering chromatin structure of the male X chromosome (Stuckenholz et al. 1999; Franke and Baker 2000). In females translational repression of msl-2 mRNA by the Sex-lethal protein (SXL) prevents formation of compensasomes and hence dosage compensation (Bashaw and Baker 1997; Kelley et al. 1997).

The processes and constraints that generate the observed distribution of compensasomes along the male X chromosome are unknown. Although the hundreds of places where compensasomes are found along the X chromosome are referred to as “sites,” they are in fact not points, but rather bands (small segments of chromosome) that roughly span the size range of salivary chromosome bands seen with DNA stains (i.e., a few tens to several hundreds of kilobases in length). Thus, both the locations and the extents of these sites are somehow specified. Furthermore, the compensasome bands do not correspond to the bands where DNA is condensed (Baker et al. 1994; Kelley et al. 1999; Demakov et al. 2003). In addition, non-dosage-compensated X-linked genes (e.g., LSP1-α) are scattered throughout the X chromosome and can reside next to dosage-compensated genes (Baker et al. 1994). Since there is no known DNA-binding component in the compensasome, and consensus DNA sequences required for binding have not yet been identified, an understanding of the distribution of compensasomes along the X chromosome needs to encompass not only how...
Table 1. Summary of the Transpositions Studied: Transpositions, Duplications, and Reciprocal Translocations

| Line | Genotype | Breakpoints | Insertion Site | ES | Number of Bands | N<sup>c</sup> |
|------|----------|-------------|----------------|----|----------------|-----------|
| I    | Tp(1;2)rb<sup>71g</sup> | 3F3–5E8 | 23A1–5 | 3–4 | 9–17 | 0 (172) |
| II   | Dp(1;3)sn<sup>13at</sup> | 6C–7C9 | 79D2–E1 | 2–3 | 8–9 | 0 (112) |
| III  | Tp(1;2)sn<sup>172d</sup> | 7A8–8A5 | 32C; 58E | 2 | 8–16 | 0 (128) |
| IV   | Tp(1;3)w<sup>co</sup> | 2C1–3C5 | 77D3; 81 | 2–3 | 4–11 | 0 (106) |
| V    | Tp(1;3)v<sup>74c</sup> | 9E4–11B2 | 80–81 | 2 | 8–19 | 0 (219) |
| VI   | Tp(1;2)w-ec | 3C2–3F1 | 37D | 1 | 4 | 0 (102) |
| VII  | Tp(1;3)f<sup>71b</sup> | 15A4–16C2 | 80–81 | 1 | 4–8 | 0 (115) |
| VIII | Tp(1;3)C153 | 16E2–4–17A–B | 99D | 0–1 | 3–5 | 0 (122) |
| IX   | Tp(1;3)sta | 1E1–2A | 89B21–C4 | 0 | 4–5 | 0 (112) |
| X    | Tp(1;3)w<sup>eh</sup> | 3C2–3C6 | 61D | 0 | 1–2 | 0 (61) |
| XI   | Dp(1;3)w<sup>67k</sup> | 3A5–3E8 | 87E1–7 | 0 | 6–8 | 0 (155) |
| XII  | T(1;3)v | 10A1–2; 93B7–10 | 0 | 0 | 0 (140) |
| XIII | T(1;2)odd<sup>1.10</sup> | 5A3; 24B | 0 | 0 | 0 (126) |
| XIV  | Dp(2;1)B19 | 25F1–24D5 | 984–C1 | 984–C1 | 0 (140) |
| XV   | Dp(3;1)2–2 | 8IF–8F10–11 | 3D | 0 | 0 (117) |

Variations in both the number of bands observed in the transpositions and their intensity are due to variable accessibility of the piece examined on the squash and the orientation of the chromosomes when flattened for observation. Similar variations were observed on the intact X. No additional MSL binding was observed into autosomal regions flanking translocated X material or onto autosome material inserted onto the X chromosome. We found a breakpoint in line VI to be at 3F1 instead of 3E7–8, and 5A instead of 4A in line XIII. Line III contains a piece of the X inserted into a pericentric inversion of the second chromosome, while line IV carries an inversion of 77D5–81.

See Materials and Methods for precise genotypes.

| Entry Site | Number of Entry/High-Affinity Sites Present in Each Transposition According to Observations and Previous Studies (Lyman et al. 1997). |
|-----------|---------------------------------------------------------------------------------------------------|
| 35–40 sites of MSL-1 and MSL-2 binding on the X seen in the first set (lines I to XI) range in size from about 1% to 15% of the length of the X, and the corresponding stretch of X chromosome for each contains 1–19 distinguishable MSL bands.

Results

The spreading model implies that a piece of the X chromosome translocated to an autosome must contain at least one of the 35–40 “entry” sites if that piece of the X is to recruit compensasomes and become dosage compensated. We looked at MSL binding in various chromosome rearrangements that inserted small pieces of X chromosome into autosomal locations. Table 1 summarizes the translocations, transpositions, and duplications examined. The insertions in the first set (lines I to XI) range in size from about 1% to 15% of the length of the X, and the corresponding stretch of X chromosome for each contains 1–19 distinguishable MSL bands. These insertions were examined in heterozygous males of either a wild-type background (Kelley et al. 1999) has come from the following findings. MSL-1 and MSL-2 represent core components of the complex: The presence of both is required for either to bind, and none of the other MSL proteins binds to the X chromosome in an msl-1 or msl-2 mutant male (Lyman et al. 1997). Furthermore, in males mutant for mle, msl-3, or mof, binding of MSL-1 and MSL-2 is only maintained at a limited number of sites (35–40) on the X chromosome, which include the roX1 and roX2 genes (Lyman et al. 1997; Kelley et al. 1999). Finally, roX transgenes inserted into an autosome retain binding of compensasomes, and in addition show compensasome binding in the autosomal region flanking the insertion site, a phenomenon termed spreading (Kelley et al. 1999). Based on these observations, a reasonable model (Kelley et al. 1999) emerged suggesting that the 35–40 sites of MSL-1 and MSL-2 binding on the X seen in mle, msl-3, or mof mutants represent nucleation sites or entry sites for the complex. From these sites, newly assembled compensasomes would spread in cis along the X to form the hundreds of final sites observed in a wild-type male. In this spreading model, roX RNAs would also be required for compensasome assembly (Park et al. 2003). However, there is to date no direct evidence that entry sites and spreading play any role in the processes that generate the normal pattern of compensasome binding along the X chromosome. We thus directly tested this model by analyzing various pieces of the X chromosome transposed or translocated to autosomal locations for their ability to bind compensasomes and initiate spreading.
the X chromosome in a wild-type male. Even the smallest piece we looked at (line X, approximately 200 kb) showed one to two MSL bands (Figure 1C). Thus, we found that any piece of the X chromosome moved to an autosomal location is able to bind compensasomes, whether or not the transposed piece of X chromosome contains an entry site. This finding suggests that each of the hundreds of MSL bands observed on the X in males carries the information necessary and sufficient to attract compensasomes, and does not require adjacent entry sites.

Interestingly, duplications showed binding both along the autosomal insertion and on the X chromosome (lines II and XI), indicating that the supply of compensasomes is not limiting in these circumstances. We also tested homozygous transpositions and duplications for MSL binding in males and found that we could recover MSL binding on each homozgyous transposed piece (unpublished data) as well as on the X. Thus, even three copies of the same segment of the X chromosome (two of the duplication plus the original piece on the X) were able to maintain MSL binding. This result extends previous data showing that, by using specific msl-2 transgenes escaping SXL repression, ectopic expression of MSL-2 in females induced binding to both X chromosomes, in a pattern identical to the single X of a wild-type male (Bashaw and Baker 1997). Therefore, binding occurs regardless of the location and number of copies of the X-linked targeted sequences.

The determinations listed in Table 1 of how many entry sites each of the transpositions contains were made by comparing the reported breakpoints of each rearrangement to the described locations of entry sites (Lyman et al. 1997). As cytological determinations can vary, we directly confirmed the presence or absence of entry sites by examining MSL binding in an msl-3 or mle mutant background for a subset of these transpositions (Figure 2). Each line used in these experiments contained the transposed region from the X inserted into an autosome and a wild-type X chromosome. For line XI we found that, in mle mutant individuals, MSL binding was undetectable in either the transposed region (3A5–E8) inserted at 87E17 (Figure 2A–2E) or in this region in the wild-type X. As expected, the same is true when only a subset of this region is duplicated: Line X did not show binding in mle mutants to region 3C2–3C6 on the X or to the transposition of that region inserted at 61D (Figure 2F–2K). These findings confirm that lines X and XI do not contain entry sites. Similarly, we confirmed that transpositions inferred to contain entry sites in two lines (IV and VI) did in fact contain such sites. Thus, for line IV in an mle mutant background we observed MSL binding to one to three sites on both the transposition and the corresponding region of the X (Figure 2N and 2P), while for line VI in an msl-3 mutant background we observed one site of MSL binding on both the transposition and the corresponding region of the X (Figure 2S). These findings are consistent with those of Lyman et al. (1997), who reported two entry sites in the region encompassed by the transposition in line VI, and one entry site in the region encompassed by the transposition in line IV. Our findings firmly establish that isolated subregions of the X chromosome display normal patterns of compensasome binding irrespective of whether they contain entry sites, and thus suggest that entry sites do not play a distinct role in the establishment of compensasome binding along the X as postulated by the spreading hypothesis. Hereafter we will refer to entry sites as high-affinity sites, their original name (Lyman et al. 1997). During the course of this study, Oh et al. (2004) have reported similar results for binding of compensasomes to transpositions from lines I, VIII, and IX. However, the scale of the analysis and the limited number of rearrangements did not yield the same conclusions.

The two high-affinity sites identified to date correspond to

Figure 1. MSL Binding to Pieces of X Chromosome Inserted into Autosomes

Salivary glands from males heterozygous for each transposition were fixed (47% acetic acid in phosphate-buffered saline, then lactic acid/water/acetic acid [1:2:3]), squashed on slides, treated with anti-MSL-1 antibodies and a secondary Cy3 anti-rabbit immunoglobulin G antibody, then counterstained with DAPI and viewed using a Zeiss Axiophot microscope. Both duplications and transpositions were able to attract compensasomes, whether or not they contained antibodies and a secondary Cy3 anti-rabbit immunoglobulin G antibody, then counterstained with DAPI and viewed using a Zeiss Axiophot microscope. Both duplications and transpositions were able to attract compensasomes, whether or not they contained entry sites. This finding suggests that each of the hundreds of MSL bands observed on the X in males carries the information necessary and sufficient to
the roX1 and roX2 genes (Kageyama et al. 2001; Park et al. 2003), and it was the fact that roX transgenes inserted into autosomal locations are able to induce spreading—binding of the MSLs to some autosomal sequences surrounding a roX transgene insertion site—that led to the hypothesis that spreading gives rise to the wild-type distributions of compensasome bands along the male X chromosome. We therefore examined whether autosomal transpositions of a piece of the X were able to induce spreading. In cells heterozygous for each of the transpositions listed above we never observed additional MSL binding to the autosomal regions either cis or trans to the insertion site (Table 1; see Figure 1). We also did not observe additional MSL binding in males homozygous for the transpositions described above.

Figure 2. MSL Binding to Autosomal Duplications of X Chromosome Pieces in mle or msl-3 Mutant Larvae

Salivary glands from w; pr mle12.17chen b w mle; Dp (1;3)msl2A10 or w; Dp (1;2)/msl2A21; msl3p/msl3p females were squashed and stained as described in Figure 1, followed by in situ hybridization with a biotinylated probe specific for regions carried by each duplication (Lavrov et al. 2004) and incubation with Oregon green-coupled streptavidin. Conditions throughout the procedure were adjusted to maximize MSL staining. Specific biotinylated probes (green bars) appear in green in merges (A, F, I, L, O, Q, and R) and as bright bands in (B, D, G, J, and M). MSL bands are shown in red in merges and in (P) and as bright bands in (C, E, H, K, N, and S). DAPI stain is blue. MSL binding is absent from duplications or the matching region on the X in line XI (3A5–3E8) (A–E) and line X (3C2–3C6) (F–K) in mle mutants, confirming that they lack any entry sites. Probe maps region 3D–E in (A–E) and 3C in (F–K). (L–P) Illustrated are the one to three bands detected in mle mutant nuclei on the duplicated region from line IV (2C1–3C5) (O and P) and on the same segment on the X (M and N). (O) and (P) are from another nucleus. (Q–S) A single band is detected at the 3F1 breakpoint of the duplication (3C2–3F1, line VI) in msl-3 mutant nuclei (S), corresponding to the weakest band of the doublet at 3F on the X. Note the weak signal on duplications compared to the same region on the X chromosome. Probe maps region 2D5–3A2 in (L–P) and 3D–E in (Q–S).

DOI: 10.1371/journal.pbio.0020341.g002
This was true irrespective of the number of high-affinity sites contained in the transpositions. Interestingly, lines I and V, which each contain several high-affinity sites, including the roX1 or roX2 gene, respectively, showed no spreading in males wild-type for the MSLs (see Figure 1B). The dichotomy between our results and those obtained with roX transgenes suggests that spreading may be a phenomenon restricted to some roX transgenes (see below) and not an aspect of dosage compensation.

To further assess if spreading in cis occurs on the X chromosome, we next asked if the complex could spread from the X onto an autosomal piece attached to the X by a reciprocal translocation. We tested two reciprocal translocations that interchanged large portions of the X and 3R or 2L (see Table 1, lines XII and XIII, respectively). Both translocations separate roX1 (3F) and roX2 (10C) genes from one another and thus both pieces of each translocation contain a roX locus. Anti-MSL-1 staining revealed the absence of any bands on either of the 3R or 2L pieces of these translocations (Figure 3), while the pattern observed on the two transposed pieces of the X was normal. These results strengthen the idea that spreading may be a phenomenon restricted to roX transgenes, since the breakpoints in line XII (10A) and line XIII (5A) are relatively close to the roX2 (10C) and roX1 (3F) loci, respectively.

We also tested two small transpositions of autosomal regions into the X (Table 1, lines XIV and XV; Figure 3C): Neither of them showed MSL binding, even weak, to any part of the inserted autosomal sequences. Furthermore, females either heterozygous or homozygous for these transpositions and expressing ectopic MSL-2 did not show any MSL bands in either of these insertions of autosomal material into the X, although they displayed normal MSL binding both to the unpaired X region (in heterozygotes) and along the paired portions of the two X chromosomes (Figure 3D). Thus, insertion of a piece of an autosome into the X does not disrupt MSL binding to either the unpaired X homologue at the insertion site or the regions of the X immediately flanking the site of insertion of autosomal material. Moreover, these results are inconsistent with the model derived from the roX transgene studies where MSL binding is observed both in the autosomal regions adjacent to the insertion site and on the wild-type autosomal homologue.

Discussion

In summary, we have used chromosome rearrangements to test two central aspects of the proposed spreading model of dosage compensation in Drosophila. It is worth noting that our experiments were a priori neutral: They could have provided compelling evidence for or against the spreading model. In both cases our results are inconsistent with the clear predictions of that model. First, we show that pieces of the X chromosome inserted into an autosome bind compensosomes in precisely the pattern characteristic of that piece of the X at its endogenous location on the X, and this property is independent of the presence of sites previously described as entry sites. Second, compensosomes do not spread from the X into autosomal pieces inserted into, or translocated onto, the X. Moreover, there is not spreading of compensosomes from autosomal insertions of pieces of the X chromosome into the autosomal regions flanking the insertion, even when such pieces contain a roX gene close to the breakpoint. These results suggest that spreading in cis is not part of the process of dosage compensation in flies. We thus propose that all of the hundreds of sites along the X...
Dosage Compensation in Drosophila

chromosome where compensasomes are found in wild-type males are competent to independently recruit compensasomes.

Our findings raise several questions regarding previous data. Are the 35–40 sites that attract partial complexes in mle or msl-3 mutants qualitatively different from the other sites at which MSL bands are found in wild-type, and if so, how? Why do roX transgenes induce additional binding to adjacent autosomal sequences?

With respect to the potential heterogeneity of compensosome binding sites, while most of the relevant data are indirect (only the roX1 and roX2 genes are identified binding sites), the data are consistent with the simple view that the binding sites are homogeneous in terms of their function, but have varying affinities for compensasomes. Our finding that pieces of X chromosome transposed to autosomal locations display normal patterns of compensosome binding, irrespective of whether or not they contain high-affinity sites, removes the one functional distinction between binding sites that had been proposed. That there are not two classes of binding sites in terms of affinity for compensasomes, but rather a continuum of affinities, is strongly suggested by the recent report of Demakova et al. (2003), who carefully characterized the number and locations of compensosome bands in mutant females expressing various limiting amounts of MSL-2. They found only four bands in the most limiting case, and progressively higher numbers of bands as more MSL-2 protein was expressed. Interestingly, the intermediate 40 sites at which complete complexes are assembled in these conditions exactly matched with the 35–40 high-affinity sites bound by partial complexes in mle or msl-3 mutants. Their data are consistent with a model in which compensosomes continue to bind site specifically to additional sites after all high-affinity sites are occupied, as opposed to spreading from high-affinity sites as previously proposed. Given these findings, a reasonable scenario as to how dosage compensation is achieved would be the following. As MSL expression begins, the high-affinity sites progressively sequester nascent partial or full complexes in the early stages of dosage compensation. When the amount of available complexes or its components increases, sites of higher affinity would accumulate more complexes, while low-affinity sites would remain undetectable, until the former have preferentially assembled sufficient amounts of complexes to make components available for sites with lower affinities. Thus, the compensosomes would progressively bind to different sites along the X according to the different affinities of these sites. Consistent with our model, we found that in mle or msl-3 mutants, duplications maintain binding of partial complexes at the high-affinity sites (Figure 2N, 2P, and 2S), though with a lower affinity than the same site on the X. The latter observation suggests that, in conditions where components of the complex are limiting, binding might also be dependent on the location of these sequences in the cell (see discussion on spreading below).

That compensosome binding sites would have a range of affinities is also consistent with what is known about DNA-binding proteins, which recognize with varying affinities a range of binding sites whose sequences are related to a common consensus. Variations from the consensus can allow temporal and quantitative modulation of individual genes, or subsets of genes. That compensosome binding sites are also likely to vary in sequence, and hence affinities, comes from what is known about sex chromosome evolution in Drosophila species (Marin et al. 1996, 2000). During the course of sex chromosome evolution in this genus there are a number of cases in which new X chromosomes have evolved, and in all cases examined to date, this has been accompanied by the new X chromosome gradually acquiring compensosome binding sites as the new Y chromosome, its former homologue, degenerates. The selective advantage of dosage compensation for each gene is determined both by the state of degeneration of the allele on the new Y chromosome and by the degree to which a gene in males requires its function, and thus its expression, to match the output of both wild-type female X chromosomes (Marin et al. 2000). Hence, one would expect individually evolved binding sites to exhibit a range of affinities for compensasomes. Finally, we note that each of the final compensosome bands on the X chromosome displays a reproducible but specific intensity, likely to reflect not only different affinities for compensasomes, but also the length of X chromosome encompassed in each band.

The last issue we wish to address is spreading. The fact that, in chromosome rearrangements that juxtapose pieces of X and autosome, we never observed spreading, even when entry sites or roX genes were near the breakpoints, suggests that spreading does not exist naturally on the X chromosome, and is not required to establish the final pattern of binding in Drosophila males. Yet spreading from roX transgenes is very well documented in a variety of situations. We therefore suggest that spreading is a phenomenon specific to the roX transgenes, and a consequence of the key function of roX RNAs in dosage compensation. In particular, we propose that the roX genes are the sites of assembly of compensosomes using newly synthesized roX RNAs, just as the ribosomal RNA genes are the sites where ribosomes are assembled. Thus, roX transgenes would generate a high local concentration of compensosomes in their vicinity, competing with other chromatin-binding factors that normally bind to nearby autosomal sequences. In some cases, compensosomes would displace these other factors, resulting in a new compensosome band in the autosomal region flanking the transgene (spreading). Several features of spreading are consistent with this proposal. First, additional bands corresponding to spreading from roX transgenes contain roX RNA and the H4Lys16 modification, suggesting that they correspond to mature complexes (Kelley et al. 1999). Second, transcription from a roX transgene is required to observe spreading of the complex onto neighboring regions (Park et al. 2002, 2003). Third, roX transgenes show variable and often no additional bands in a wild-type background, suggesting that spreading is largely dependent on the insertion site and its environment on the autosomes. One possibility would be that these roX transgenes lacking spreading are inserted next to sites bound by factors normally counteracting the effect of compensosomes on the autosomes. Such a view is supported by recent data showing that association of compensosomes at some roX1 transgenes can overcome the effect of methylation-mediated silencers (Kelley and Kuroda 2003). Finally, MSL-1 and MSL-2 co-overexpression leads to mislocalization of partial MSL complexes to the autosomes and the centromere, as well as a dramatic decompaction of the X (Oh et al. 2003), a male-specific phenotype also observed in both iswi or nurf mutants, two chromatin regulators (Deuring et al. 2000;
Molecular studies of dosage compensation in flies, worms, and mammals have revealed some striking similarities between these systems. In all three systems dosage compensation is achieved by a widespread modification of the structure of X chromosome chromatin, and in mammals and flies this involves specific modifications of histones. Dosage compensation in mammals and flies is also similar in that noncoding RNAs are essential components of the dosage compensation machinery. With respect to the other components of the dosage compensation machinery, the situation is less clear. While compensosome-related complexes might be present in mammals (orthologs of msl-1, -2, -3, mle, and mof genes exist in mammalian genomes), some of them have identified functions not related to dosage compensation, and orthologs of msl-1, -2, and -3 were not found in Caenorhabditis elegans (Marin 2003). Up until now it had also been thought that spreading was involved in dosage compensation in all three systems (Park et al. 2002; Oh et al. 2003; Csankovszki et al. 2004; Okamoto et al. 2004). However, our findings indicate that in flies each of the bands on the X chromosome at which compensosomes are found in males is able to independently attract those complexes. Thus, at the interband level spreading does not appear to be part of the dosage compensation process in flies. However, it should be noted that our results do not address either how compensosomes are distributed across the tens of kilobases of DNA that likely comprise individual compensosome bands in salivary gland chromosomes, or how that distribution is achieved; it is possible that at the level of single bands, spreading may be part of the process of dosage compensation.

Materials and Methods

Flies strains and genetic crosses. Flies were raised on standard cornmeal-yeast-agar medium. Fly stocks containing transpositions or deletions were obtained from the Bloomington Drosophila Stock Center. Their genotypes are: Tp(1)2b/Y; w1, y1 C(1)DX, y1 w1 f1 (line I); Df(1)1/49, F(1)1/49, Df(1)2/74, f1 (line II); Tp(1)2/74 F(1)1/49, Df(1)3/124 F(1)3/124, Df(1)2/20 (line III); Tp(1)3/124 F(1)2/20 (line IV); Tp(1)3/124 F(1)3/124 F(1)74 FM4 (line V); Tp(1)2/20 F(1)2/20 F(1)74 FM4 (line VI); Tp(1)3/124 F(1)3/124 F(1)1/49 (line VII); Tp(1)3/124 F(1)3/124 F(1)1/49 F(1)1/74 (line VIII); Tp(1)3/124 F(1)3/124 F(1)1/49 F(1)1/74 FM4 (line IX); Tp(1)3/124 F(1)3/124 F(1)1/49 F(1)1/74 FM4 (line X); Df(1)1/49 F(1)1/74 F(1)2/74 F(1)3/124 F(1)2/20 (line XI); Tp(1)2/20 F(1)2/20 F(1)74 FM4 (line XII); Tp(2)/21 F(2)/21 F(2)1/49 F(2)1/74 F(2)1/21 (line XIII); Tp(2)/21 F(2)/21 F(2)1/49 F(2)1/74 F(2)1/21 F(2)1/21 (line XIV); Tp(2)/21 F(2)/21 F(2)1/49 F(2)1/74 F(2)1/21 F(2)1/21 F(2)1/21 (line XV). Breakpoints and insertion site are referred in Table 1. Some lines contain additional rearrangements referenced in Lindsey and Zimm (1992). Depending on their genotype, each line was crossed to Canton-S males or females for studies of MSL binding in their male progeny. For homozygous transpositions studies, stocks were balanced to give w; Tp(1)2b/CyoGFP or w; Tp(1)3/124 F(1)3/124 FM4-GFP stocks. Non-GFP third instar male larvae were dissected for autosomal-to-X transpositions, females from lines XIV and XV were mated with w; msl2A3–21/TyGFP or Dp(A1);1/0; msl2A3–21/TyGFP males. Non-GFP female larvae were dissected. For mle and msl-3 mutant analysis, stocks were balanced to give w; Tp(1)2b/CyoGFP, Tp(1)3/124 F(1)3/124 FM4-GFP or w; msl2A3–21/TyGFP, Tp(1)3/124 F(1)3/124 FM4-GFP stocks. Females were crossed to w; Tp(1)2b/CyoGFP, Tp(1)3/124 F(1)3/124 FM4-GFP, or mle ecd1 dpo2 cl1 females. Non-GFP third instar female larvae were dissected for salivary glands polytene chromosomes analysis. Lines expressing MSL-2 from transgenes msl2A3–21 and msl2A3–10 are described in Bashaw and Baker (1995). Mle and msl-3 mutants are described in Fukunaga et al. (1975), Kuroda et al. (1991), and Gorman et al. (1995). All crosses to generate larvae for immunostaining were carried out at 18°C.

Polytene chromosome immunostaining. Glands from male third instar larvae were dissected in PBS/0.7% NaCl, prefixed in 45% acetic acid for 10 s, and then fixed for 2–3 min in lactic acid/20% acetic acid (1:2.3) on siliconized coverslips. Glands were squashed and coverslips flipped off after freezing the slides in liquid nitrogen. Slides were then incubated in PBS for 15 min followed by incubation with affinity-purified anti-MSL-1 antibodies (dilution 1:100) as described previously (Gorman et al. 1995). Chromosomes were viewed under epifluorescence optics on a Zeiss Axioskop microscope or a confocal microscope; pictures were taken using Spot software and colored.

Immunofluorescent in situ hybridization of polytene chromosomes. Clones RP-98 17E:2, RP-98 03D:13, and RP-98 48O:22 were obtained from the Drosophila melanogaster BAC library (BACPAC Resources, Oakland, California, United States) were used to map regions 3D–E, 3C, and 2D–3A2, respectively. Specific probes were obtained from BAC clone DNA preparations using the Bionick Labelling System (Invitrogen, Carlsbad, California, United States) according to the manufacturer’s instructions. Squashes were prepared as described above. Immunostaining with affinity-purified anti-MSL-1 antibodies was followed by incubation with the appropriate biotinylated probe according to the method of Lavrov et al. (2004).

Supporting Information

Accession Numbers

The LocusLink (http://www.ncbi.nlm.nih.gov/LocusLink) accession numbers for the genes and gene products discussed in this paper are jil-1 (LocusLink 39241), mle (LocusLink 35523), mof (LocusLink 31180), msl-1 (LocusLink 35121), msl-2 (LocusLink 35563), msl-3 (LocusLink 38779), roX1 (LocusLink 43963), roX2 (LocusLink 44673), and SXL (LocusLink 44872).

Acknowledgments

We thank Greg Bashaw for the msI2A10 and msI2A21 lines, and would like to express our gratitude to Victoria Muller, Joseph Lipsick, Liqun Luo, and the Bloomington Stock Center for providing stocks, and to Marc Tessler-Lavigne for the use of the microscope. Zienia Fertil gave inestimable help with squashes, and Guennet Bohm with skillful preparation of fly media. We thank members of the Bruce Baker and Mike Simon labs for helpful discussions and encouragement, and Mark Siegal and Dev Manoli for comments on the manuscript. This work is supported by grants from the National Institute of General Medical Sciences.

Conflicts of interest.

The authors have declared that no conflicts of interest exist.

Author contributions.

DF and BSB conceived and designed the experiments, performed the experiments. DF and BSB analyzed the data. DF and BSB wrote the paper.

References

Akhtar A, Becker PB (2001) The histone H4 acetyltransferase MOF uses a GH2 zinc finger for substrate recognition. EMBO Rep 2: 113–118.

Amrein H, Axel R (1997) Genes expressed in neurons of adult male Drosophila. Cell 88: 450–469.

Badenhorst P, Vos M, Rebay I, Wu C (2002) Biological functions of the ISWI chromatin remodeling complex NURF. Genes Dev 16: 3186–3198.

Baker BS, Gorman M, Marin I (1994) Dosage compensation in Drosophila. Annu Rev Genet 28: 491–521.

Bashaw GJ, Baker BS (1995) The msl-2 dosage compensation gene of Drosophila encodes a putative DNA-binding protein whose expression is sex specifically regulated by Sex- lethal. Development 121: 3245–3258.

Bashaw GJ, Baker BS (1997) The regulation of the Drosophila msl-2 gene reveals a function for Sex- lethal in translational control. Cell 90: 789–798.

Corona DF, Clapier CR, Becker PB, Tamkun JW (2002) Modulation of ISWI chromatin-remodeling protein is required for gene expression and the function by site-specific histone acetylation. EMBO Rep 3: 242–247.

Castrignanii G, McDonald SE, Ayerley JH (2004) R-mutant and Ring of the C. elegans dosage compensation complex along X chromosomes. Science 303: 1182–1185.

Demakova OV, Koliakov IV, Gordanz PE, Alekseyenko AA, Kuroda MI, et al. (2005) The MSL complex levels are critical for its correct targeting to the chromosomes in Drosophila melanogaster. Chromosoma 112: 105–115.

Deuring R, Fant I, Armstrong JA, Sarte M, Papoulos O, et al. (2000) The ISWI chromatin-remodeling protein is required for gene expression and the Dosage Compensation in Drosophila.
maintenance of higher order chromatin structure in vivo. Mol Cell 5: 355–365.
Franke A, Baker BS (1999) The rox1 and rox2 RNAs are essential components of the compensosome, which mediates dosage compensation in Drosophila. Mol Cell 4: 117–122.
Franke A, Baker BS (2000) Dosage compensation rox1. Curr Opin Cell Biol 12: 351–354.
Fukunaga A, Tanaka A, Oishi K (1975) Maleless, a recessive autosomal mutant of Drosophila melanogaster that specifically kills male zygotes. Genetics 81: 135–141.
Gorman M, Franke A, Baker BS (1995) Molecular characterization of the male-specific lethal-3 gene and investigations of the regulation of dosage compensation in Drosophila. Development 121: 465–475.
Hilfiker A, Hilfiker-Kleiner D, Pannuti A, Lucchesi JC (1997) mof, a putative acetyl transferase gene related to the Tip60 and MOZ human genes and to the SAS genes of yeast, is required for dosage compensation in Drosophila. EMBO J 16: 2054–2060.
Kageyama Y, Mengus G, Gilfillan G, Kennedy HG, Stuckenholz C, et al. (2001) Association and spreading of the Drosophila dosage compensation complex from a discrete roX1 chromatin entry site. EMBO J 20: 2236–2245.
Kelley RL, Kuroda MI (2003) The Drosophila roX1 RNA gene can overcome silent chromatin by recruiting the male-specific lethal dosage compensation complex. Genetics 164: 565–574.
Kelley RL, Wang J, Bell L, Kuroda MI (1997) Sex lethal controls dosage compensation in Drosophila by a non-splicing mechanism. Nature 387: 195–199.
Kelley RL, Meller VH, Gordadze PR, Roman G, Davis RL, et al. (1999) Epigenetic spreading of the Drosophila dosage compensation complex from roX RNA genes into flanking chromatin. Cell 98: 513–522.
Kuroda MI, Kernan MJ, Kreber R, Ganetzky B, Baker BS (1991) The maleless protein associates with the X chromosome to regulate dosage compensation in Drosophila. Cell 66: 935–947.
Lavrov S, Dejardin J, Cavalli G (2004) Combined immunostaining and FISH analysis of polytene chromosomes. Methods Mol Biol 247: 289–303.
Lyman LM, Copps K, Rastelli I, Kelley RL, Kuroda MI (1997) Drosophila male-specific lethal-2 protein: Structure/function analysis and dependence on MSL-1 for chromosome association. Genetics 147: 1743–1753.
Marin I (2005) Evolution of chromatin-remodeling complexes: Comparative genomics reveals the ancient origin of “novel” compensosome genes. J Mol Evol 56: 527–539.
Marin I, Franke A, Bashaw GJ, Baker BS (1996) The dosage compensation system of Drosophila is co-opted by newly evolved X chromosomes. Nature 383: 160–163.
Marin I, Siegal ML, Baker BS (2000) The evolution of dosage-compensation mechanisms. Bioessays 22: 1106–1114.
Meller VH (2000) Dosage compensation: Making 1X equal 2X. Trends Cell Biol 10: 54–59.
Meller VH, Wu KH, Roman G, Kuroda MI, Davis RL (1997) roX1 RNA paints the X chromosome of male Drosophila and is regulated by the dosage compensation system. Cell 88: 445–457.
Oh H, Park Y, Kuroda MI (2003) Local spreading of MSL complexes from roX genes on the Drosophila X chromosome. Genes Dev 17: 1334–1339.
Oh H, Bone JR, Kuroda MI (2004) Multiple classes of MSL binding sites target dosage compensation to the X chromosome of Drosophila. Curr Biol 14: 481–487.
Okamoto I, Orte AP, Allis CD, Reinberg D, Heard E (2004) Epigenetic dynamics of imprinted X inactivation during early mouse development. Science 303: 644–649.
Park Y, Kelley RL, Oh H, Kuroda MI, Meller VH (2002) Extent of chromatin spreading determined by roX RNA recruitment of MSL proteins. Science 298: 1620–1623.
Park Y, Mengus G, Bai X, Kageyama Y, Meller VH, et al. (2003) Sequence-specific targeting of Drosophila roX genes by the MSL dosage compensation complex. Mol Cell 11: 977–986.
Smith ER, Pannuti A, Gu W, Steurnagel A, Cook RG, et al. (2000) The Drosophila MSL complex acetylates histone H4 at lysine 16, a chromatin modification linked to dosage compensation. Mol Cell Biol 20: 312–318.
Stuckenholz C, Kageyama Y, Kuroda MI (1999) Guilt by association: Non-coding RNAs, chromosome-specific proteins and dosage compensation in Drosophila. Trends Genet 15: 454–458.
Wang Y, Zhang W, Jin Y, Johansen J, Johansen KM (2001) The JIL-1 tandem kinase mediates histone H3 phosphorylation and is required for maintenance of chromatin structure in Drosophila. Cell 105: 433–443.