Dosage Compensation Regulatory Proteins and the Evolution of Sex Chromosomes in Drosophila

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ABSTRACT

In the fruitfly Drosophila melanogaster, the four male-specific lethal (msl) genes are required to achieve dosage compensation of the male X chromosome. The MSL proteins are thought to interact with cis-acting sites that confer dosage compensation to nearby genes, as they are detected at hundreds of discrete sites along the length of the polytene X chromosome in males but not in females. The histone H4 acetylated isoform, H4Ac16, colocalizes with the MSL proteins at a majority of sites on the D. melanogaster X chromosome. Using polytene chromosome immunostaining of other species from the genus Drosophila, we found that X chromosome association of MSL proteins and H4Ac16 is conserved despite differences in the sex chromosome karyotype between species. Our results support a model in which cis-acting regulatory sites for dosage compensation evolve on a neo-X chromosome arm in response to the degeneration of its former homologue.

The prevailing theory for the evolution of sex chromosomes is that they are derived from former homologues that have evolved to become morphologically and genetically distinct X and Y (or Z and W) chromosomes (Westergaard 1958; White 1973; Bull 1983). In the genus Drosophila, males are heterogametic (XY) and females are homogametic (XX). The Y chromosome is postulated to be the former homologue of an ancestral X chromosome, which over time degenerated to retain only a few functional genes, most notably a cluster of rRNA genes and those loci encoding male-fertility functions (Charlesworth 1978; Steinemann et al. 1993). Dosage compensation, in the form of increased expression of genes on the X chromosome in males, is thought to be a response to the decline in the function of genes on its former homologue (see Charlesworth 1978; Lucchesi 1987; Steinemann et al. 1993; Charlesworth 1996 and references therein).

The products of the male-specific lethal genes are required for dosage compensation of the male X chromosome in Drosophila melanogaster. The MSL proteins colocalize at hundreds of sites on the polytene male X chromosome and are associated with an approximate twofold up regulation in transcription of most of the resident genes (Kuroda et al. 1991; Bone et al. 1994; Bashaw and Baker 1995; Gorman et al. 1995; Kelley et al. 1995; Zhou et al. 1995). In addition to the MSL proteins, an acetylated isoform of histone H4 is predominantly associated with the male X (Turner et al. 1992). This histone H4 (H4Ac16), acetylated at lysine 16, exhibits an immunostaining pattern largely coincident with the MSL proteins and is dependent on function of the msl genes (Bone et al. 1994). Thus, the biochemical basis for dosage compensation in Drosophila may depend on sex-specific alterations in chromatin structure, a hypothesis put forward in studies that predated molecular characterization of the process (Dobzhansky 1957; Belote and Lucchesi 1980).

Multiple cis-acting regulatory sites for dosage compensation in D. melanogaster are thought to be dispersed throughout the X chromosome, but despite substantial genetic evidence for their existence, these hypothetical cis-acting sites have yet to be isolated or defined (reviewed in Lucchesi and Manning 1987; Baker et al. 1994). They are presumed to be analogous to the hundreds of sites of interaction of the MSL proteins with the X chromosome (Kuroda et al. 1991). No consensus sequence associated with X-linked genes has been documented, but discrete portions of the X chromosome are still up-regulated in males when transposed to another chromosome, demonstrating that dosage compensation acts at least partially at a very local level (Scholnick et al. 1983; Spradling and Rubin 1983; Hazelrigg et al. 1984; Levis et al. 1985; Gutierrez et al. 1989; Cooper et al. 1994; Roseman et al. 1995; Qian and Pirrotta 1995). Presumably, such multiple sites would not arise simultaneously, but were acquired over time, as homologous gene functions on the proto-Y chromosome degenerated (Charlesworth 1996).

Within the genus Drosophila, there are naturally occurring variations in sex chromosome karyotype (Figure 1) that can be surveyed to test the idea that multiple regulatory sites for dosage compensation are acquired.
when a chromosome arm becomes functionally hemizygous in males. For example, *D. melanogaster* has an X chromosome consisting of a single acrocentric arm, which is thought to be homologous to the acrocentric X chromosome of the primogenitor Drosophilid species (Müller’s element A) (PATTERSON and STONE 1952). Several Drosophila species, however, possess metacentric X chromosomes that are the apparent result of fusion events between element A and an autosomal element in an ancestral species. Such a fusion event would result in males with one metacentric X chromosome, the original Y chromosome, and a neo-Y chromosome consisting of a freely segregating homologue of the newly fused neo-X chromosome arm. Depending on the amount of time since the fusion event, the neo-Y chromosome may still be active, may be partially degenerated, may be inert or even absent. The degeneration of this homologue would lead to a requirement for dosage compensation of the formerly autosomal neo-X chromosome arm.

In this study, we examine Drosophila species with alternative sex chromosome karyotypes to determine whether or not the MSL proteins participate in dosage compensation of a previously autosomal arm, which during evolution was rendered hemizygous in males. Our results are consistent with the hypothesis that cis-acting sites for the MSL proteins can be acquired on previously unrelated chromosome arms during evolution. A correlation can be made between the age of the fusion event creating a neo-X chromosome arm, the state of degeneration of the former homologue (the neo-Y), and the acquisition of sites for MSL protein and histone H4Ac16 association.

**MATERIALS AND METHODS**

**Fly stocks:** Larvae were raised at 25°C on standard cornmeal-yeast-agar-molasses medium, containing propionic acid as an antifungal agent. Stocks were obtained from the National Drosophila Species Resource Center at Bowling Green State University. The *D. miranda* Ma32 strain was obtained from Dr. Brian Charlesworth.

**Preparation of antisera:** Affinity-purified anti-MLE and anti MSL-1 antibodies were prepared essentially as described previously (KURODA et al. 1991; PALMER et al. 1993). Preparation and characterization of antisera specific for acetylated histone H4 isoforms can be found in TURNER and FELLOWS (1989) and TURNER et al. (1989).

**Immunostaining of polytene chromosomes:** Polytenic chromosome preparation was performed as published previously (BONE et al. 1994). Chromosomes were viewed using epifluorescence optics with a Nikon Microphot-FXA microscope and photographed with Kodak Ektachrome 400 slide film. The resulting slides were scanned onto Kodak Photo CD (National Photo Lab, Houston, TX) and composite figures were prepared using Photoshop 3.0 (Adobe Systems, Mountain View, CA) and printed out on a Phaser IISDX printer (Tektronix Corp., Seattle, WA).
Evolution of Dosage Compensation

Western blot analysis: Western blot analysis was performed as previously described (PALMER et al. 1993).

RESULTS

Within the genus Drosophila, there are several naturally occurring variations in sex chromosome karyotype. To determine whether dosage compensation regulatory proteins are restricted to chromosomes that are homologous to the *D. melanogaster* X or are found on any chromosome arm that is functionally hemizygous in males, we performed polytene chromosome immunostaining on a variety of Drosophila species. The species included in this study were selected based on their chromosome arrangement and evolutionary distance from *D. melanogaster* (Figure 1). Immunostaining was performed using antisera raised against the *D. melanogaster* maleless (MLE) and male-specific lethal-1 proteins (MSL-1) and antibodies to a synthetic histone H4 peptide acetylated at lysine 16 (H4Ac16) (KURODA et al. 1991; TURNER et al. 1992; PALMER et al. 1993). The species examined fall into four general categories with regard to their sex chromosome karyotype (Figure 1).

**Chromosome staining of *D. simulans* and *D. virilis***: The first group of species examined have karyotypes similar to *D. melanogaster*, with an acrocentric X chromosome corresponding to MULLER'S element A, and a single Y chromosome that carries male fertility functions. Within this group are species that are highly related to *D. melanogaster*, such as *D. simulans*, and much more distant relatives, such as *D. virilis* (PATTERSON and STONE 1952). Figure 2 shows immunolocalization of MLE, MSL-1 and H4Ac16 in polytene chromosome squashes from *D. simulans* (Figure 2, A–C) and *D. virilis* (Figure 2, D–F). The respective antisera generate a strong signal on the polytene X chromosome in males of both species, as is seen for *D. melanogaster* (KURODA et al. 1991; TURNER et al. 1992; PALMER et al. 1993). These results demonstrate that the X chromosome proteins associated with dosage compensation in *D. melanogaster* are sufficiently conserved to allow significant antibody cross-reaction. The evolutionary distance between *D. melanogaster* and *D. virilis* is estimated to be 60,000,000 yr (SPICER 1988).

**Chromosome staining of *D. a. americana* and *D. pseudoobscura***: The second type of sex chromosome configuration examined, represented by *D. americana americana*, consists of species that carry a metacentric X chromosome with one arm corresponding to MULLER'S element A and the second arm homologous to MULLER'S element B. Element B corresponds to chromosome 2L in *D. melanogaster* (PATTERSON and STONE 1952). The fusion of element B to the X chromosome in *D. a. americana* is thought to be a quite recent event, estimated to have taken place a few hundred thousand years ago (THROCKMORTON 1982; SPICER 1991; TOMINAGA et al. 1992; NURMINSKY et al. 1996). The free homologue of element B (the neo-Y) is present in males and shows no sign of degeneration (B. CHARLESWORTH, personal communication). Because element B is not
functionally hemizygous in males, it should not require dosage compensation. Consistent with this hypothesis, anti-MLP, anti-MSL-1, and anti-H4Ac16 all stain the D. a. americana X chromosome arm that is homologous to element A, but no immunostaining is detectable on the arm related to element B (Figure 3, A–C). This result confirms previous observations that dosage compensation in Drosophila is a local rather than chromosome wide process, as the linkage of large pieces of autosomal material, or in this case a whole autosomal arm, does not result in detectable spreading of the process to the translocated genetic material (MULLER and KAPLAN 1966; LAKHOTIA 1970; ROEHRDANZ et al. 1977).

The third group of species examined, represented by D. pseudoobscura, carry a metacentric X chromosome with one arm (XL) corresponding to MULLER’s element A and the second arm (XR) homologous to MULLER’s element D (autosomal 3L in D. melanogaster). This appears to be the result of a much older translocation estimated to have taken place 13,000,000 yr ago (RUSSO et al. 1995). In species with this sex chromosome configuration, there is no apparent remnant of the former homologue of element D in males, which has presumably degenerated and been lost. Thus, element D in D. pseudoobscura is hemizygous in males and is subject to dosage compensation (ABRAHAM and LUCCHESI 1973, 1974; MUKHERJEE and CHATTERJEE 1975; PIERCE and LUCCHESI 1979; SASS and MISELSON 1991). Consistent with the need to dosage compensate two X chromosome arms in this species, we detect MLE, MSL-1, and histone H4Ac16 on both XL and XR in D. pseudoobscura (Figure 3, D–F). Similar results, using the same source of histone H4Ac16 antibodies, have been reported independently (STEINEMANN et al. 1996). These results are in striking contrast to the restriction of dosage compensation proteins to only one arm of the metacentric X chromosome in D. a. americana, and are consistent with a model in which selective forces and/or a long period of time are required for the accumulation of MSL binding sites on a newly acquired X chromosome arm (CHARLESWORTH 1996).

**Chromosome staining of D. miranda:** The fourth category is represented by D. miranda, a species that is closely related to D. pseudoobscura and carries the same type of metacentric X chromosome (element A fused to element D) without any remnant of the former free homologue of element D. However, D. miranda also contains a chromosome referred to as X2, or MULLER’s element C, that exists in two copies in females, and in one copy in males. The homologue of element C in males is fused to the heterochromatic Y chromosome, but retains stretches of euchromatin (MACKNIGHT 1939). The fusion event is thought to have occurred ~2,000,000 yr ago (NORMAN and DOANE 1990; RUSSO et al. 1995). There is evidence that the Y-linked C element is in the process of degeneration (STEINEMANN 1983; GANGULY et al. 1992; STEINEMANN and STEINEMANN 1992) and that the X2 chromosome in males is heterogeneous for hypertranscription, with distinct regions that are fully or partially dosage compensated.
and a few regions that fail to compensate (Strobele et al. 1978; Das et al. 1982). We found that anti-ML-, anti-ML-1, and anti-H4Ac16 stain both XL and XR chromosome arms of D. miranda in a densely banded pattern, as in D. pseudoobscura (Figure 4). In addition, ~20 strong or moderately staining sites are distributed along the length of the X2 chromosome arm (Figures 4, arrows, and 5), consistent with the partial dosage compensation measured previously (Strobele et al. 1978; Das et al. 1982). Detection of histone H4Ac16 on the X2 of D. miranda has been reported independently by M. Steinemann, S. Steinemann, and B. M. Turner (Steinemann et al. 1996). Significantly, we found that histone H4Ac16 and the MSLs colocalize at a majority of the sites on the X2 chromosome (Figure 5 and data not shown). The patterns are nearly identical, but differences in signal intensity exist when the two patterns are compared side by side. This is similar to the comparison of MSL and H4Ac16 patterns on the D. melanogaster X chromosome and may be a result of the sensitivity of acetylated histones to the polytene chromosome staining procedure (Bone et al. 1994).

Polytene chromosome squashes from several other Drosophila species were also immunostained, and the results are summarized in Figure 6. In each case, the arms of the hemizygous male X chromosome stained positively for MLE, MSL-1, and histone H4Ac16. No specific X chromosome immunostaining was observed in chromosome squashes of females from any of the Drosophila species studied (data not shown). However, scattered autosomal sites for MLE staining were observed in both sexes in all species examined. Autosomal sites for MLE are also seen in D. melanogaster. The significance of these sites is not known, but the majority of sites are absent in mle mutants, suggesting that they are not simply due to cross-reaction of anti-ML- antibodies with related proteins (Kuroda et al. 1991). In addition to the autosomal MLE sites observed in all species, staining of the chromocenter was seen in all species from the subgenus Sophophora, but not in species from the subgenus Drosophila. This staining was most striking in species from the Obscura group (arrowheads, Figures 3D and 4A). Whether this immunostaining represents MLE or is a cross-reaction is not known.

**Detection of MLE and MSL-1 by Western blot:** Western blot analysis was performed using whole extracts from third instar larvae of selected species. As seen in Figure 7A, a strong band that is the same apparent size as the D. melanogaster MLE protein is observed in both males and females of every species studied. We com-
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**Figure 6.**—Phylogenetic relationships and summary of chromosome staining. Only data from male larvae are included. Next to each species is the arrangement of the metaphase X chromosome. In most cases, XL (XS in *D. azteca*) refers to Muller's element A. In the case of *D. ananassae*, both XL and XR refer to Muller's element A, which underwent a telomere-centromere fusion and a subsequent chromosome breakage event, generating two chromosome arms from one (Fatterson and Stone 1952). For all species except *D. americana americana*, XR (XL in *D. azteca*) refers to Muller's element D. In *D. a. americana*, XR and X2 refer to the two homologues of Muller's element B. X2 refers to Muller's element C in *D. miranda*. Chromosome arms that stain positively with the antisera for MLE, MSL-1, and H4Ac16 are listed in the column under the particular antisera. N.D., not determined.

It is concluded that MLE is highly conserved throughout the genus Drosophila and that it is not regulated at the level of sex-specific expression in any of the species tested. The male-specific X chromosomal association of MLE in all species is likely to be due to protein-protein interactions with the sex-specific MSL proteins such as MSL-1 and MSL-2, as postulated to occur in *D. melanogaster* (Gorman et al. 1993; Palmer et al. 1994; Kelley et al. 1995).

Antibodies to MSL-1 were reacted with a duplicate Western blot (Figure 7B). MSL-1 is detected in *D. melanogaster* males, but is in extremely low abundance in females (lanes 1 and 2) as seen in previous studies. A strong band of approximately the same mobility is seen in the males (*D. melanogaster* males in Figure 7B).

**Figure 7.**—Western blot analysis of several Drosophila species using antibodies specific for either the *D. melanogaster* MLE protein (A) or MSL-1 protein (B). Male and female extracts are shown for each species. The migration distance of the *D. melanogaster* proteins are indicated by arrows in each panel.
in *D. simulans* males and is only weakly detected in females (lanes 3 and 4), suggesting that the sex-specificity of MSL-1 is conserved in this species. However, in other species studied, we were unable to identify a male-specific band of similar mobility as *D. melanogaster* MSL-1 (data not shown). It is possible that the degree of conservation of MSL-1 in the more distantly related species is not high enough to allow detection by Western blot. The difficulty to detect MSL-1 in heterologous species may be exacerbated by its relative instability in protein extracts (R. Richman and M. I. Kuroda, unpublished results).

**DISCUSSION**

We have found a correlation between the functional hemizygosity of chromosome arms in *Drosophila* males, and the acquisition of sites for MSL and H4Ac16 protein association, indicative of dosage compensation. In species with distinct sex chromosome karyotypes, the length of time since the fusion event creating neo-X and neo-Y chromosome arms correlates with the state of degeneration of the neo-Y and the acquisition of MSL and H4Ac16 binding sites on the neo-X. Our results are consistent with a model for the co-evolution of degenerated Y chromosomes with dosage compensation of the remaining X chromosome (reviewed in Charlesworth 1996).

We studied three types of sex chromosome karyotype that are distinct from the *D. melanogaster* arrangement. Two of the examples are straightforward. *D. pseudoobscura* has an acquired X chromosome arm with no cytological or genetic evidence of its former homologue. The relatively ancient (ca. 13,000,000-yr-old) neo-X chromosome arm is known to be dosage compensated and exhibits a density of MSL and H4Ac16 binding sites on the neo-X. Our results are consistent with a model for the co-evolution of degenerated Y chromosomes with dosage compensation of the remaining X chromosome arm. At the other extreme, *D. americana americana* has an acquired Y chromosome arm that has a functional free homologue. This configuration is thought to have arisen relatively recently (several hundreds of thousands of years ago), and *D. a. americana* exhibits no MSL or H4Ac16 binding sites on this neo-X arm, despite its fusion to the primordial X chromosome.

Only one intermediate situation was available to examine dosage compensation on an evolving X chromosome. In *D. miranda*, a free neo-X chromosome is estimated to have appeared at most 2,000,000 yr ago. Its homologue is fused to the Y chromosome, and signs of its presumed degeneration have been observed (Steinemann and Steinemann 1991, 1992; Ganguly et al. 1992). Several studies indicate that the X2 is composed of many regions that exhibit dosage compensation interspersed among regions that do not dosage compensate (Strobel et al. 1978; Das et al. 1982; Norman and Doane 1983, 1990; Forsyth and Cobbs 1984; Krishnan et al. 1991). We found that the MSL proteins and histone H4Ac16 are detected at multiple discrete sites on the neo-X (X2) of *D. miranda*. The regions of the X2 that stain with MSL antibodies correspond to many regions previously found to be partially or fully dosage compensated (Strobel et al. 1978; Das et al. 1982). A more detailed analysis will be required to determine the extent of the correlation, but our data strongly support the hypothesis that dosage compensation is evolving on the X2 chromosome as its former homologue degenerates.

Our results are in agreement with the idea that dosage compensation is acquired piecemeal, under gene-by-gene selection as the neo-Y chromosome degenerates (Charlesworth 1996). Understanding how this occurs will require a much better understanding of the hypothetical cis-acting sites that govern dosage compensation. That dosage compensation was acquired by regions of the *D. miranda* X2 chromosome over a relatively short period of time suggests that the sequence of the cis-acting sites may be very simple. Interestingly, a correlation has been observed between the acquisition of dosage compensation on chromosome arms in *Drosophila* species and enrichment for specific dinucleotide repeat sequences (Pardue et al. 1987; Lowenhaupt et al. 1989). In future studies, a more detailed analysis of the *D. miranda* X2 chromosome may determine whether the enrichment for a particular sequence composition or repetitive element maps specifically to segments where dosage compensation has evolved.

There is a growing body of evidence that regulation of chromatin composition and structure provides the basis, at least in part, for hypertranscription of the male X chromosome. The presence of a specific acetylated form of histone H4 on the male X could be a critical component of this chromatin difference, but it has not been possible to test this compelling idea experimentally. However, by studying the evolution of dosage compensation, we have found that histone H4Ac16 is coincident with the acquisition of MSL association in all *Drosophila* species tested. Thus, our results provide further support for the hypothesis that histone acetylation is integral to the dosage compensation mechanism.

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