RESEARCH ARTICLE

Cold-induced alteration in the global structure of the male sex chromosome of $In(1)B^{M2}(reinverted)$ of *Drosophila melanogaster* is associated with increased acetylation of histone 4 at lysine 16

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Abstract

In *Drosophila melanogaster*, dosage compensation occurs through hypertranscription of sex-linked genes in males. The hypertranscription involves acetylation of histone 4 at lysine 16 (H4K16) on a male X-chromosome, brought about by a histone acetyltransferase encoded by the dosage compensation gene, *males absent on the first* (*mof*). We report a phenomenon in the strain $In(1)B^{M2}(reinverted)$ of *D. melanogaster* where the global structure of the male X-chromosome can be altered at the third instar larval stage through a 4-h cold shock at $12\pm1°$C. We show that the cold shock results in a transient hyperacetylation of H4K16 and an increased expression of *MOF*. Control proteins H4 acetylated at lysine 5, and the dosage compensation gene *msl-2*, do not show any change in expression after cold shock. Cytology of the male X-chromosome at different time points during cold shock and recovery, suggests that the hyperacetylation of H4 at lysine 16 causes the X-chromosome to corkscrew into itself, thereby achieving the cold-induced change in the higher order structure of the male polytene X-chromosome. Our studies suggest a role for H4K16 in maintaining the structure of the male X-chromosome in *Drosophila*.

Introduction

The strain $In(1)B^{M2}(reinverted)$ of *Drosophila melanogaster* originated from a spontaneous reinversion of the X-chromosome region 16A1-5; 20 F in $In(1)B^{M2}$ (Mazumdar *et al*. 1978). Chromosome prepared from a single pair of salivary glands show extremely puffy and diffuse male X-chromosomes ($18\pm1°$C; figure1,a) in about 25% to 30% polytene nuclei (table 1). The global alteration in the structure of the male polytene X-chromosome occurs only when the cultures are reared at $18\pm1°$C. The sex-specific and chromosome-specific structural alteration of the male X-chromosome can also be induced at the third instar larval stage through a 4-h cold shock at $12\pm1°$C, followed by recovery at room temperature for 2 h (Kar *et al*. 2000, table 1). The structure of the female X-chromosomes and autosomes remains unaffected. The transcription of these puffy X-chromosomes remains at the wild-type state, despite the chromosome resembling a giant puff (Kar and Pal 1995). Lack of perturbation in transcription is reflected in the fact that there is no larval lethality, and the sex ratio remains unaffected, even after continuous development at $18\pm1°$C. The adult flies are normal in morphology and fecundity. However, the strain shows marked developmental retardation and female semilethality when reared on medium containing 0.2 M sodium butyrate (Dey-Guha and Kar 2001). Earlier studies suggest that the phenotype arises due to position effect variegation (Bose and Duttaroy 1986).

The sex-specific and chromosome-specific alteration in the global structure of the male X-chromosome in $In(1)B^{M2}(reinverted)$ larvae is reminiscent of the phenotype seen in dosage compensation mutants, where loss-of-function mutations result in perturbation of the structure/function of the male X-chromosome (Lucchesi *et al.* 2005). Dosage compensation and the phenomenon of

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Table 1. Proportion of nuclei manifesting alteration in the structure of the X-chromosome in polytene nuclei of third instar *In(1)B^{M2}(reinverted)* larvae reared at 24±1°C, 18±1°C, or subjected to cold shock for 4 h at 12±1°C followed by recovery at room temperature for 1 h.

Rearing temperature	Male		Female	
	Normal X's	Puffy X's	Normal X's	Puffy X's
24±1°C	526	1 ± 1.4	530	0
18±1°C	341	33 ± 4.0	475	0
12±1°C	323	11 ± 4.0	307	0

equalization of sex linked gene products is brought about in *Drosophila* by hypertranscription of genes on the male X-chromosome (Mukherjee and Beermann 1965). Increased transcription is achieved through a ribonucleoprotein complex made up of the protein products of the *male-specific lethal* genes, *MSL1* (Palmer *et al*. 1993), *MSL2* (Zhou *et al*. 1995), *MSL3* (Gorman *et al*. 1995), *MLE* (*maleless*; Kuroda *et al*. 1991), *MOF* (*males absent on the first*; Hilfiker *et al*. 1997) and two untranslated RNAs (Akhtar 2003). This complex localizes on the male X-chromosome, where proteins involved in chromatin modification facilitate the two-fold increase in transcription of the sex-linked genes of the male relative to that of the female (reviewed in Lucchesi *et al*. 2005; Straub *et al*. 2005; Mendjan and Akhtar 2006). A key modification of the X-chromatin is brought about by the histone acetyltransferase encoded by the *mof* gene which effects a unique acetylation of histone 4 at lysine 16 on the male X-chromosome. Other proteins involved in chromatin remodelling in this context include the histone kinase encoded by the *jil-1* gene (Jin *et al*. 2000), and protein products of chromatin remodelling proteins, *ISWI* (Corona *et al*. 2002), Trl (Greenberg *et al*. 2003), and the supercoiling factor (Furuhashi *et al*. 2006).

Acetylated H4K16 is a general exception to the observed functional relationship between histone acetylation and gene activation. The exact role of acetylated H4K16 in achieving hypertranscription remains unknown (Straub *et al*. 2005). Genomewide studies to correlate transcription and histone acetylation have found a negative correlation between acetylated H4K16 and transcription (Kurdistani *et al*. 2004). A role for H4K16 in maintenance of the structure rather than transcription of the chromatin is supported by recent structural studies suggesting that H4K16 acetylation modulates higher order chromatin structure by inhibiting the formation of the 30-nm chromatin fibre (Shogren-Knaak and Peterson 2006; Shogren-Knaak *et al*. 2006). This role for acetylated H4K16 in chromatin structural organization is also evident from the diffuse chromatin morphology of yeast where nearly 80% of the genome contains this acetylated isoform of histone 4. A similar observation can be made from the cytology of the male X-chromosome in flies. The male X-chromosome is exclusively acetylated at lysine 16

of histone 4 and appears puffy and diffused when compared with other chromosomes. In this report, we show that cold shock of *In(1)B^{M2}(reinverted)* larvae results in an increased acetylation of H4 at K16. The increased acetylation is accompanied by a higher order alteration in the structure of the male X-chromosome, suggesting that the increased acetylation may be responsible for the global alteration in the male X-chromosome structure in this strain.

Materials and methods

Strain

The original strain was recovered as a spontaneous reinversion in the strain *In(1)B^{M2} (In(1)B;16A1-5;20F)* (Mazumdar *et al*. 1978), and has been described earlier (Kar and Pal 1995; Kar *et al*. 2000; Dey-Guha and Kar 2001; Dey-Guha 2002; Kulkarni-Shukla 2006).

Temperature shift experiments

In(1)B^{M2}(reinverted) routinely reared at 24±1°C were cold shocked by reducing the temperature to 12±1°C at the time points mentioned in the text. Recovery from cold shock was allowed at room temperature (24±1°C). The polytene chromosomes were prepared using routine methods (Ashburner 1989).

Western blot analysis

Histones were isolated using the method of Turner *et al*. (1992). Total soluble proteins were extracted in buffered Ringer (6.5 M NaCl, 0.14 M KCl, 0.2 M NaHCO$_3$, 0.01 M NaH$_2$PO$_4$.2H$_2$O, 0.12 M CaCl$_2$.2H$_2$O) containing 1mM phenyl-methyl sulphonyl fluoride. Histones (60 µg) were separated on 15% SDS-PAGE mini-gels, whilst total soluble proteins were separated on 10% SDS-PAGE. After electrotransfer, nitrocellulose membranes were probed with antibodies to either histone H4 acetylated at lysine 16 (Serotec, Oxford, UK), histone H4 acetylated at lysine 5, (Serotec, Oxford, UK), or anti-*mof* or anti-*msl-2* antibodies (Santa Cruz, California, USA). Signal detection was done using the Sigma Chemiluminescence detection kit (Sigma-Aldrich, St Louis, USA).

Results

The structure of the male X-chromosome and autosomes of third instar male *In(1)B^{M2}(reinverted)* larvae reared at 24±1°C are identical in morphology to that of Oregon-R larvae. Structural alteration of the male X-chromosome could be induced in 1% to 7% polytene nuclei by exposing third instar *In(1)B^{M2}(reinverted)* larvae to cold shock at 12±1°C for 4 h (figure 1,b). Permitting salivary glands to recover at 24±1°C after cold-shock resulted in an increase in the number of nuclei manifesting puffy X-chromosomes (figure 1,b). The transition from normal to puffy X-chromosome morphology was reversible, since by 6 h of recovery at room

temperature from a 4-h cold shock, all male larvae manifested X-chromosomes with normal morphology. There was no larval lethality associated with the process of cold shock and recovery from cold shock (data not shown). Prolonged exposures of third instar larvae to cold shock at $12\pm1^\circ$C for 24 h did not result in an increase in the number of nuclei manifesting puffy X-chromosomes (figure 1,b). The structure of autosome or the female X-chromosome was unaffected by cold-shock demonstrating, sex-specificity and chromosome-specificity of the phenotype.

Based on our earlier reports of butyrate-sensitivity of *In(1)B^{M2}(reinverted)*, we checked the acetylation of H4 at lysine 16 using Western blotting with anti-acetylated H4K16 antibodies (figure 2,a&b). Anti-acetylated H4K5 antibodies were used as control (figure 2,c&d), since this acetylated isoform of histone 4 is distributed on the euchromatin of the fly genome (Turner *et al.* 1992). Results of Western blotting showed that acetylation of H4K16 (figure 2,a&b), but not H4K5 (figure 2,c&d) was increased when *In(1)B^{M2}(reinverted)* third instar larvae (figure 2,a) or adults (figure 2,b) were subjected to 4 h of cold shock (figure 2,a&b, lane 6). There was no change in acetylation of H4K16 in Oregon-R male larvae after cold shock (figure 2,a; lane 5). The observation of the hyperacetylation of H4K16 in adults suggested that the enhanced acetylation of H4K16 was not exclusively a larval phenomenon. During recovery from coldshock (figure 2,e&f), a detectable increase in the acetyla-

tion of H4K16 could be observed after 2 h of recovery from cold shock (figure 2,e, lane 4) but not after 1 h or 3 h of recovery from cold shock (figure 2,e, lanes 3 and 5). The acetylation of H4K5 remained unaltered (figure 2,f). Identical results were seen in adults (data not shown). Based on the above results the expression of *mof* gene was checked using Western blotting with anti-*mof* antibodies. The results of the experiments were identical to those observed for H4K16, i.e. *mof* expression increased after cold shock (figure 3,a; lane 4) and during recovery for 1 h (figure 3,a; lane 6). There was no change in *mof* expression in Oregon-R larvae at identical time points (figure 3,a; lanes 3 and 5).

In all experiments, cold shock did not result in any lethality, as expected from our early observations that cold shock affected the structure but not the transcription of the male X-chromosome. However, since cold shock increased *mof* expression, the expression of one of the dosage compensation proteins, MSL-2 was checked after cold shock and recovery (figure 3,b). The results showed that *msl-2* expression was not affected by cold shock of male third instar *In(1)B^{M2}(reinverted)* larvae, implying that cold-shock selectively resulted in the increased expression of *mof*.

The mechanism by which hyperacetylation of H4K16 induced puffy X-chromosomes during the process of cold shock and recovery was suggested from the cytology of polytene chromosomes. During this process, many X-chromosomes demonstrated spiralized regions associated
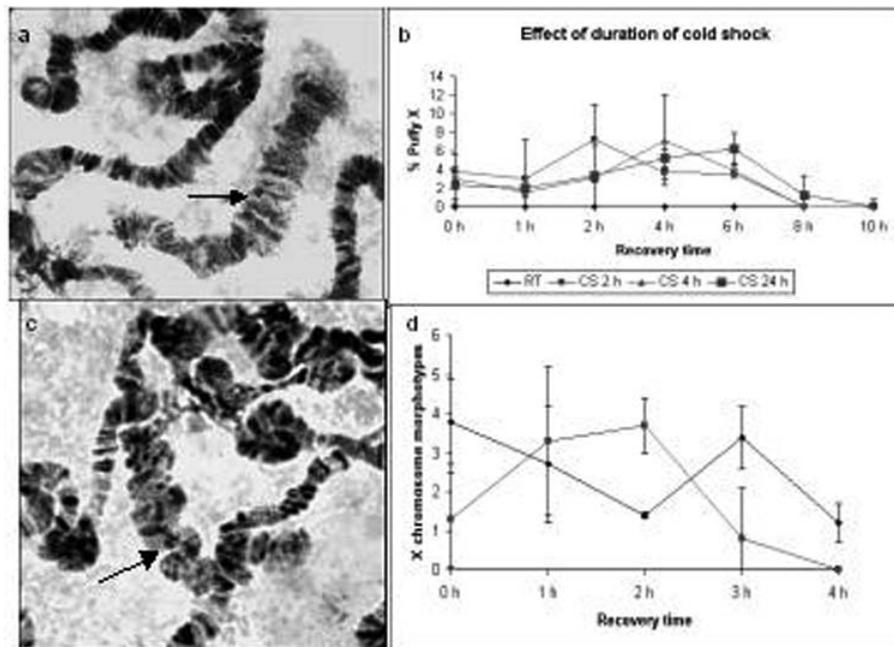
Figure 1. (a) Puffy male X-chromosome (arrow) of *In(1)B^{M2}(reinverted)*. (b) Induction of puffy X-chromosomes after cold shock for 2, 4 or 24 h followed by recovery at $24\pm1^\circ$C for the time indicated. (c) Regions of spiralization (arrow) associated with puffy region of the X-chromosome of *In(1)B^{M2}(reinverted)*. (d) Number of puffy (circles) and spiralized (boxes) chromosomes in preparations from single pair of salivary glands at different time points during recovery from cold shock.
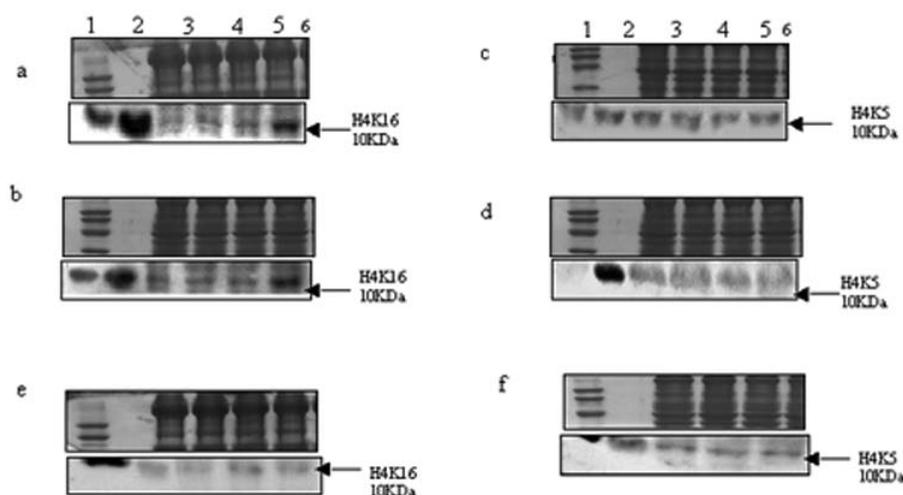
Figure 2. Immunoblotting using antibodies against acetylated H4K16 (left panel) and H4K5 (right panel) in larvae (a, c, e and f) and adults (b and d). The upper panels in figures a–f are coomassie blue stained gels, representing loading control. The level of acetylated H4K16 is increased in $In(1)B^{M2}(reinverted)$ larvae (a) and adults (b) subjected to cold shock (lane 6). The signal intensity remains unchanged in Oregon R (lane 3) and $In(1)B^{M2}(reinverted)$ (lane 4) larvae (a) or adults (b) reared at room temperature, or Oregon R larvae (a) or adults (b) subjected to cold shock (lane 5). The right panel shows that the levels of acetylated H4K5 which remain unchanged in Oregon R or $In(1)B^{M2}(reinverted)$ larvae (c) or adults (d) either reared at room temperature (lanes 3 and 4) or after cold shock (lanes 5 and 6). Immunodetection of (e) H4K16 and (f) H4K5 in $In(1)B^{M2}(reinverted)$ third instar larvae subjected to cold shock for 4 h followed by recovery at room temperature for 1 h (lane 3), 2 h (lane 4) and 3 h (lane 5). Lane 1 represents control protein molecular weight marker, lane 2 represents calf thymus H4 marker.
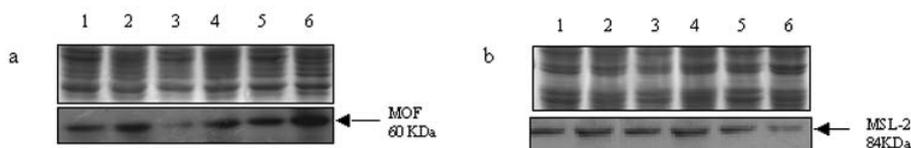
Figure 3. Immunodetection of *mof* (a) and *msl-2* (b) in Oregon R larvae (lanes 1, 3 and 5) or $In(1)B^{M2}(reinverted)$ third instar larvae (lanes 2, 4 and 6), reared at room temperature (lanes 1 and 2) or subjected to cold shock for 4 h followed by recovery at room temperature immediately after cold shock (lanes 3 and 4), or after recovery from cold shock for 1 h (lanes 5 and 6). The upper panels in (a) and (b) are coomassie blue stained gels, representing loading controls.

with regions where the chromosome appeared puffy (figure 1,c). There was an inverse relationship between the number of such spiralized chromosomes and the number of puffy X-chromosomes, when the numbers of these X-chromosome morphotypes were scored at different time points during the process of recovery from cold shock (figure 1,d). These results suggested that the global alteration in male X-chromosome structure was brought about by the X-chromosome corkscrewing into itself, resulting in the puffy and diffuse structure.

Discussion

The results of this study suggest a mechanism by which cold shock for 4 h at the third instar larval stage can induce

global alteration in the architecture of the male polytene X-chromosome of $In(1)B^{M2}(reinverted)$. The results suggest that cold shock deregulates the activity of *mof* histone acetyltransferase, resulting in an increased acetylation of histone 4 at lysine 16. Although we cannot demonstrate through Western blotting that this enhanced hyperacetylation is restricted to the male X-chromosome, the cold shock induced spiralization of the male X-chromosome immediately after 4 h of cold shock would seem to suggest that the enhanced acetylation of H4K16 results in alteration of the chromatin structure, bringing about the higher-order alteration in chromosome structure after cold shock. The reversion of the male X-chromosome structure by 4 h of recovery from cold shock, from a diffuse and puffy morphology to wild-type morphol-

ogy, could possibly occur due to turnover of MOF at room temperature.

One of the significant findings of this study is that cold shock resulted in specific deregulation of *mof*, but not for another dosage compensation gene, *msl-2*. This observation is noteworthy since both proteins are a part of the dosage compensation complex that brings about hypertranscription. The findings of this study suggest that *mof* is independently deregulated from that of the other dosage compensation proteins. The current study does not address the question of how the deregulated *mof* activity brings about alteration in the structure of only the male X-chromosome but not the autosomes. The specific cold shock mediated deregulation of *mof* and its phenotypic effect of structural alteration of the male X-chromosome without affecting transcription (as seen by absence of lethality after cold shock) serves to dissect out the functional roles of *mof* and *msl-2*. Acetylated H4K16 along with MSL proteins appear enriched on the 3′ coding region rather than the promoter suggesting a role for this histone isoform in transcription elongation, rather than transcriptional activation (Smith *et al.* 2001; Kind and Akhtar 2007). Our observations reiterate the role of H4K16 in creating the chromatin architecture that facilitates hypertranscription. The results also demonstrate a delinking between H4K16 acetylation and transcription, since enhanced acetylation of H4K16 after cold shock did not result in increased transcription, as evidenced by lack of cold-shock-induced lethality of *In(1)B^{M2}(reinverted)* males.

Our ongoing studies have shown the absence of mutations in the coding sequence, promoter or 3′UTR of the *mof* gene of *In(1)B^{M2}(reinverted)* (Vartak R. unpublished data). This raises the issue of how cold shock deregulates *mof* activity. Our earlier results (Kar and Pal 1995), and those of Bose and Duttaroy (1986) had demonstrated that the expression of the puffy X-chromosomes of *In(1)B^{M2}(reinverted)* was regulated by the 15F-16A reinverted breakpoint of the X-chromosome, and that the puffy X phenotype was a consequence of position effect variegation (PEV). It may be possible that cold shock enhances PEV, resulting in silencing of a histone deacetylase that regulates the male specific acetylation by *mof*.

Acknowledgements

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