Nucleus (2016) 59:141–154
DOI 10.1007/s13237-016-0177-0

ORIGINAL ARTICLE

Heterochromatin-binding proteins regulate male X polytene chromosome morphology and dosage compensation: an evidence from a variegated rearranged strain [*In (1)BM*^{2},(*rv*)] and its interactions with hyperploids and *mle* mutation in *Drosophila melanogaster*

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Received: 10 May 2016 / Published online: 21 July 2016
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Abstract The reinverted *In*(*1*)*BM*^{2} strain of *Drosophila melanogaster* alter the global architecture of the male X chromosome in ~30 % salivary nuclei, while rest of the nuclei show a perfectly normal haplo-X morphology. We show that, in the aberrant morphology of male X chromosome bearing nuclei, the chromocenter was grossly abnormal, exhibiting either loose morphology or reduced in size. Rearing flies at lower temperature (16 °C) enhanced both frequency and severity of variegated phenotype in the X chromosome and chromocenter. In variegated X chromosome, many intercalary heterochromatic sites showed cytologically visible link (ectopic pairing) at higher frequencies than the X chromosome of Oregon R males. When duplication for the segment 18A-20F, 16F-20F, 8C-20F and 1A-17F were combined with rearranged X chromosome of male, the rearranged X chromosome ceases to show variegating phenotype. Similarly, when a seperate *maleless* mutation (either *mle*^{1} or *mle*^{ts}—a dosage compensation regulatory gene mutation) was introduced in homozygous condition in the rearranged strain, variegated phenotypes of the male X chromosome were not only modified, bloated appearance of male X chromosome was also partially reduced. On the basis of the results, we suggest that (1) like many other re-arrangements involving pericentric heterochromatin, reinverted *In*(*1*)*BM*^{2} X chromosome induce long-distance heterochromatin spreading into juxtraposed euchromatic sequences of the X chromosome, (2) X limited distribution of intercalary heterochromatin (either SR sequences or transposable elements bearing heterochromatic sites) function as relay elements for 'spreading' of heterochromatic factors to the entire X chromosome, and (3) the termination of heterochromatin spreading on the male X chromosome by different genomic context indicate that there is an inherent mechanism for movement of heterochromatin-binding proteins in the X chromosome from one class of site to another and back, for regulation of X chromosome organization.

Keywords Position effect variegation · Dosage compensation · Heterochromatin · Epigenetics

Introduction

Higher order organization of the X chromosome in male is induced epigenetically for regulation of dosage compensation in *Drosophila* [11, 13, 52, 53]. To date, the male specific lethal mutations provide an avenue to understand the role of the genetic elements in establishing of higher order organization of the polytene X chromosome in male for dosage compensation. Current hypothesis is that, in *Drosophila,* male X specific dosage compensation complex (DCC), consist of five proteins called MSLs (MSL1, MSL2, MSL3, MLE and MOF) and two non-coding RNAs, roX1 and roX2 are instrumental for higher rate of transcription of the male X chromosome. The expression of MSL2 in males, mediates the assembly of DCC specifically

The paper is dedicated to Prof. A. K. Sharma, as inspirational and integrative leader in the field of chromosome research.

Electronic supplementary material The online version of this article (doi:10.1007/s13237-016-0177-0) contains supplementary material, which is available to authorized users.

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on the X chromosome in combination with the roX1 and roX2 RNAs. This results an enrichment of acetylation of histone H4 at lysine 16 (H4K16ac) on the male X chromosome, due to MOF activity. The acetylation of histones is linked to transcriptional hyperactivation because histone acetylation decreases inter-nucleosomal interaction and the interaction of nucleosome 'tails' with linked DNA, thereby allowing greater accessibility of RNA polymerase for faster rate of RNA chain elongation [12, 16]. In females, the *Sex lethal* gene turn off the dosage compensation system by repressing the MSL-2 translation.

Conversely, evidences of X chromosome modulation by other autosomal gene mutations are rapidly accumulating. For example, hypomorphic or loss of function mutations at *Jil-1*, a histone H3S10 kinase, [5, 20, 38, 84, 85], enhance abnormal polytene X chromosome morphology in males. Comparable phenotype of male X chromosome was noted when reduction in the dose of other euchromatin associated proteins, e.g. the Nurf complex [4], histone [59], and ISW1 [19, 21, 81] were generated in cells, due to partial loss of function of these genes. The structural components of heterochromatin SU(VAR)205 heterochromatic protein 1 (now called HP1a), SU(VAR)3-7 also enhance or suppress the position effect variegation (PEV) in male X chromosome, depending on their dose [73]. These data together suggest that a dynamic balance in the amount of the products of these genes are essential for establishment of distinct X chromatin environment of males for dosage compensation. In fact, an integrated output from these genes are essential for establishment of a genetic system to specify the level of higher order X chromosome structure in male for dosage compensation.

Several lines of evidence indicated that both PEV phenotype and hyperactive organization of male X chromosomeare epigenetically regulated [24, 53], although two different types of chromatin state are established by two different pathways. The PEV effect is the repression of gene expression and for PEV phenotype the coordinated function of several enzymes is required for removing euchromatin- specific histone modification marks before the transition to heterochromatin packaging can take place. On the other hand, an enrichment of acetylation of histone H4 at lysine 16 (H4K16ac) on the male X chromosome, due to MOF activity cause hyperactive male X chromosome organization. Spierer et al [73] however, noted contrasting effects of genetic interaction between mle^{1} mutations, a gene coding for the RNA helicase component of the DCC [44], and the PEV induced male X chromosome, resulting from hypomorphic mutation of *su*(*var*)3-7 gene. They showed that in absence of mle^{1} function, not only the PEV effect of male X chromosome was suppressed, but the bloated appearance of male X chromosome was also collapsed. This observation reveals a new intriguing genetic interaction between epigenetic silencing and the dosage compensation machinery. Therefore, reexamination of the interaction between DCC activity and altered appearance of the male X chromosome in a rearranged strain (where there is PEV like phenotype), will be useful for understanding the precise DCC-X chromatin interaction in PEV induced X chromosome.

Earlier, in this laboratory and elsewhere [39, 45, 56, 60] have shown that in $In(1)BM^{2}$,(*rv*), rearrangement strain of *D. melanogaster,* the chromatin packing along the entire male X chromosome is variably modified (pompon-like) resulting mosaic organization with respect to X chromosome morphology at the level of polytene chromosomes. This atypical X chromosomal structure in males is enhanced when flies were reared at cold (i.e. 10–18 ± 1 °C) [39, 45, 60]. What could explain the specific sensitivity of the male X chromosome by X chromosomal rearrangements? Since, in $In(1)BM^{2}$ rearrangements, physical breakpoints are located on 16A4 and 20E, and since severity of the variegated phenotype in male X chromosome is displayed in $In(1)BM^{2}$, compare to $In(1)BM^{1}$ rearrangements due to the difference of one break-point within pericentric heterochromatin [45, 49], it is considered that difference in heterochromatic breakpoint is the main cause of enhancing PEV to the male X chromosome. Since reinverted $In(1)BM^{2}$ rearrangements cause PEV like phenotype of the male X chromosome [8], and since the severity of packaging varied from cell to cell basis, it is expected that the study of remodeling action of X chromosome in DCC null genetic background will be useful to know whether heterochromatinized marked X chromosome of $In(1)BM^{2}$ (*rv*) males will be interacted differently in absence of DCC function or not. Such an analysis is moreover necessary since conflicting results are recorded between two groups workers on the transcriptive activity of the PEV induced X chromosome [39, 45, 60] of $In(1)BM^{2}$ (*rv*) males. So far our knowledge is concerned, no attempt has made to understand the genetic interaction between dosage compensation regulatory gene mutations, *mle* and the variegating rearrangement strains $In(1)BM^{2}$,(-*rv*) in *D. melanogaster.* Furthermore, relatively little is known about the effect of $In(1)BM^{2}$(*rv*) rearrangements on chromocentric heterochromatin organization of the polytene chromosomes in males.

In view of these reasons, in present investigation, we firstly, reexamined the PEV pattern of the polytene male X chromosome of third instar larvae of $In(1)BM^{2}$,(*rv*) strain at both normal (23 °C) and cold (16 °C) temperature. We also analyzed the phenotypes of the reinverted X chromosome of male under different genomic context to understand the nature of genetic factor(s) that can modify PEV phenotype of the X chromosome.

Materials and methods

Fly stocks

The following strains and mutants of *Drosophila melanogaster* were used: (1) The wild type Oregon R strain; (2) *In*(*1*)BM^2*rv*, strain of *D. melanogaster,* originally derived from *In*(*1*)BM^2 inversion with a breakpoint at 16A4 and 20E. At present, the X chromosome shows no visible mark of rearrangements [39, 60]. However, frequently ectopic pairing between 15F-16A4 and β heterochromatin at 20E were noted in some polytene nuclei [60].

The *male lethal* mutant stocks were: (3) *w,* mle^1*/CyO*; the mle^1 is a null mutation and (4) mle^{ts}*/CyO,* a temperature sensitive allele of *mle* generated by EMS mutagenesis [6]. This temperature sensitive allele kills homozygous males at 23–29 °C but allows some escapers (adult mle^{ts}/mle^{ts} homozygous males) to survive at 16 °C. The thin male X chromosome of the salivary gland nuclei is the characteristic feature of *mle* mutation [6].

(5) Translocation stocks were: T(X;Y)B18/Y; (6) T(X;Y)B50/Y; and T(X;Y)J8 [77]. Males of these stocks have a free Y chromosome besides the one involved in the translocation. In these translocations, the X chromosome is broken at 18A; 16F and 8C respectively. The X chromosome involved in the translocation was marked by the recessive mutation *yellow* (*y*) body colour and bristles and in larvae with brownish mouth parts. The long arm of the Y chromosome (Y^L) involved in the X;Y translocation is marked with B^s, expressed in adult as strip-bar eyes. The short arm of the Y chromosome (Y^S) is marked with piece of the X is marked with y^+.

All marker mutations and aberrations are described in Lindsley and Zimm [49] and http://wwwflybase.org [27].

Culture conditions

All fly stocks were raised on standard *Drosophila* food medium containing agar-cornmeal-brown sugar–yeast [3]. Propionic acid was added as a mold inhibitor. Methyl paraben was added to the medium to suppress mold growth. The culture medium was supplemented with live yeast for better nourishment. All developmental stages were reared in a BOD incubator at either 16° ± 1 °C or 23 ± 1 °C in 80 % relative humidity, unless otherwise specified. All flies were kept in uncrowded condition and the culture medium was changed in every 10–15 days.

Crosses

Aneuploid males and females carrying X chromosome of *In*(*1*)BM^2 (*rv*) strain were constructed from the above mentioned translocation stocks of *D. melanogaster* by the method of Chatterjee [10]. Cross between T(X;Y)50/Y males and *In*(*1*)BM^2 (*rv*) virgin females produced aneuploid males with duplication and female with deficiency for the segment 18A-20. Dp(18A-20)/*In*(*1*)BM^2,(*rv*) males are viable and sterile. Df((18A-20F)/*In*(*1*)BM^2,(*rv*) female are viable and fertile. Cross between T(X;Y)B18/Y males and *In*(*1*)BM^2 (*rv*) virgin females produced aneuploid males with duplication Dp(16F-20F)/*In*(*1*)BM^2 (*rv*), that were viable and sterile. Similarly, cross between T(X;Y)J8 males with *In*(*1*)BM^2,(*rv*) virgin females produced aneuploid males carrying duplication (Dp(8C-20F)/ *In*(*1*)BM^2,(*rv*), that were viable till late third instar larval stages.

Two crossing schemes (see supplement Fig. 1a, b) were used to generate either *In*(*1*)BM^2,(*rv*), mle^1/mle^1 or *In*(*1*)BM^2(*rv*) mle^{ts}/mle^{ts} males for studying morphological phenotypes of X chromosome of the variegated strain in the background of *mle/mle* mutations. For all crosses, just enclosed virgin females of appropriate genotype were collected, check for sex under a binocular microscope, crossed to appropriate young males (4–5 days old) in 15:20 proportion in culture bottles, unless it was specified. The flies were transferred to fresh food bottles, after 4–5 days. After 3–4 such changes, parental flies were discarded and desired progenies were collected for further crosses. Briefly, we crossed, the virgin *In*(*1*)BM^2,(*rv*) females with *w,* mle^1*/CyO* male (that carries the *CyO* balancer) (P_o) to obtain *In*(*1*)BM^2,(*rv*), +*/CyO* males (F_1). These males were again crossed with homozygous *In*(*1*)BM^2,(*rv*) females to obtain *In*(*1*)BM^2,(*rv*)+*/CyO* females (F_2). These females were further crossed with *w,* mle^1*/CyO* males to obtain *In*(*1*)BM^2,(*rv*)*/w;* mle^1*/CyO* female (F_3) and further crossed with *w,* mle^1*/CyO* males (F_4) in ten vials. The cross yield among others *In*(*1*)BM^2,(*rv*)*,* mle^1/mle^1males (see supplement Fig. 1a). It was observed that from the cross about 25 % of eggs did not hatched. Therefore, the number of viable progenies of the crosses were small. Only seven *In*(*1*)BM^2,(*rv*)*, Y;* mle^1/mle^1 males (non-*white Cy*) were identified as escaper at pharate adult stage. These flies die soon after emerged. Most mle^1/mle^1males (either *In*(*1*)BM^2, (*rv*)/Y; mle^1/mle^1 or *w/Y;* mle^1/mle^1) remained as larvae for several days after other pupated. These larvae and/or prepupae die at that stage. The larvae of *In*(*1*)BM^2(*rv*)*,w,* mle^1/mle^1 male were separated from others by absence of inversion and colour of malphigin tubules (yellow) [48]. It may be noted here that Fakunaga et al. [26] also recorded some unidentified escaper flies from their crosses.

Similarly, *In*(*1*)BM^2,(*rv*) mle^{ts}/mle^{ts} males were generated as per crossing design (supplement Fig. 1b). The larvae were separated as mentioned above.

Cytological preparation

The polytene chromosomes were prepared as per methods of Ashburner [3]. Salivary glands from mature well nourished third instar larvae were excised in *Drosophila* Ringer solution at pH 7.2, fixed in aceto-ethanol, stained in aceto-orcein and squashed in a drop of lacto-orcein for temporary preparation. Chromosomes were photographed in an Olympus photomicroscope, Japan, when necessary.

For width measurement, 11 segments of X chromosome (viz. 1F, 3C, 5D, 9A, 10C, 11C, 12 F, 16A, 17C, 18C, and 19F) and seven segments of the tip of 2R (viz. 48A, 50C, 51A, 55C, 56F, 59A, and 60F) were identified and captured images of the chromosomes from different preparations using Olympus photomicroscope, Japan connected to a computer. Image analysis software of Olympus was used for image capturing and the analyses of the images [14].

Results

In(1)BM^2*(rv)* rearrangements have global effect on the structure male X chromosome and cold sensitive

As noted earlier [60], in reinverted *In(1)BM*^{2} strain, the inverted segment has been rearranged to its original sequences and no visible change in banding pattern can be identified, although frequently ectopic pairing between 15F-16A5 and the β heterochromatin at 20EF were noted [60; our unpublished observation]. Yet, it has been observed from squash preparations of salivary glands at 23 °C reared larvae that the reinverted *In(1)BM*^{2} strain display varying degrees of alterations in X chromosome morphology, although autosomes of these nuclei do not show any differences from those in male nuclei with a normal X chromosome. In fact, the male X chromosome of the strain behaves like any other heterochromatic variegating rearrangements [8, 39]. We noted that out of 989 nuclei observed from nine salivary glands of *In(1)BM*^{2},*(rv)* males larvae reared at 23° ± 1 °C, 183 (18.5 %) nuclei had variegated X-chromosome, while the remaining 806 (81.5 %) nuclei showed the “normal male” X (Table 1). The mosaic phenotype of male X chromosomes in a salivary gland may be determined during differentiation of silencing at third instar larva. Furthermore, critical analysis of the X chromosomes show that, in PEV induced nuclei, the banding pattern of entire X chromosome was blurred and somewhat diffused throughout their length (Fig. 1b–d). Not all euchromatic region of X chromosome show the same efficiency in generating PEV (Fig. 1b, c). Some discrete sites of the X chromosome display different chromatin modification. In some nuclei, the X chromosome was not hold together with the chromocenter (Fig. 1d) and the chromocenter morphology was loose net of thin fibrils and aggregates of granules (inset Fig. 1d’). We also noted that generally the degree of spreading of heterochromatin depends on the level of compaction of heterochromatic factors at the break points (Fig. 1c’). A reduction in level of compaction of heterochromatin near the breakpoint and pericentric heterochromatin cause increasing potential for heterochromatic spreading and silencing of the X chromosome. Thus, it appears that a physical ‘spreading’ of heterochromatin from the breakpoint into the euchromatin results PEV phenotype of the X chromosome.

Since the male X chromosome of cold grown *In(1)BM*^{2} *(rv)* larvae exhibit varying degree of alterations in morphology [60], we further reexamined the PEV phenotypes of the X chromosomes of the larvae reared at cold (16 °C), *In(1)BM*^{2} *(rv)* males. As noted earlier, we also recorded that X chromosome of male is greatly perturbed in cold reared larvae (Fig. 1e–h). The heterochrotinization is cytologically visible in the polytene X chromosome as a shift from a banded to an amorphous structure of the X chromosome arm. In some nuclei, the male X chromosomewas shorten, folded with a non-orderly intermixing of euchromatin and compacted chromatin characteristic of banded regions, (pompon like) (Fig. 1f–h). The entire male X chromosome was completely disorganized and no bands can be easily identified except for the very prominent landmarks at 3C and 11A regions (Fig. 1f–h). As mentioned above, the autosomes in these nuclei do not show significant difference from those in male nuclei with a “normal looking” X. The ‘pompon like’ forms of X chromosome are also seen in low polytene nuclei (Fig. 1h). In cold rearing flies, the frequency of ‘pompon like’ X chromosome bearing nuclei were higher than that of normal rearing flies. Out of 991 nuclei observed from salivary glands of nine males, reared at 16 °C, only 364 (36.73 %) had variegated X chromosome while (63.26 %) nuclei showed ‘normal’ looking X chromosome (Table 1). The organization of the X-chromosome in *In(1)BM*^{2}*(rv)* female was similar to that observed in cold-reared Oregon R (Fig. 4e).

Interestingly, we noted that when polytene X chromosome morphology was greatly perturbed at cold temperature at 16 °C, chromocenter phenotypes became smaller and/or loose variegated (Fig. 1e′–h′). In fact, severity of PEV phenotype of male X chromosome of cold rearing larvae was correlated with loosen chromocenter morphology of salivary gland nuclei. It, therefore, appears that alteration within the heterochromatic domain of chromocenter has some bearing on the alteration of X chromatin packaging in males.

Table 1 Frequency distribution of variegated male nuclei of *In(1)BM*^{2}(*rv*) at different rearing temperature and genetic background

Genotype	Temp.	Total nuclei observed	No. of nuclei with normal X (hyperactive) chromosomes	Percentage of normal X bearing nuclei	No. of nuclei with variegated/partially vari. X chromosome	Percentage variegated X bearing nuclei	Percentage of thin/partially thin X bearing nuclei
In(1)BM2,rv/Y; +/+	23 ± 1 °C	989	806	81.50	183	18.50	–
In(1)BM2,rv/Y; +/+	16 ± 1 °C	991	627	63.26	364	36.73	–
w/Y; mle^1/mle^1	23 ± 1 °C	909	114	12.54	–	–	87.46
In(1)BM^2*,rv/Y*; mle^1/mle^1	23 ± 1 °C	886	131	14.78	109	12.30	72.91
In(1)BM^2*,rv/Y*; mle^1/mle^1	16 ± 1 °C	821	142	17.30	126	15.35	67.36
+/Y; mle^{ts}/mle^{ts}	23 ± 1 °C	735	111	15.10	–	–	84.90
In(1)BM2,rv/Y; mle^{ts}/mle^{ts}	23 ± 1 °C	781	137	17.54	89	11.39	71.06
In(1)BM2,rv/Y;mle^{ts}/mle^{ts}	16 ± 1 °C	853	451	52.87	325	38.10	9.03

Homozygous mle^1 male larvae were generated from cross can be separated each other by colour of malphigian tubules

Variegated X chromosome induce high frequency ectopic pairing for heterochromatinization

While examining aceto-orcein stained squash preparations of salivary male X chromosome of *In(1)BM*^{2},(*rv*) larvae, we noticed that, many X chromosomal intercalary heterochromatic regions displayed the property of ectopic pairing (association of non-homologous regions). In fact, the linear continuity of the PEV induced chromosome was often disrupted by the pairing (ectopic pairing) because adjacent bands which normally were arranged in tandem, join side by side to form either a single unit of double length or a V shaped configuration (Fig. 2a–d). The regions in which pairing were mostly detected, were 1A, 1DE, 2B, 3C, 4DE, 7BC, 8BC, 9A, 11A, 12DE, 16A, 17A, 19A and 19E of the X chromosome. As it appears from Fig. 3, the different regions of the X chromosome of *In(1)BM*^{2},(*rv*) male induce approximately ten times higher frequency of ectopic pairing than that of the Oregon R male X chromosome. Among them 1A, 3C, 11A, 12DE and 19E regions are weak points and reported to carry transposable elements. Interestingly, these sites also displayed ectopic pairing in our preparations (Fig. 2b, d). Furthermore, as it appears from Fig. 1f, 1B1 band usually has tendency to pair with the bands of an autosome or with chromocenter, while 2B region occasionally pair with 3C region (Fig. 2b), 1DE displayed ectopic pairing occasionally with other telomeric regions (Fig. 2c) so on. Since the ectopic pairing (non-specific pairing) as a criterion for location of intercalary heterochromatic regions of the chromosome [23, 40, 89–91], We believe that the high frequency ectopic pairing between these intercalary heterochromatin regions in PEV induced X chromosome of *In(1)BM*^{2} (*rv*) males play some role in spreading of heterochromatic proteins to the entire chromosome.

Duplicated segment of X alter variegated phenotypes in male X chromosome

To know whether the additional segment of X and a fraction of Y chromosome material can modify PEV phenotypes of the X chromosome of *In(1)BM*^{2},(*rv*) males, we performed the cytogenetic experiment, using the technique devised by Stewart and Merrium [77]. Our results showed that when *In(1)BM*^{2},(*rv*) strain carry an additional proximal segment of the X chromosome up to 8C-20F, with an extra Y chromosome fragment including B^s, the PEV phenotype of the male X chromosome was not only suppressed, but the width of the *In(1)BM*^{2},(*rv*) male was reduced (Fig. 4a–c). In these aneuploids, the chromocenter phenotypes were condensed or variegated (Fig. 4a, b).

In contrast, when an aneuploid carrying deficiency 18A-20F bearing X chromosome and a rearranged X chromosome of *In(1)BM*^{2},(*rv*), the two X chromosomes were set at ‘female’ level organization (Fig. 3d). Interestingly, we noted that chromocenter morphology of the nuclei were strongly reduced. These results together indicate that the major heterochromatin markers of PEV induced X chromosome may be redistributed to the aneuploidy segment of the X chromosome due to competition of heterochromatin factors, resulting suppression of PEV mark into the rearranged X chromosome. However this explanation could not explain the whole story. Curiously, two rearranged *In(1)BM*^{2},(*rv*) X chromosomes of females set ‘female’ level organized (Fig. 4e) with normal level of chromocenter morphology and compaction. The reason is not

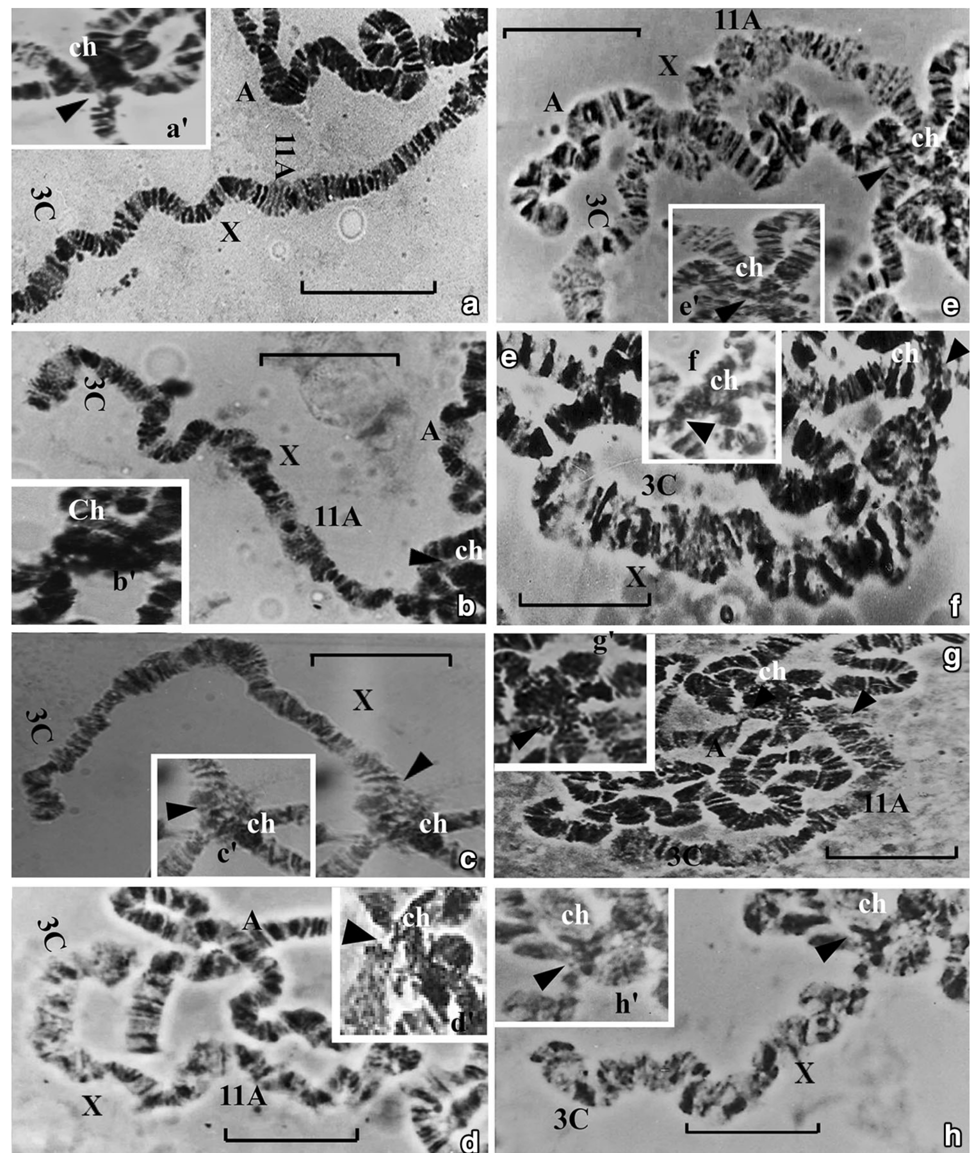

Fig. 1 a–h Photomicrographs showing salivary gland X chromosome configurations of: **a** Oregon R male (control) larva, **b–d** *In*(*1*)*BM*^{2},(*rv*) male larvae, reared at 23 °C; **e–h** *In*(*1*)*BM*^{2},(*rv*) male larvae reared at 16 °C. Note the polytene X chromosome morphology of cold reared larvae (16 °C). Clearly cold reared polytene male X chromosome was highly inflated stumpy compared with control lavae. The chromocenter morphology of the *In*(*1*)*BM*^{2},(*rv*) male became larger than wild type (*inset* (**c**′, d′, **g**′) and the X chromosome was not held together as tightly (*inset* (**d**′). *Solid arrow* indicate the chromocenter. *Bar* in all figures represent 10 μm. *X* X chromosome, *A* autosome

clear. Possibly, male specific lineage of X chromatin materials may have some bearing on the suppression of PEV phenotype of *In*(*1*)*BM*^{2} (*rv*) male X chromosome [11].

Effect of null function of *mle* on the variegated phenotype of male X chromosome

The thin male X chromosome of the salivary gland nucleus is the characteristic phenotype, of the *mle* mutation [6]. To examine the effect of null mutation of *mle* on the variegated phenotype of the X chromosome of *In(1)BM*^{2},(*rv*) males, we cross *In(1)BM*^{2},(*rv*) females with *mle*^{1}/*CyO* males (Suppl. Figure 1a). From the progeny of the cross, we selected *In(1)BM*^{2}, *mle*^{1}/*mle*^{1} larvae for examining the phenotypes of *In(1)BM*^{2},(*rv*) *mle*^{1}/*mle*^{1} polytene X chromosome. To our surprise, we noted that homozygous *mle*^{1} mutation could not be able to suppress variegated phenotype of all polytene X chromosomes of *In(1)BM*^{2},(*rv*) salivary gland male nuclei. Table 1 shows that *mle*^{1} cause reduction of bloated phenotypes of about 67 % of nuclei in *In(1)BM*^{2},(*rv*) males. Photomicrographs presented in Fig. 5a–c further support the contention that although a general decrease of male X chromosomal width was noted in *In(1)BM*^{2},(*rv*); *mle*^{1}/*mle*^{1} males, the frequency of variegated X chromosome had never been dropped below 10–15 % (Table 1). Such attainment of the morphology by *In(1)BM*^{2},(*rv*) X chromosomes clearly suggest that variegated morphology can not be completely suppressed in response to null function of *mle*^{1} in the nuclei although width of male X chromosome has been reduced considerably from all nuclei of the genotype. The decondensed chromocenter morphology was also noted in some nuclei

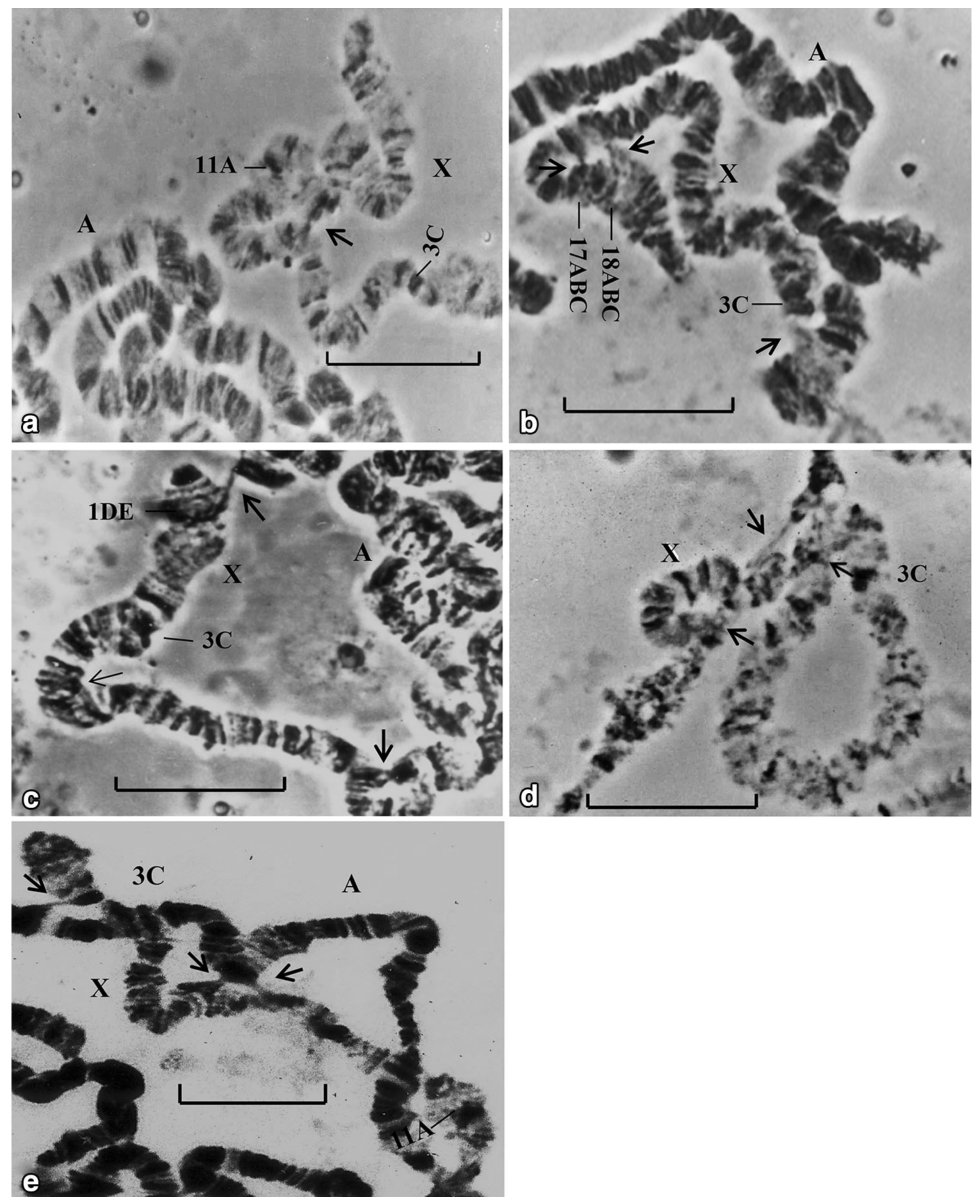

Fig. 2 **a–e** Photomicrographs showing some examples of non-homologous association of the bands (ectopic pairing): **a–d** within X chromosomal regions, and **e** between X and the autosomal regions in the variegated X chromosomes of *In*(*1*)*BM*^{2},(*rv*) males. *Symbols* and *scales* as in Fig. 1

(Fig. 5b). Curiously, seven *In*(*1*)*BM*^{2},(*rv*) *mle*^{1}/*mle*^{1} adults males were recovered as an escaper males in our crosses (data not included). These data clearly suggest that total suppression of variegated morphology of male X chromosome can not be realized in absence of functional MEL protein.

To ascertain that the effect was due to mutation of *mle*^{1}, we repeated this experiment with the *mle*^{ts} allele both 16 ± 1 °C and 23 ± 1 °C. Our results with *mle*^{ts} mutation at 23 ± 1 °C were almost the same as *mle*^{1} mutation (Table 1). From these data we conclude that null function of *mle* can not be able to counteract totally the variegated phenotype of the X chromosome of *In*(*1*)*BM*^{2},(*rv*) males.

Discussion

The results presented in the paper demonstrated that like many other X chromosomal rearrangements involving pericentric heterochromatin, the *In*(*1*)*BM*^{2},(*rv*) rearrangements also induce cell-to-cell phenotypic variation in salivary X chromosome of males, although the variegated phenotype of the male X chromosome was frequently differ in penetrance. We further noted that as reported earlier [30, 43], the phenotypes of male X chromosome of the larvae reared at 16 °C were more perturbed (‘pompon’ like) (Fig. 1e–h) compared to the X chromosome of the larvae reared at 23 °C (Fig. 1b–d).

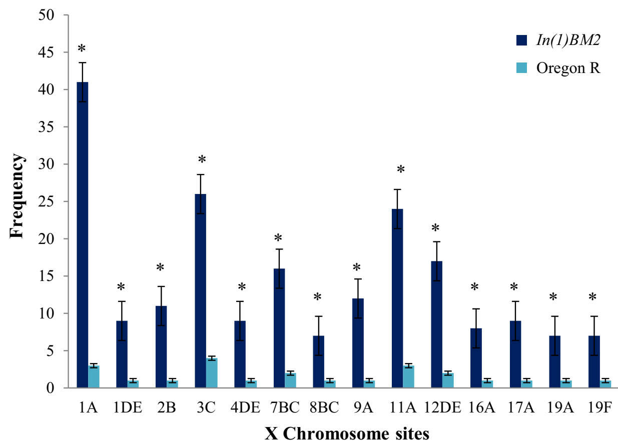

Fig. 3 Histogram showing the pattern of non-homologous association of the chromosomal regions in PEV induced X chromosome of *In*(*1*)BM^2 (*rv*) males. *P < 0.001, when compared with Oregon R male

Since frequently, ectopic pairing occurs between 15F-16A4 and the β heterochromatin at 20E in reinverted *In*(*1*)BM^2, male X chromosome, it is expected that a small fraction of pericentric heterochromatic materials remain separate in the stock, although no visible mark of rearrangements in banding pattern can be identified using light microscope. Since displacement of the pericentric heterochromatin of any chromosome can lead to PEV phenotype [30], it is expected that in *In*(*1*)BM^2,(*rv*) rearrangement, the separation of pericentric heterochromatic materials is the primary cause of variegated phenotype of the X chromosome in males. Furthermore, since rearrangements involving 20E region of pericentric heterochromatin induce PEV phenotype in the X chromosome, we conclude that variegating induce alleles of the X chromosome are located at the cytological position 20E.

Various lines of evidence indicate that PEV phenotypes resulted from a physical 'spreading' of heterochromatin from the break point into euchromatic regions of the chromosome [82, 87]. Although the mechanism of spreading is as yet incompletely understood, there is abundant evidence of spreading of chromocentric heterochromatin to the euchromatic segment of rearranged chromosome depending on a series of molecular reactions within euchromatic arm of the chromosome [78]. In addition, the variegated phenotype can be modified by changing histone dosage of genetically altered level of histone acetylation. The pattern of variegation of the X chromosome of *In*(*1*)BM^2,(*rv*) males suggest that the cis-spreading model of heterochromatin is not the actual cause of PEV phenotype of entire X chromosome. Precisely, global effect of such magnitude are difficult to explain by strict linear propagation of a chromatin state along the chromatin fiber. This view is strengthened from the observations that the deletion of a significant portion of centric heterochromatic region of X enhanced PEV in some distally located genes of the X chromosome [63]. This may imply that breakpoint itself did not cause the PEV phenotype. Furthermore, the data presented in the paper also documented some trans-interactions between different heterochromatic regions in PEV induced X chromosome of *In* (*1*)BM^2,(*rv*) males (Fig. 2a–d). Importantly, the PEV phenotype of X chromosome of the males is generally correlated with decrease level of heterochromatin formation/deposition in the chromocenter (Fig. 1c–e, h). It, therefore, appears that cells normally maintain higher level of heterochromatic proteins through some machinery in the centromere (a part of chromocenter, see below) of the chromosomal regions that may serve as a reservoir. Chromosome rearrangements with heterochromatin-euchromatin breakpoints or any other signal (physical, chemical or genitical) that can cause destabilization of the variegating induce alleles cause spreading of heterochromatic packaging into euchromatin domain of the X chromosome of male [89–91] because of its high affinity heterochromatic protein binding sites.

The salivary gland nuclei chromocenter where all pericentric and Y heterochromatin coalesces, is composed of highly repetitive, middle repetitive, short satellite sequences and transposon fragment [31, 79]. The region is gene poor [72]. Specialized RNAi system is involved in compaction of the chromocenter rich heterochromatin [32, 62, 67, 72]. Although we do not have a clear picture of macromolecular assemblies in pericentric heterochromatin, it is generally accepted that a discrete set of heterochromatic promoting factors are involved in chromocenteric heterochromatic formation and concomitant gene silencing in the region. Principle components of chromocenter are HP1a and the product of the *su*(*var*)3-7 gene. They interact with SU(VAR)3-9, histone H3K9 HKMT for formation of a stable pericentric heterochromatin. Since alteration in the dose of the HP1a, and/or SU(VAR)3-7 can enhance or suppress PEV phenotype in male X chromosome [73, 74], it is generally believe that formation of stable pericentric heterochromatic protein compaction is necessary for sequestration of heterochromatic components at centromeric region including variegating inducing allele of the chromosome.

Our works on aneuploidy interaction assays monitoring polytene X chromosome morphology defect, associated with *In*(*1*)BM^2,(*rv*) rearrangements further indicate that the PEV effect in male X chromosome is not the permanent changes in genes [88]. The PEV effect in rearranged X chromosome of male can be modified by changing the amount of X and/or Y chromosome materials in the genome. Precisely, addition of proximal segment of X chromatin upto 8C-20F (i.e. with the increase number of intercalary heterochromatin binding sites) in the

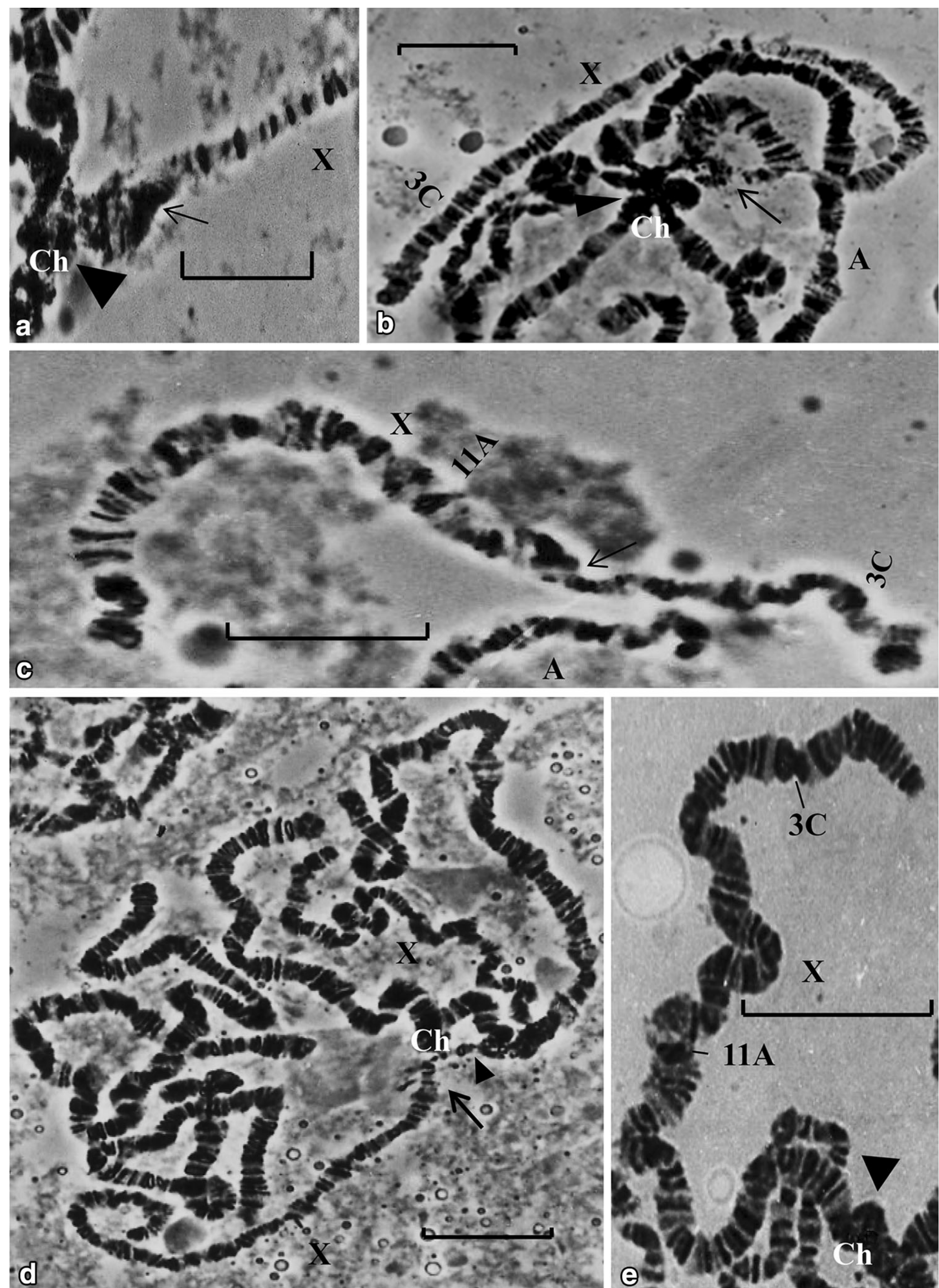

Fig. 4 a–e Photomicrographs showing X chromosome configurations of *In*(*1*)*BM*^{2},(*rv*) segmental aneuploids and a female; **a** a male nucleus with Dp(18A-20F); **b** a male nucleus with Dp(16A-20F); **c** a male X chromosome with Dp(8C-20F), **d** a nucleus with Df(18A-20F) aneuploid and **e** a female nucleus with two reinverted *In*(*1*)*BM*^{2}(*rv*) *X* chromosome. Note the configurations of the X chromosome of *In*(*1*)*BM*^{2} (*rv*) male aneuploids. In (**b**), *B*^{s}. Y is not cytologically recognizable except the ectopic pairing with chromocenter and the differential width of deficiency bearing X (*arrow*). *Arrows* indicate the end of the duplicated segment of X. *Symbols* as in Fig. 1. *Bar* represent 10 µm

In(*1*)*BM*^{2},(*rv*) male chromosome complement not only suppress PEV phenotype of the X chromosome, but also cause repression of 'male' level organization in the aneuploids, although not all euchromatic duplication show similar efficiency in generating reduced level of X chromosome organization [10]. When duplicated segment include more than 85 % distal segment of the X chromosome, both the rearranged chromosome and aneuploidy segment set 'female' level organization (Fig. 4d) and chromocenter morphology of the nuclei was drastically reduced and condensed. Interestingly, the two rearranged X chromosomes of *In*(*1*)*BM*^{2},(*rv*) females established 'female' level organization and the chromocenter of the nuclei were compacted and normal in appearance (Fig. 4e). These data together suggest that the redistribution of the major heterochromatin markers from the PEV induced X chromosome to the duplicated segment of X chromosome cause reduced or negligible heterochromatin marks on the PEV induced male X chromosome. However, this explanation can not ruled out that possibility that male specific lineage

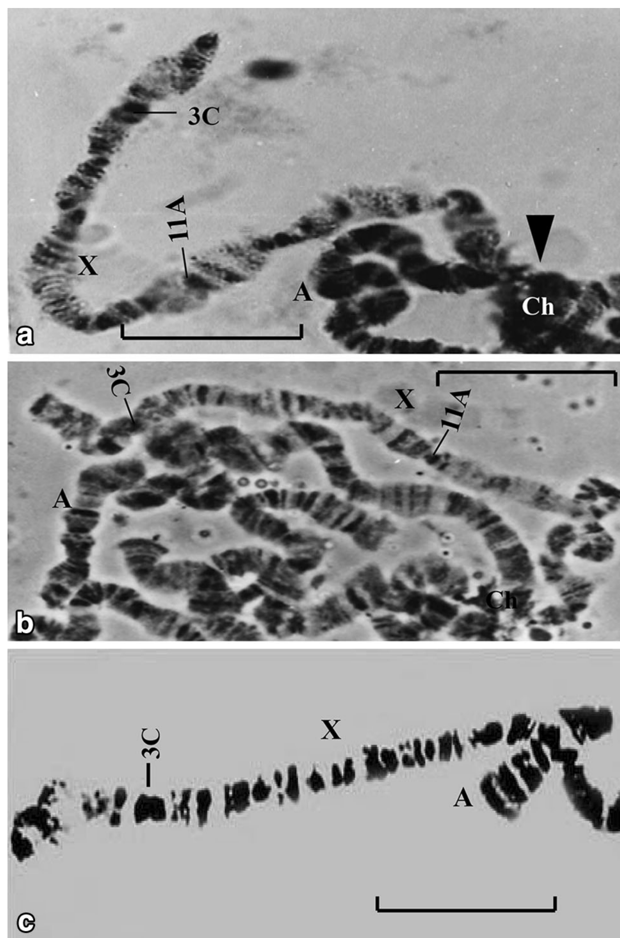

Fig. 5 Photomicrographs showing some examples of the X chromosome configuration of *In*(*1*)*BM*^{2},(*rv*) males in the background of *mle*^{1}/*mle*^{1} mutation (see Text); **a** a male nucleus with slightly variegated X chromosome and condensed chromocenter, **b** a male nucleus with partially thin X chromosome and decondensed chromocenter and **c** a male nucleus with condensed X chromosome. *Symbols* as in Fig. 1. *Bar* represent 10 µm

of X chromatin material may have some bearing on suppression of PEV phenotype of *In*(*1*)*BM*^{2}(*rv*) male X chromosome in combination of the additional X chromatin material [11].

Since spreading does not seem to be simple matter of mass action, an obvious question arise how *In*(*1*)*BM*^{2}(*rv*) rearrangements induce variegated position effects globally to the euchromatic segment of X chromosome? A satisfactory molecular explanation to account for the propagation of the ON versus OFF state of long distance PEV effects of such magnitude is still missing. However, the existence of 15 % more (40 %) repetitive sequences in the euchromatic segment of the X chromosome compared to second and third chromosomes (25 %) have been elucidated by a series of studies [22, 37, 50, 51, 64, 86]. Three different families of repetitive sequences that have been identified so far, from the X chromosome. Among them one families (1.688 g/cm^3) are exclusively localized on the X chromosome [22, 86]. To our current understanding, the roles of these selfish DNA in the X chromosome are not clear. It is generally believed that during evolution, X chromosome sequences has recruited these repetitive sequences for greater degree of regulatory events required for establishment of dosage compensation machinery in males. This view is strengthened by observations that acquisition of dosage compensation mechanism by the neo-X chromosome of *D. miranda* (X_2), is correlated with progressive recruitment of higher levels of these repeats on the X_2 chromosome during evolution [64, 75, 76]. The sequence is referred to as satellite related (SR) arrays (intercalary heterochromatin or 1.688/cm^3 sequences) and are distributed in different discrete location in the X chromosome in reverse repeat orientation and associated with heterochromatin associated proteins [7, 22, 28, 35, 86]. Earlier, it has been noted that occasionally ectopic pairing occurs between these reverse repeat SR array sequences [7, 71]. Our cytogenetic studies also showed that in PEV induced *In*(*1*)*BM*^{2}(*rv*) male X chromosome, most of these intercalary regions displayed the property of ectopic pairing in higher frequency compare to Oregon R male X chromosome (Figs. 2a–d, 3). We believe that these intercalary heterochromatic sites might serve as a platform for heterochromatin associated proteins including HP1a, for regulating various subsequent processes such as chromosomal organization and chromatin long range interaction. However, Menon et al. [57, 58], believed that the siRNA pathway of these repeats help to recognize the DCC for hyperactivation of male X chromosome.

Another repetitive sequences are transposable elements (TEs) that are major structural component of the X chromosome in most *Drosophila* somatic cells. A fundamental question therefore arise, what set the patterns that determine the region of the X chromosome should contain transposable elements, assemble into a heterochromatic or euchromatic state, and once determined, how that state is maintained? Despite these events, the evidence supporting a role of these elements in *Drosophila* remains indirect. Available data indicate that many but not all, TEs can be targets for heterochromatin formation in X chromosome [25, 67, 83]. The small piRNAs produced from TEs may play a crucial role in initiation of heterochromatin formation at selected sites (i.e. a subset of TEs) in the genome of somatic tissues at early embryos by chromosomal protein interactions [9]. Most pi-RNAs are derived from particular genomic sites termed piRNA clusters which contain a large number of various types of TEs. In reality, most piRNA cluster in *Drosophila* are within cytologically defined heterochromatic regions. It, therefore, reasonable to consider that X chromosome is not a passive collections of genes rather it contains an internal structure that has a role

in the regulation of gene expression. Furthermore, the requirements of TEs in the X chromosome are at least twofold: (1) a mechanism that recognizes such a diverse set of TE types, and (2) a mechanism that distinguishes them from other cellular genes and selectively targets them for silencing. The piRNAs produced from TEs in gonads (where they silence through a feed back regulatory mechanism) serve as cis-acting targets for heterochromatin assembly [36, 66, 70]. Furthermore, the TEs belong to gypsy retrotranspos on family, may provide a means to compartmentalize the genome and prevent heterochromatic spreading into active euchromatic regions [47, 61]. This gypsy insulator locus could be prototype for TE transposition landing pads. Ahmad and Golic [1] further noted that variegating P-insertion in euchromatic regions act as a intercalary heterochromatin. Variegating insertions were also recovered in or near the telomeres of all chromosomes [30]. Several similar experiments demonstrated that PEV generated by P-element insertions in the non-telomeric locations behave like chromosomal rearrangement PEV, although inserts in the telomeric region have different properties. Importantly, analysis of EST libraries from *Drosophila* embryos indicate that most TE families are transcribed [18]. Here, we also noted that some of the cytological defined regions of the X chromosome that contain interstitial heterochromatin, weak points, and piRNA cluster sites (i.e. reportedly identified as TE symbiosis sites), displayed 'ectopic pairing' in PEV induced X chromosome in *In(1)BM*^{2},(*rv*) males. We believe that these regions also play crucial role to disperse and/or reassemble the heterochromatic factors in the X chromosome by tethering complementary nascent TE transcripts and guiding heterochromatic factors recruitment and methylation [69, 70]. It is, therefore, reasonable to speculate that host cells may have taken advantage of the universal property of TEs, their transposition ability to trap them in specific genomic locations and subject them to a silencing program, which employs small RNA based immunity to selectively silence homologous elements [54]. Given that many heterochromatic regions is largely composed of transposable elements, PEV can be seen as a breakdown in the normal process by which transposable elements and host genes are effectively sequestered from each other. Thus, it appears that a complex network of system play a crucial role for spreading of heterochromatic proteins from high affinity disperse sites and reassemble for establishment, maintenance or function of X chromatin structure. In fact, the release of DNA binding proteins at mitosis has been documented [15, 55, 65], and their movement from one class of sites to another and back in every cell cycle is a regular phenomenon in dividing cells. Earlier, Kellum et al. [41], noted that in late embryos, where significant fractions of HP1a and SU(VAR)3- disperse from chromosomes during metaphase and then reassemble on the chromatin at telophase during mitosis. Furthermore, the physiological correlation of the structural alterations of the X chromosome in different phase of meiosis indicated that sex chromosomes might have distinct allocyclic properties by specific regulatory mechanisms. This view is strengthened from the observation of Hoskins et al. [34] who noted that heterochromatin account of the X chromosome of *D. melanogaster* is almost twofold [estimated to 19.9 million base pairs (Mb) out of 41.8 Mb] compared to autosomes and have been recruited not only to tolerate being in a heterochromatic region but to actually require for its normal function.

In addition, di-nucleotide repeats (especially dC-dA/dG-dT and CG) [37, 64], are present in high frequency in the X chromosome compared to autosomes. Since PEV phenotype resulted from a physical spreading of heterochromatin from the breakpoint to the euchromatic segment of X chromosome, we speculate that the binding and release of specific proteins occur on the simple sequence repeats, by a self assembly mechanism. Since the sequences are not X chromosome specific, we noted that different X chromosomal sites connect ectopically with autosomal segments also (Fig. 2e).

A curious unanswered question is therefore, why PEV is not induced in female X chromosome by the rearrangements? A large body of data indicated that specific depletion of HP1a in the female germ line results over expression of some (but not all) TEs, indicating a role of TE elements for heterochromatin in silencing [33]. Therefore, germ line depletion of Piwi leads to a loss of silencing of this group of TEs, with concomitant loss of HP1a and H3K9me2 association. Thus, female germ line X chromosome is normally hyperactive [11]. Since, females receive X chromosomes from both the parents and since epigenetic state of male X chromosome follow germ line gene silencing pathways, it is expected that one X chromosome in female is upregulated and other X chromosome is silenced to neutralize the differences in X linked gene dose between male and female. Furthermore, the final phenotype of female X chromosome, is determined by the type of heterochromatin binding and replacement of canonical histones with other variants in zygotes [80], routed through male lineage for 'basal level' organization of female X chromosomes [11].

Evidence of the genetic interaction between one of the dosage compensation machinery, *mle* and PEV induced male X chromosome of *In(1)BM*^{2}*rv* indicated that in absence of RNA helicase component of the DCC [44, 46] the hyper active organization of male X chromosome was not fully collapsed (Table 1; Fig. 5a, b). Generally, mutants of this gene suppress bloated phenotype of male X chromosome necessary for dosage compensation and

therefore, die at the third-instar larval stage [6]. A small fraction of progenies of genotype *In(1)BM*^{2}(*rv*), *mle*^{1}/*mle*^{1} males were also recovered as pharate adults (data not included). These observations together indicate that, loss of a component of DCC could not counteract totally the PEV phenotypes of all nuclei of the salivary gland in males. Furthermore, apart from regulation of male X chromosome dosage compensation, MLE has involved in diverse regulatory pathways namely, regulation of the level of sodium channel activation [42], RNA processing, HP1a deposition and the NuRD complex [17]. In addition, despite the analogies of function of a DEAD box containing *homeless* helicase (a modifier of PEV and transposon silencing) and the DEAH box containing RNA helicase subfamily of MLE, the evidence supporting a role of these two helicase in regulating X chromosomal organization in *Drosophila* remain indirect [29]. Therefore, secondary level of regulation by DCC/X chromatin interaction, has not been ruled out. Moreover, Alekeyenko et al [2] noted that MSL complex selectively identifies active genes on the X chromosome.

In sum, our data provide evidences that like other pericentric rearrangements, reinverted *In(1)BM*^{2} rearrangements of the X chromosome also induce PEV phenotype on the entire male X chromosome in a mosaic fashion. However, the spreading of heterochromatin packaging from the break point to the euchromatic segment of X chromosome was not a simple matter of mass action which implies that a built-in mechanism for spreading of heterochromatic proteins might be functional in the euchromatic segment of X chromosome. We also noted some signature of heteochromatic protein dispersion mechanism (using intercalary heterochromatic sites and many families of TEs symbiosis sites), in PEV induced X chromosome of *In* (*1*)*BM2*(*rv*) rearrangements. Additional evidences of heterochromatic proteins involving dosage compensation come from the recent discovery of the role of adaptive divergence of *D. melanogaster* and *D. simulans* heterochromatic proteins that contribute to speciation of the two species by destabilizing dosage compensation mechanism using hybrid incompatibility gene of *D. melanogaster, Hybrid male rescue* (*Hmr*$^+$) and *D. simulans, Lethal hybrid rescue* (*Lhr*$^+$) [14, 68]. The mutant form of the two genes, *Hmr* and *Lhr* suppress a broad range of heterochromatic repeats for restoring dosage compensation [14, 68] and rescue interspecific hybrid viability. Aneuploid interaction data further indicated that PEV effect of X chromosome of *In(1)BM*^{2},(*rv*) males can be modified by adding amount of X chromosome material to male that can compete heterochromatic factors. On the basis of the data we conclude that there is an inbuilt mechanism within X chromosome for movements of heterochromatin and other proteins from one class of sites to another and back for regulation of X chromosomal organization. In the context, it may be noted here that, in eutherian mammals, *Xic* act as a ‘switch gene’ for spreading of heterochromatic proteins to the X chromosome, necessary for dosage compensation, although *Drosophila* and mammals might reflect considerable functional and/or structural differences of the silencing complex. We, therefore, favour the possibility that the complex patterns of protein dispersion and reassembly pathways at various stages of cell cycle may be implicated for regulation of X chromosomal organization for dosage compensation [10] in *Drosophila*.

Acknowledgments We are thankful to Bloomington *Drosophila* stock center, for generously sending the *Drosophila* stocks. The work has been supported by UGC Emeritus Fellowship [Sanction No. F. 6-6/2015-17/EMERITUS-2015-17-GEN-5478(SA-II) dt.21.09.2015] to RNC.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicting interest.

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