

Butyrate induced alterations during development of the strain *In(1)B^{M2}(reverted)* of *Drosophila melanogaster*

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The polytene male X chromosome of *D. melanogaster*, has a unique morphology, which is correlated with the property of increased transcription of the sex-linked genes of the male X chromosome. This ensures equalization of X-linked gene products between males (XY) and females (XX). Till date, an invariable correlation between the structure and transcription of the male X chromosome has been reported. However, the strain *In(1)B^{M2}(reverted)* of *D. melanogaster* presents a caveat to this invariable correlation. In this strain, although the male X chromosome appears puffy and diffuse, the transcription remains at the wild type level. This observation suggests the perturbation in the function of a regulator that controls the structure of the male X chromosome. In this report the response of the strain to butyrate, an inhibitor of histone deacetylase, has been studied, with specific reference to development, sex ratio and chromosome morphology of the strain. Two important conclusions arise from these experiments : (a) exposure to butyrate has more severe consequences on the development of the mutant strain and on the survival of females. (b) rearing on butyrate induced a temporal series of structural alteration of the polytene chromosome of the wild type, with the male X chromosome being most vulnerable to structural changes. The butyrate-interaction of *In(1)B^{M2}(reverted)* together with our current biochemical analyses of a chromosome coiling protein recovered from this strain, provide data for a working hypothesis explaining the sex and chromosome specific alteration of the structure of the male X chromosome of *In(1)B^{M2}(reverted)*.

Dosage compensation is the phenomenon whereby males with a single X chromosome have the same amount of X-linked gene products as females with two X chromosomes¹. In *Drosophila*, dosage compensation is brought about by a two fold increase in the level of X chromosome transcription in males, relative to a single female X chromosome². The increased transcription of the genes on the male X chromosome is brought about by four dosage compensation genes, termed *male specific lethal-1, 2, 3 (msl-1, msl-2, msl-3)* and *maleless (mle)*, collectively referred to as the *male specific lethals (msls)*. The MLE and MSLs preferentially associate with the X chromosome of males^{3,4}. Subsequent to the binding of these proteins, histone 4 is specifically acetylated at lysine 16 on the male X chromosome⁵. This specific acetylation is dependent on the dosage compensation genes⁶. The gene *male absent on X (mof)* encodes a putative acetyltransferase^{7,8} that may be responsible for the sex specific acetylation of histone 4. Two RNA's, *rox-1* and *rox-2* also specifically associate with the male X chromosome⁹.

The increased transcription of the male X chromosome of *Drosophila* is correlated with the unique cytology of the polytene X chromosome of male larvae. The male X chromosome appears diffuse and puffy and twice as wide as that of a single female

X chromosome or autosome. Various studies demonstrate the invariable correlation between the structure and function of the male X chromosome, suggesting a functional association. For example, in mutants of the dosage compensation gene *mle^Δ*, the transcription of X chromosome is reduced by 30% and the chromosome appears narrow and condensed¹⁰. Mutant combinations of other regulators of the dosage compensation pathway also manifest this structure-function relationship¹¹.

A caveat to this invariable relationship between the transcription and cytology of the male X chromosome is seen in a strain of *Drosophila melanogaster* termed *In(1)B^{M2}(reverted)*^{12-15,17}. The phenotype of the strain *In(1)B^{M2}(reverted)* is significant since in male third instar larvae reared at 18°C, about 25% of salivary gland nuclei show unusually diffuse polytene X chromosomes, that appear twice as wide as the X chromosome of wild type male larvae (Fig. 1a). The morphology of the female X chromosome and the autosomes of both sexes remain unaltered, demonstrating the sex and chromosome specificity of the phenomenon. Furthermore, despite the male X chromosome appearing puffy and twice as wide as the autosomes, the transcription is not enhanced¹⁴, that is, the ³H-uridine incorporation on the mutant X chromosome remains identical to that of the paired

female X chromosomes and the wild type male X chromosome. The absence of correlation between structure and transcription of the mutant X chromosomes indicate that in this strain, the chromosomal rearrangement disrupts a pathway that controls the structure, but not transcription of the polytene male X chromosome. The puffy X phenotype of *In(1)B^{M2}(reverted)* arises due to position effect variegation (PEV)¹⁵. Position effect variegation is a eukaryotic phenomenon whereby a chromosomal rearrangement juxtaposes a euchromatic region to a heterochromatic region, resulting in inactivation of the normal euchromatic loci¹⁶.

The structural alteration of the male X chromosome of *In(1)B^{M2}(reverted)* is only observed at $18^{\circ}\pm 1^{\circ}\text{C}$. At $24^{\circ}\pm 1^{\circ}\text{C}$, all male X chromosomes manifest normal

morphology. Recently it has been reported that temperature shifts of third instar larvae of *In(1)B^{M2}(reverted)*, from $24^{\circ}\pm 1^{\circ}\text{C}$ to $18^{\circ}\pm 1^{\circ}\text{C}$ as well as recovery from cold shock can result in structural alteration of the male X chromosome¹⁷. On the basis of these results, a chromosome coiling protein has been partially purified from male adult, pupae and third instar larvae of *In(1)B^{M2}(reverted)*¹⁷. *In vitro*, this protein can induce coiling and subsequent condensation of polytene chromosomes. These novel findings provide preliminary evidence of a hitherto unreported system that is male specific and affects chromosome structure.

In this article, the butyrate response of this strain is reported. Butyrate is an inhibitor of histone deacetylase¹⁸⁻¹⁹ and is a known suppressor of PEV²⁰.

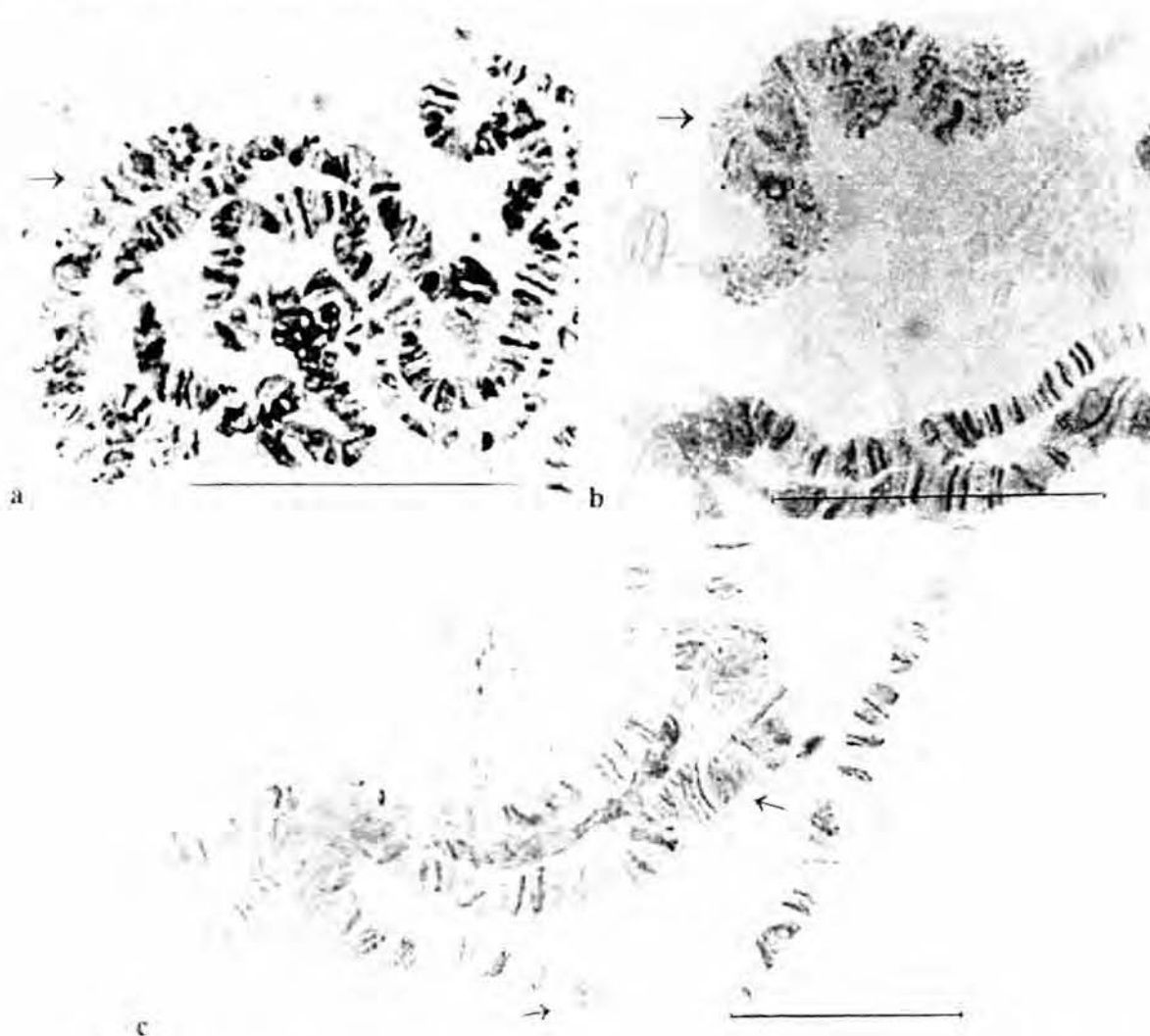


Fig 1—(a) Puffy male X chromosome (\rightarrow) of *In(1)B^{M2}(reverted)*. (b) Identical structural alteration of the male X chromosome (\rightarrow) of third instar Oregon R larvae, reared on medium containing butyrate. (c) Alteration in structure of the female X chromosome (\leftarrow) and autosomes of third instar Oregon R larvae. (\rightarrow) represents the normal width of the chromosome. Bar $10\mu\text{m}$

Butyrate has been routinely used as a tool for studying histone acetylation. In all eukaryotic organisms, the histones undergo a variety of post-translational modifications, like acetylation, methylation, phosphorylation, ubiquitination and ADP-ribosylation²¹. Changes in gene activity are correlated with specific changes in acetylation of core histones²². Histone acetylation destabilizes the histone-DNA association, resulting in an altered nucleosome conformation, thereby rendering the DNA template more easily accessible to the transcriptional machinery. The objective of this study is to document butyrate response in order to determine whether the PEV of *In(1)B^{M2}* (reverted) inactivates a regulator of chromatin structure.

Materials and Methods

Flies of Oregon R and *In(1)B^{M2}*(reverted) were reared at either 25° ± 1°C or 18° ± 1°C in culture boxes containing standard *Drosophila* medium. Nipagin (*n*-methylparaben) was used as a mold inhibitor. Medium containing sodium butyrate was prepared by adding the chemical to medium cooled to 45°C to 50°C. Butyrate sensitivity was determined by comparison of development on medium containing different concentrations of sodium butyrate (0.2, 0.1, 0.05 and 0.001M) to that on control medium (without butyrate). Polytene chromosome spreads were prepared from slow developing third instar larvae reared on butyrate containing medium using routine methods. ³H-uridine incorporation and autoradiography were performed as described

earlier¹⁴.

Results and Discussion

The response of Oregon R (wild type) and *In(1)B^{M2}*(reverted) to butyrate stress was tested by the following experiments. To test the effect of butyrate on development, adult Oregon R flies and those of *In(1)B^{M2}*(reverted) were allowed to lay eggs on medium containing 0.2M sodium butyrate at 25° ± 1°C and at 18° ± 1°C. The duration of various developmental stages was compared to that on control medium (without butyrate). Rearing on medium containing 0.2M butyrate resulted in prolonging development in both wild type and mutant strains, at both temperatures as reported^{23,24}. Both embryogenesis and larval development were affected, as shown by late emergence of third instar larvae and delay in third instar larval to pupal transition (Table 1). However, there was no delay in the time required for metamorphosis of pupae to flies in butyrate containing medium (Table 1). This observation is consistent with reports that the butyrate effect can be reversed once the organism is removed from the butyrate stress²¹, the latter occurring due to encapsulation of the pupae by the pupal case. As expected, developmental delay was increased for both Oregon R and *In(1)B^{M2}*(reverted) strains reared at 18° ± 1°C as compared to that at 25° ± 1°C. The most important experiments were repeated by rearing the embryos on medium containing different concentrations of butyrate, and monitoring third instar larval to pupal transition (Fig. 2). The results showed

Table 1—Development of wild type (Oregon R) and *In(1)B^{M2}*(reverted) on normal culture medium and on medium containing 0.2M sodium butyrate

[Values expressed in days are mean ± SD]

Strain		25°C		
		E → LS3*	LS3 → B.P.*	B.P. → F*
Oregon R	C	5.0 ± 0.2	2.0 ± 0.3	3.0 ± 0.5
	E	8.0 ± 1.5	4.0 ± 1.7	2.0 ± 0.2
<i>In(1)B^{M2}</i> (reverted)	C	5.0 ± 0.4	2.0 ± 1.3	3.0 ± 0.2
	E	12.0 ± 2.4	6.0 ± 2.0	2.0 ± 0.4
18°C				
Oregon R	C	9.0 ± 0.7	4.0 ± 1.3	7.0 ± 0.3
	E	17.0 ± 1.4	9.6 ± 1.5	9.0 ± 0.5
<i>In(1)B^{M2}</i> (reverted)	C	9.0 ± 0.7	4.0 ± 1.3	7.0 ± 0.3
	E	21.0 ± 2.6	12.0 ± 2.2	8.0 ± 0.2

*E=egg, LS3= third instar larvae, B.P.= brown pupae, F= fly;
C=Control ; E=Experimental

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Table 2—Scores of adults of Oregon R and *In(1)B^{M2}*(reverted) emerging after development on normal culture medium or medium containing varying concentrations of butyrate

Conc. of butyrate (M)	Oregon R			<i>In(1)B^{M2}</i> (reverted)		
	25°C			18°C		
	Male	Female	F:M	Male	Female	F:M
0.0	1004	1049	1.0	1191	1181	1.0
0.2	769	535	0.7	915	344	0.37*
0.1	913	728	0.8	608	361	0.6
0.05	634	536	0.8	655	410	0.6
0.001	1390	1194	0.8	923	671	0.7
0.0	1086	1092	1.0	1109	1107	1.0
0.2	651	314	0.48	727	191	0.27**
0.1	705	444	0.65	795	468	0.58
0.05	733	514	0.7	714	421	0.58
0.001	806	640	0.8	783	577	0.7

P values: * < 0.03; ** < 0.02

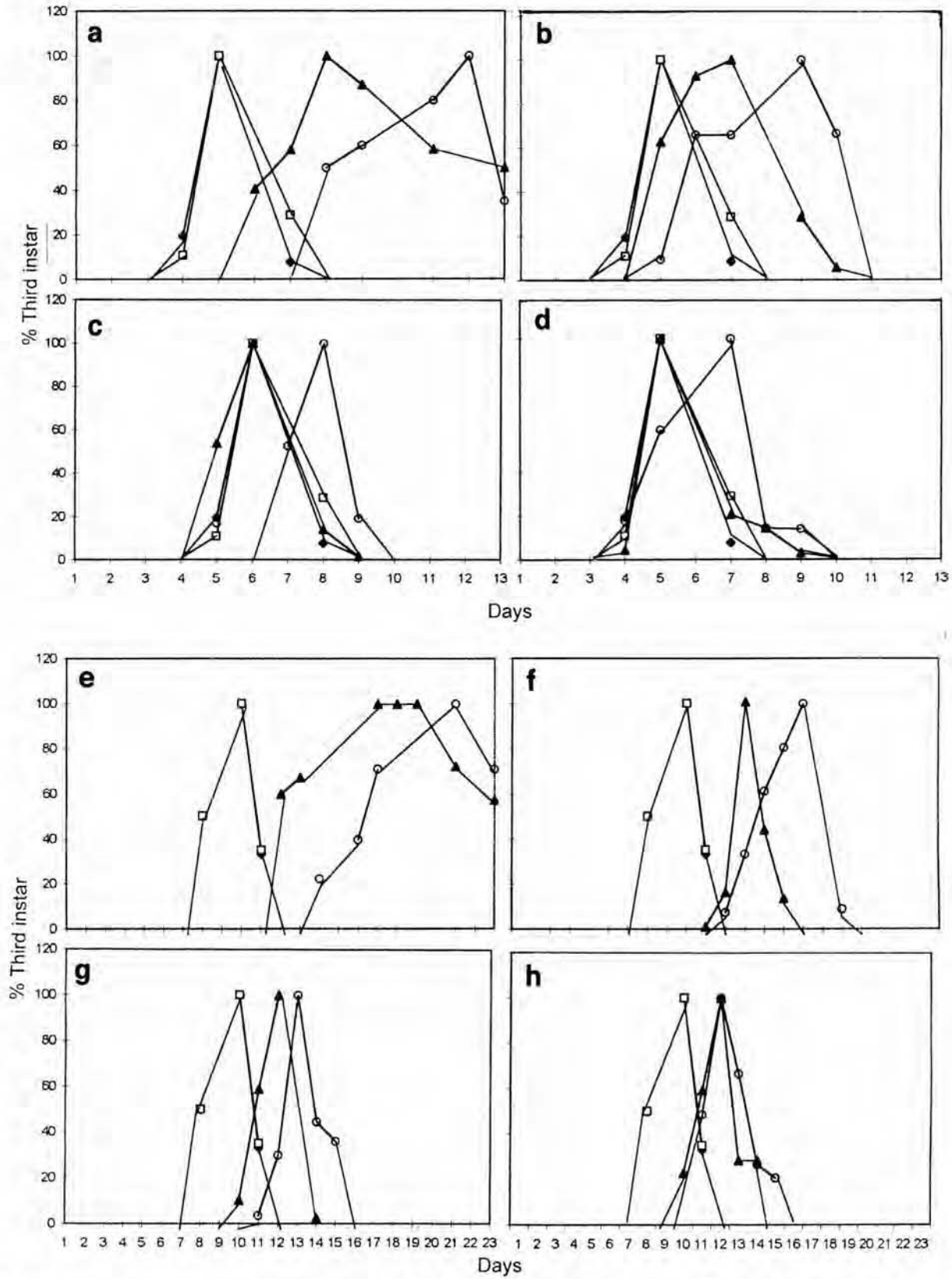


Fig. 2—(a-d) Development of third instar larvae of Oregon R (◆, ▲) and *In(1)B^{M2}(reinverted)* (□, ○) on medium without butyrate (◆, ○), and on medium containing different concentrations of butyrate (▲, ○), at 25°C, (a-d) and at 18°C, (e-h). a,e: 0.2M; b,f: 0.1M; c,g: 0.05M; d,h: 0.001M.

that the butyrate effect could be reproduced at concentrations of 0.1 and 0.05M, although at concentrations of 0.001M butyrate, there was no difference between the wild type and mutant strains on medium with and without butyrate, at either $25^{\circ} \pm 1^{\circ}\text{C}$ (Fig. 2a-d) or at $18^{\circ} \pm 1^{\circ}\text{C}$ (Fig. 2e-h). The results further confirmed that at higher butyrate concentrations, the emergence of third instar larvae of *In(1)B^{M2}(reverted)* was delayed (Fig. 2a-c, e-g), as compared to Oregon R. At $18^{\circ} \pm 1^{\circ}\text{C}$, third instar larvae could be recovered as late as 32 days (for Oregon R) and 36 days [for *In(1)B^{M2}(reverted)*] after laying on medium containing butyrate.

The sex ratio of the flies that emerged from medium containing butyrate at $25^{\circ} \pm 1^{\circ}\text{C}$ and $18^{\circ} \pm 1^{\circ}\text{C}$, was scored. Exposure of wild type and mutant strains to butyrate stress resulted in a marked female semilethality (Table 2). The lethality was observed as a failure of the eggs to hatch on butyrate-containing medium. Temperature was a compounding factor, since the female-specific lethality was enhanced at $18^{\circ} \pm 1^{\circ}\text{C}$ as compared to $25^{\circ} \pm 1^{\circ}\text{C}$ in both wild type and mutant strains. However, as previously observed, the effect was marked in *In(1)B^{M2}(reverted)* as compared to Oregon R.

Butyrate treatment is known to result in accumulation of acetylated histones in treated cells²¹. To determine whether butyrate induced any alteration in the cytology of polytene chromosomes, slow developing third instar larvae from Oregon R reared at $18^{\circ} \pm 1^{\circ}\text{C}$, were harvested daily and chromosome spreads were prepared. It was observed that butyrate induced increased widths in polytene chromosomes (Fig. 1b,c). The series of morphological alterations were remarkably specific for the sex and chromosome type and could be summarized for Oregon R as follows : the first chromosome to manifest morphological alteration was the X chromosome of male third instar larvae. The alteration, which was evident by day 20, resulted in the induction of X chromosomes that resembled the puffy X chromosomes of *In(1)B^{M2}(reverted)* (Fig. 1b). Structural alteration of the autosomes of male third instar larvae appeared later, and at the same time that the polytene X chromosomes and autosomes of female third instar larvae manifested alteration in morphology.

The morphology of width increase differed significantly between the sexes. Whereas in males increased width resulted in X chromosome appearing puffy and diffuse with disruption in the banding

pattern (Fig.1b), the female X chromosomes and autosomes showed increased width, whilst retaining a distinct band identity (Fig. 1c). Further, butyrate induced structural alteration of the male X chromosome, resulted mostly in a global alteration, affecting the entire chromosome. In contrast, the female X chromosome and autosomes more frequently manifested width increase in localized random regions along the chromosome.

The slow developing third instar larvae of *In(1)B^{M2}(reverted)* exhibited puffy X chromosomes on butyrate medium but the number of puffy X's was extremely variable in polytene chromosome spreads of individual larvae (data not shown). Due to mosaic expression of the mutant X chromosomes in third instar male larvae that had been reared on medium without butyrate, it could not be statistically concluded, whether butyrate enhanced the number of puffy X chromosomes of *In(1)B^{M2}(reverted)* or not. Although transcription autoradiographic studies were performed for the butyrate induced alterations in polytene chromosomes of Oregon R, the results were inconclusive due to the random nature of the width increase (data not shown).

The strain *In(1)B^{M2}(reverted)* arose as a spontaneous reversion in the strain *In(1)B^{M2}(In(1)16A1-5;20F)*. At the level of light microscopy, the cytology of the reverted 15F/16A breakpoint is identical to that of the wild type X chromosome. However, the expression of the X chromosome morphotypes can be altered in combination with mutations in modifiers of position-effect variegation¹⁵. These observations suggest that the X chromosome phenotype arises due to PEV. The consequences of exposure of *In(1)B^{M2}(reverted)* to butyrate stress suggests an interaction of the PEV of this strain with *Su(var)* effect of butyrate. As compared to the wild type strain, butyrate induced a more pronounced delay in development, while butyrate-induced female semi-lethality was more marked in cultures of *In(1)B^{M2}(reverted)* than in Oregon R. Although butyrate induced developmental delay has been reported in *Drosophila*^{21,24} and is a possible consequence of the known effect of butyrate in affecting cell cycle progression, female semi-lethality of *Su(var)* strains, (strains *Su-var(2)1*^{23,24}) has been reported to arise due to the interaction with the known effect of butyrate as a suppressor of position effect variegation²⁰. Thus, these evidences explain the differences in the butyrate response between wild type and mutant strains.

One of the significant observations of these experiments related to the butyrate-induced sequence of morphological alterations of the polytene chromosomes bears a striking similarity to our current investigations on the biochemical basis of alteration of the structure of the male X chromosome of *In(1)B^{M2}(reverted)*. A heat-labile chromosome coiling activity has been purified from males of *In(1)B^{M2}(reverted)*, that can induce coiling and subsequent condensation and alteration in the structure of polytene chromosomes *in vitro*¹⁷. Interestingly, upon incubation of salivary glands of wild type larvae in the chromosome coiling factor, polytene chromosomes undergo a series of changes that show a striking similarity to that observed in the slow-developing larvae, reared on butyrate. In both these instances, the first chromosome to undergo alteration in its morphology was the male X chromosome. The female X chromosome and the autosomes underwent subsequent morphological alterations suggesting that the male X chromosome structure is most vulnerable to changes.

Extrapolating these results to those of ongoing biochemical investigations, it may be possible that the PEV of *In(1)B^{M2}(reverted)* perturbs a pathway, resulting in the accumulation/modification of a chromosome coiling factor in males. The quantitative level of this factor is such, that *in vivo*, only the structure of the male X chromosome is affected. This hypothesis would explain the basis of alteration of the structure of the male X chromosome of *In(1)B^{M2}(reverted)*. Further studies, aimed at determining the validity of this hypothesis are in progress.

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