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Nucleolus organizer-suppressed position-effect variegation in *Drosophila melanogaster*

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Summary

The *white* locus is inactivated in a cell-by-cell variegated pattern when juxtaposed with the proximal or distal parts of the nucleolus organizer region (*NO*) by X chromosome inversion. Recombinants for two such inversions, w^{m51b} and w^{m4}, were obtained and randomized for genetic background. *White* locus activity was much higher in the w^{m4} recombinant duplicated for most of the *NO* and much lower in the w^{m51b} recombinant deficient for it. Although there may be other molecular differences between the heterochromatic regions of the recombinants, the most obvious is the dosage of *NO*. Suppression of a *NO* region-evoked variegated phenotype by additional *NO* doses is discussed in relation to four different classes of models for position-effect variegation (PEV): chromatin structure, nuclear geometry, incomplete transposition of mobile elements, and heterochromatin promoter-driven transcription. A corollary of the structural model is functional subdivision of heterochromatin, which would enable the use of PEV as a tool for its study.

1. Introduction

Variable loss of function, termed position-effect variegation (PEV), is the most common *cis* effect of heterochromatin on ordinary genes brought close by chromosome rearrangement, best documented in *Drosophila melanogaster* (reviewed by Lewis, 1950; Hannah, 1951; Baker, 1968; Spofford, 1976; Eissenberg, 1989). In *trans*, extra copies of the largely heterochromatic Y chromosome suppress PEV and extra copies of parts of the Y suppress PEV to a lesser extent (Dimitri & Pisano, 1989) while lack of a Y exaggerates PEV. PEV-suppression may be a general *trans* function of heterochromatin. Here we ask whether PEV-suppression is a *trans* function of a segment of the X heterochromatin that includes the *cis* factors evoking the PEV whose level is monitored.

The specific base sequences that might be responsible for either these *cis* or *trans* effects are unknown, as is whether they may be alike in one or all heterochromatic regions. In *Drosophila*, satellite DNA sequences are nearly limited to the centromere-associated non-polytenizing α-heterochromatin (Lohe & Roberts, 1988). Moderately repeated sequences occur in both α-heterochromatin and the variably under-replicated β-heterochromatin bordering it (Hilliker, Appels & Schalet, 1980; Miklos *et al.* 1984; Donnelly & Kiefer, 1986; John, 1988). There are few essential genes, most scattered sparsely in β-heterochromatin (Schalet & Lefevre, 1976; Hilliker, 1976; Miklos *et al.* 1988; Devlin, Bingham & Wakimoto, 1990). There are roughly 200 copies of the ribosomal RNA (rDNA) cistrons in each nucleolar organizer (*NO*), on both X and Y chromosomes, apparently subdivided into clusters by spacers of various lengths (Endow & Glover, 1979; Indik & Tartof, 1980; Coen, Thoday & Dover, 1982; DeCicco & Glover, 1983; Sharp, Gandhi & Procunier, 1983; Williams *et al.* 1990). On the X chromosome they are flanked proximally by the 1·688 satellite (Hilliker & Appels, 1982), and distally by a cluster of imprecise repeats homologous to the Type I insert found in roughly half of the 28S cistrons (Appels & Hilliker, 1982).

Not all heterochromatic regions appear to be equivalent in evoking PEV (see Spofford, 1976, for review). For example, of the 68 transmissible rearrangements variegating for *white* (w^ms, or *white-mottleds*) listed in 1968 (Lindsley & Grell), nearly twice as many bring that locus next to heterochromatin of the 4 as of the X. Yet the late pro-metaphase *Xh* is 3·5–4 times the length of the entire fourth chromosome.

Four of the five cytologically localized *Xh* w^m breaks are near the borders of the *NO*. In particular, w^{m4} is near the distal edge of *NO*: the secondary constriction

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remains uninverted (Cooper, 1959, and below) but rDNA hybridizes *in situ* heavily to the uninverted and weakly to the inverted segment (Appels & Hilliker, 1982). One only (w^{m51b}) is proximal to the *NO*: rDNA hybridizes *in situ* weakly to the uninverted and heavily to the inverted segments (Appels & Hilliker, 1982). No breaks eliciting *white* variegation are in the more proximal or distal segments of the long arm of *Xh*, although these regions elicit variegation at other X-linked loci such as *scute*.

Thus factors evoking *white* variegation reside in the vicinity of the *NO* in *Xh*. We asked whether that part of the *NO* lying between the w^{m4} and w^{m51b} proximal break positions contains a PEV-suppressing *trans* function as well. In these experiments, hyperploidy for this region suppressed PEV (i.e. increased the fraction of cells with wild-type expression) of the *white* locus brought near the interrupted *NO*, and its deficiency did the reverse.

2. Materials and methods

(i) *Genetic materials*

The two X chromosome inversions employed, the recessive markers *cin* (1–0·0), *y* (1–0·0), *dor* (1–0·3), pn^{51h8} (1–0·7), *cv* (1–13·7), *m* (1–36·1), *f* (1–56·7), *car* (1–62·5), *mel* (1–64·1), and *mal* (1–64·8), the dominant genes *B* and *Su(var)3–41.3*, the variegating duplication *Dp(1;3)* $w^{m264-58}$, the w^+Y, and the Oregon-R wild-type strain are described in Lindsley & Grell (1968). Males from the stock *C(1)DX*, *y f/In(1)*w^{m4}, *y* w^{m4} *cv m f* ('w^{m4}' hereafter) were used as gratefully received from the Drosophila Stock Center at the California Institute of Technology. Homozygous *In(1)* w^{m51b} ('w^{m51b}'), originating from an irradiated Oregon-R X chromosome, was provided by Dr William K. Baker. The marked w^{m4} and the Oregon-R X chromosome, from which w^{m51b} originated, have been calculated to have the same number of rDNA cistrons (Procunier & Tartof, 1978). The *In(1)dl-49*, *v* sn^{X2} mal^2/*In(1)*sc^8, sc^8 *B* mal^{12}/y^+Y mal^{106} stock was kindly sent by Dr George Lefevre, Jr.; pertinent features of *Df(1)*mal^{12} and y^+Y mal^{106} are described by Schalet & Lefevre (1976). The w^+Y was derived from a stock kindly sent by Dr Burke Judd; *dor*, by the Mid-America Drosophila Stock Center at Bowling Green State University.

(ii) *Matings and culture conditions*

Stocks were maintained and single-pair matings of virgin flies kept at 24±1 °C. Flies to be compared were reared in shell vials containing aliquots of the same batch of standard cornmeal–agar–malt–sucrose medium. Three replicate series of crosses, with a lapse of 6 months between nos. 1 and 2 and of 2 years before no. 3, were made to form flies duplicated or deficient for the heterochromatic region between the right breakpoints of the two inversions. All were initiated by several w^{m51b} females singly paired with *y f m cv* w^{m4} males (markers listed in their inverted order). Virgin F_1 females were pair-mated either to unrelated *B* males (series 1), to Bx^{46} males (series 3), or to their w^{m51b} brothers (series 2). Their sons were scored for recessive markers before measurement of their eye pigments. Larvae for karyotypic analysis were offspring of pair matings of *y w* females to recombinant males from series 2: *y* + + + males for putative deficiencies and + *f m cv* males for putative duplications. Also, two males from each recombination class in series 2 were test mated first with harems of *C(1)RM*, *y w/Y* females for 5 days, then the harems of *C(1)RM*, *y w/sc*^{v1} Y^s females autosomally co-isogenic with the first females.

(iii) *Drosopterin-affecting loci on* w^{m4} *chromosome*

The pepper-and-salt type of mosaicism due to both w^ms dictates use of a convenient pigment assay, such as drosopterin content, for large numbers of individuals. We have previously found that the amount of the red eye pigment drosopterin correlates linearly with visual estimates of percentage of eye area pigmented for another w^m that gives large clonal pigmented areas (Spofford, 1967). Extracted drosopterins have commonly been used as an index of *white* gene expression (Baker & Spofford, 1959; Sinclair, Mottus & Grigliatti, 1983). However, their usefulness here depends on the presence of phenotypically similar alleles at all drosopterin-affecting loci in the two inversion chromosomes.

The correlation of drosopterin pigmentation with total eye pigment is visually evident. Mosaic eyes can be examined under a drop of mineral oil to eliminate surface reflection from the facet corneas. The first hint of variegation is the loss of bright red pigment from one or more scattered primary pigment cells surrounding the 'lenses' just beneath the corneas and from the more superficial part of a few secondary pigment cells below the primaries. The duller ommochromes in the interior section of the secondary cells are thus obliquely exposed to view, creating the impression of a 'dark fleck' on a wild-type background. More intense variegation removes the bright red pigments (surface ommochrome as well as drosopterin) from contiguous primary and secondary pigment cells in more extensive areas. Then loss of the duller ommochromes from the underlying portion of the secondaries creates a truly 'white' patch. The most extreme w^m variegation, short of complete loss of all pigments from the eye (= 'white'), is a single ommochrome-containing secondary, usually in the posterior angle of the eye. Eyes from several flies in each of the recombination classes listed in Table 1 were examined and found to follow the normal pattern relating extent of visible red pigmentation and extent of totally 'white' areas. Far less than 50% of

the w^{m4} secondaries contained dull ommochrome, and there was very little bright red pigment. This particular w^{m4} chromosome is not associated with the concentric distribution pattern of pigmentless ommatidia noted for w^{m4} typified by higher levels of w^+ expression (cf. Koliantz & Hartmann-Goldstein, 1984*b*).

We confirmed that the w^{m4} chromosome used did not harbour a hypomorphic allele at any of the drosopterin-influencing loci in interval (1) – *cin*, *dor* and *pn* distal to Xh^L and *mal* and *car* proximal to it (Phillips & Forrest, 1980). We applied several different tests, as follows.

(1) The w^{m4} chromosome completely complemented *car*, *dor*, mal^2 and pn^{51h8} in females confirmed by progeny test to be heterozygous for $y\,f\,m\,cv\,w^{m4}$ and each of the following four chromosomes: *dor*; $In(1)sc^{L8L}\,sc^{8R}$, *car*; *In(1)dl-49*, $v\,sn^{X2}\,mal^2$; and pn^{51h8} *spl*.

(2) The w^{m4} chromosome also completely complemented the *mal*, *mel* and *mell* (both also dull-eye mutants) effects of $Df(1)mal^{12}$, whose extent (19A1-20) was confirmed in polytene chromosome preparations from the mal^{12} stock at the time it was used. On visual comparison, $y\,f\,m\,cv\,w^{m4}/sc^8\,mal^{12}\,B$ females were equally red-eyed whether or not they also carried $Y.mal^+$. In contrast, $sc^8\,mal^{12}\,B/v\,sn^{X2}\,mal^2$ females have brownish eyes unless $Y.mal^+$ is present.

(3) We looked for a weak allele of *cin* or any other locus with an effect on xanthine dehydrogenase (XDH) activity strong enough to reduce drosopterin levels, by comparing the staining intensity of the XDH bands after electrophoresis of whole-fly extracts on a single gel. The XDH activity was as high or higher in $y\,f\,m\,cv\,w^{m4}$ homozygotes as in Oregon-R or w^{m51b}. On the same gel, meanwhile, no activity was detected in mal^2/mal^{12} and very little in $cu\,kar\,ry^{54}/In(3)MKRS$, $kar\,ry^2\,Sb$, mutants known to lower drosopterin levels by reducing XDH activity. Hence the w^{m4} chromosome has wild-type XDH-affecting loci.

(4) $In(1)w^{m4L}\,sc^{8R}$, $y\,f\,m\,+\,+/Y$ males and $In(1)w^{m4L}\,sc^{8R}$, $y\,f\,m+\,+/y\,f\,m\,cv\,w^{m4}$ females bearing recombinants from $y\,f\,m\,cv\,w^{m4}/sc^8\,mal^{12}\,f$ have fully wild-type eye colour. At the base of the recombinant chromosome, the variegating white locus has been replaced by the w^+ allele in sc^{8R}. The chromosome lacks *NO* and is lethal in $Y\,bb^-$ males. Although duplicated for the chromosome section 1B3-3C1, it derives the remainder of interval (1) – 1A1-1B2 and 15F3-20 – from w^{m4} only.

(5) The w^+ *Y* contains a duplication genetically extending from pn^+ to spl^+ but not including any other drosopterin locus with the possible exception of cin^+; $y\,f\,m\,cv\,s^{m4}/w^+$ *Y* males have wild-type eyes.

(6) The fragment $sc^{v1}\,Y^S$ carries the tip of the X including $cin^+\,y^+$. It has previously been found similar to a normal Y in variegation-suppressing effect on w^m drosopterin amounts (Baker & Spofford, 1959). We crossed several of the w^{m4} males and recombinant males successively to $Y/C(1)RM$, $y\,w$ females and to $sc^{v1}\,Y^S/C(1)RM$, $y\,w$ females, coisogenic for the major autosomes. There was no detectable difference between the resulting half-brothers with or without the extra cin^+.

(7) The $y^+Y\,mal^{l06}$ chromosome, carrying a *NO* and a duplication for sections 19 and 20 and possibly for ABO (Pimpinelli *et al.* 1985), but mutant for *mal*, greatly suppresses variegation in $y\,f\,m\,cv\,w^{m4}$ males, giving fully wild-type pigments in extensive regions of their eyes. The implications of this will be noted in a later section.

Thus drosopterin level is a valid measure of the extent of variegation in this experiment.

(iv) *Eye pigment measurement*

Drosopterin was measured by densitometry following chromatography of single heads. The procedure described previously (Spofford, 1967) was followed. In brief, males were aged at least five days before decapitation. Up to 70 heads were squashed on each sheet of Whatman No. 3 filter paper. Usually eight sheets were processed at a time. Six Oregon-R heads were squashed at intervals on each sheet to monitor sheet-to-sheet or day-to-day variation in the measurement process.

The drosopterin values for the control Oregon-R male heads on each of the 20 sheets we chromatographed showed considerable sheet-to-sheet variation, accounting for 18% of the total control variance in arbitrary O.D. units ($F = 2{\cdot}27$ with 19 and 99 D.F.). The correlation between sheet averages of Oregon-R readings and sheet averages for any given w^m genotype approached significance (maximal for the non-crossover w^{m51b} progeny, $r = 0{\cdot}498$ with 19 D.F.) at the 5% level. However, the distribution of raw scores for the various recombinant genotypes fell so clearly into certain patterns that we did not attempt numerical conversion of raw scores.

(v) *Cytological preparation*

Salivary gland chromosomes were prepared and stained with lacto-aceto-orcein, by the technique outlined by Yoon, Richardson & Wheeler (1973).

Neuroblast metaphase and prometaphase cells were fixed by an adaptation of the techniques outlined by Gatti, Tanzarelli & Olivieri (1974) and by Holmquist (1975). Dissected neural ganglia were transferred to 1% Na citrate for 10 min, fixed in 1:1 methanol:acetic acid for 3 min, and transferred to a drop of 60% acetic acid warmed to 38 °C for just 30 s before the drop was rolled across the slide to leave a spiral trail of isolated cells. The slide was air-dried and then stained, either by the silver nitrate procedure outlined by Goodpasture & Bloom (1975) or by a drop of aceto-orcein (3 g Gurr's orcein per 100 ml 70% acetic acid) on the tissue. A coverslip was applied and cells

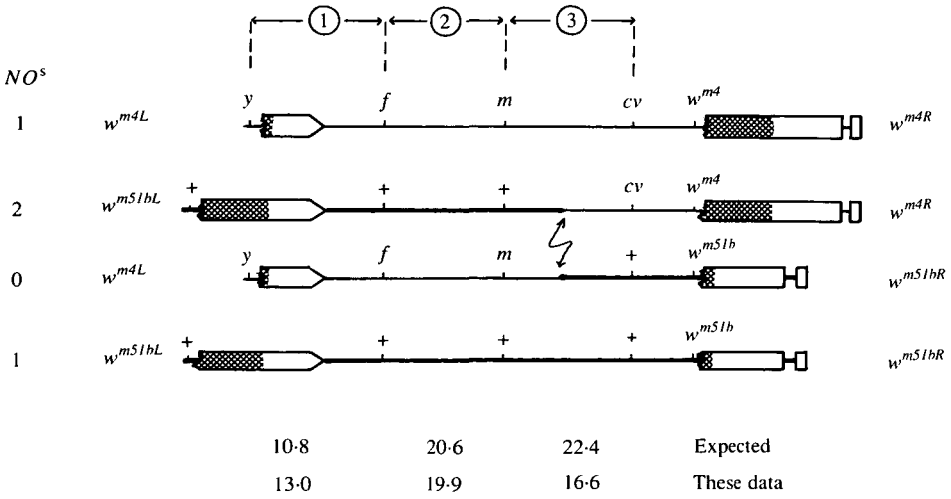

Fig. 1. Diagram of chromosomes prior to and after crossing over indicating genetically marked intervals, and location of *NO* (cross-hatching). 'Expected' values of crossing over for these intervals in normal chromosomes at 25 °C are presented above the values obtained here (at 24 °C).

were flattened by thumb pressure. The coverslip was removed by passing the slide through a graded ethanol series (70–95–95 %). After air-drying, a drop of Euparol and a new coverslip were added.

Cytological preparations were examined and photographed on Kodak Pantomic X film through a Zeiss standard 14 MO1 microscope equipped with planapochromat 100/1·3 phase oil immersion objective, Optovar magnification changer, and 35 mm camera body.

(vi) *Gel electrophoresis*

Xanthine dehydrogenase (XDH) activity was demonstrated on gels prepared electrophoretically as described by Hubby & Lewontin (1966), and stained as described by Prakash, Lewontin & Hubby (1969). All samples, each a single-fly homogenate, were run in duplicate on the same gel.

3. Results

The two inversions used, and their single recombination products, are diagrammed in Fig. 1. In particular, the w^{m4} and w^{m51b} inversions bring the same locus, *white*, to different heterochromatic positions. Single crossovers between these two inversions generate chromosomes permitting two sets of comparisons. One comparison is between one and two doses of *NO*: between just one dose (proximal) in the non-recombinant w^{m4} chromosome and two, both proximal and distal, in the recombinant w^{m51bL} w^{m4R}. Both cases retain the same juxtaposition of *white* with variegation-eliciting chromatin in the proximal w^{m4R}. The other comparison is between one dose of *NO* and none: between the single distal dose in the non-recombinant w^{m51b} chromosome and its absence in the recombinant w^{m4L} w^{m51bR}. Again, both retain the same proximal w^{m51bR} juxtaposition of the *white* gene with a (different) variegation-eliciting region.

Comparisons can be made only between chromosomes sharing the same proximal end, since w^{m4R} and w^{m51bR} differ both in euchromatic and heterochromatic contributions to the junction sequence. The euchromatic break in w^{m4} is 3 kb more distal to the white gene (Tartof, Hobbs & Jones, 1984); sequences affecting variegation propensity may lie between the w^{m4} and w^{m51b} breakpoints. Despite its greater distance from the centromere, w^{+} is inactivated more severely in w^{m4R}. Most of the eye is white in the w^{m4} stock employed, whereas only a few pigment cells are colourless in the eyes of w^{m51b} stock males (Tartof *et al.* 1984; and authors' observations).

The only variable systematically differentiating the genotypes compared was the dosage of the region lying between the variegation-inducing breakpoints of the two inversions. This was assured as well as possible by cytological examination and by the crossing protocol.

(i) *Cytological boundaries of the inversions and their recombinants*

The euchromatic boundary of w^{m51b} clearly lay between bands 3C1 (*lethal(1)zw9*) and 3C2 (*white*), as reported by Gersh, in Lindsley & Grell (1968).

In neuroblast pro-metaphases, roughly two-thirds of *Xh* is moved distally in w^{m51b}. In contrast, less than one-third of *Xh* is moved distally in the marked w^{m4} chromosome. All four *Xh* segments – w^{m4L}, w^{m51bL}, w^{m4R}, w^{m51bR} – were clearly recognizable in the recombinant chromosomes. The total length of *Xh* was the same for w^{m51b} and the Oregon-R wild-type X (Spofford & DeSalle, 1978). The boundaries of distal *Xh* are difficult to determine for w^{m4}, chromatid tips often completely separated from each other. Hence the apparent deficit of *Xh* in w^{m4} noted previously may

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Table 1. *Eye pigment variegation in sons* of* w^{m51b}/w^{m4}, y f m cv *females*

Karyotype	No. NOs	Crossover class†	Phenotype	Number counted	Number measured	Drosopterin‡ mean ± S.E.‖	Between family s^2 (%)§
w^{m4L} w^{m4R}	1	NCO	*y f m cv*	454	236	31·8 ± 1·5	14
w^{m51bL} w^{m4R}	2	SCO-1	*+ f m cv*	66	35	71·7 ± 3·7	—
		SCO-2	*+ + m cv*	151	98	71·7 ± 3·9	31
		SCO-3	*+ + + cv*	148	94	76·8 ± 2·6	—
						73·8 ± 2·4	21
w^{m4L} w^{m4R}	1	DCO-1,2	*y + m cv*	7	1	23·9 ±	—
		DCO-1,3	*y + + cv*	21	8	43·1 ± 7·9	—
		DCO-2,3	*y f + cv*	7	5	30·1 ± 4·9	—
						37·1 ± 4·2	
w^{m51bL} w^{m51bR}	1	NCO	*+ + + +*	659	373	113·0 ± 1·7	14
w^{m4L} w^{m51bR}	0	SCO-1	*y + + +*	158	85	60·8 ± 2·0	—
		SCO-2	*y f + +*	227	149	59·7 ± 3·0	13
		SCO-3	*y f m +*	134	83	63·6 ± 4·4	34
						61·0 ± 2·2	16
w^{m51bL} w^{m51bR}	1	DCO-1,2	*+ f + +*	0	—	—	—
		DCO-1,3	*+ f m +*	14	5	116·1 ± 13·1	—
		DCO-2,3	*+ + m +*	16	13	105·1 ± 5·0	—
						108·2 ± 5·1	
w^{m4L} w^{m51bR}	0	TCO	*y + m +*	2	0	—	—

* Since the proportions in all but the rarest categories of sons agreed in the first two series (c^2 = 10·9 for 9 D.F.), only pooled counts presented.

† Interval (1), *y–f*; interval (2), *f–m*; interval (3), *m–cv*, as in Fig. 1.

‡ On scale giving 100 ± 1·98 for average of 119 control Oregon-R wild-type measurements.

§ Percent of total variance due to between-sibship factors (genetic or measurement) when analysis of variance indicated significance at 5% level. Between-sheet variation within large sibships strongly suggests that much of the among-smaller-sibship variation within any single recombinant class reflected the inclusion of entire sibships on single chromatograph sheets. Thus, probably, measurement error dominates the inter-sibship variation.

‖ Standard error of mean computed to allow for between-sibship variance when sibships vary in size (Snedecor & Cochran, 1967, pp. 289–294).

be spurious. Hilliker and Appels (pers. comm.) find no difference in total *Xh* in their preparations from these same stocks.

The secondary constriction between hB and hC (Cooper, 1959) has been the classic marker for the position of *NO*, although it probably represents but a fraction of the entire rDNA-rich segment of the *Xh* or *Yh*. The secondary constriction was difficult to see in brain pro-metaphase chromosomes of the homozygous w^{m51b} stock using preparative methods (with or without colchicine) that were successful in other stocks. In w^{m4} the secondary constriction remained proximal as figured by Cooper (1959) and by Pimpinelli *et al.* (1985). Secondary constrictions could be seen on both heterochromatic segments in w^{m51bL} w^{m4R}. Thus both tips could be clearly diagnosed in both the parental and recombinant chromosomes.

(ii) *Crossovers*

The crossover flies arose in three series of crosses. The first preliminary and third confirmatory series demonstrated a tolerably low level of nondisjunction, evaluated so as to avoid confounding *X-NO* dose with the PEV-suppressing effect of a matroclinous Y chromosome. Not only would an extra Y suppress PEV in any *XYY* sons, but there might also be a maternal effect on PEV in both *XY* and *XYY* sons of a *XXY* mother (see Spofford, 1976). Thus, in series 1, only one non-disjunctional fly, a B^+ female, appeared among the 1298 offspring of 12 females. There were no non-disjunctional exceptions among the 734 offspring in series 3. Hence, the 3862 offspring in series 2 were produced by mating 90 w^{m51b}.*y f m cv* w^{m4} virgin females individually to their w^{m51b} brothers.

The number of sons in each category is presented in Table 1; the resulting calculated map distances are presented in Fig. 1.

None of the crossovers in interval (1), *y–f*, were found distal to Xh^L, the inverted block of *Xh*, among the 14 *+ f m cv* recombinants karyotyped, all of which had w^{m51bL} tips, nor among the 50 *y + + +*, all of

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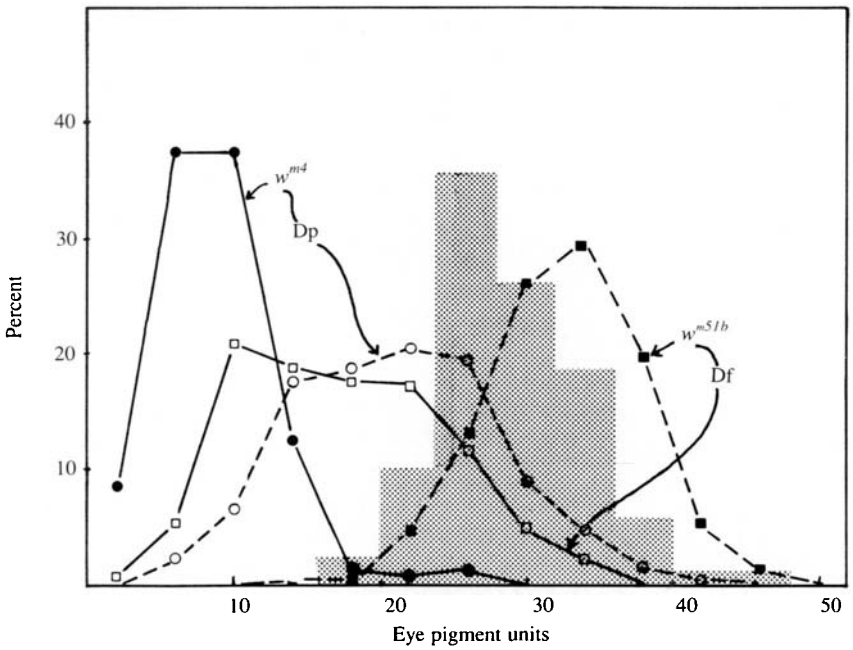

Fig. 2. Distribution of drosopterin levels in non-crossover and single crossover classes. Abscissa: optical density reading of chromatographically developed drosopterin spot intensity on arbitrary scale. Ordinate: percentage of indicated recombinant-type in 4 O.D. unit classes. Symbols: w^{m4} (solid circles), w^{m51b} (solid squares), $w^{m4L}\ w^{m51bR}$ (open squares), $w^{m51bL}\ w^{m4R}$ (open circles). Oregon-R control males, cross-hatched.

which had w^{m4L} tips. The distal segment, from *y* to the left breakpoint, would normally be expected to have 13·9% of the 10·8 map units conventionally expected for interval (1), or a probability $(1-0{\cdot}139)^{64} = 0{\cdot}00007$ of finding no recombinants distal to Xh^L. The absence of these recombinants probably reflects a true and sharp reduction in distal crossing over with structural heterozygosity. Hence, *y* provides an excellent marker for $w^{m4}\ Xh^L$.

Parenthetically, the 40% increase over 'normal' levels of crossing over in interval (1) is comparable to values found by Merriam (1967), Grell (1970) and Szauter (1984) in w^{m4} structural homozygotes. Both the increase in interval (1) crossing over and the decrease in interval (3) represent the usual effect of distance from the centromere, as discussed by Yamamoto & Miklos (1978).

The centromere effect makes *cv* a good though not perfect marker for the proximal w^m component of the inversion, including the *white* locus itself. Not all the *cv*–w^m recombinants were expected to be reliably detectable because of overlap in the levels of pigmentation in the two w^ms. In series 1, only 7 of 485 sons had phenotypes so deviant as to be clearly recombinant for *cv* and w^m.

(iii) *Drosopterin amounts*

Phenotypic data are presented for the F_2 flies in the second (largest) series. The qualitative comparisons agree with the much smaller sets of measurements made for series 1 and 3.

Table 1 presents mean and standard error of drosopterin values for each recombinant class except the triple-crossovers (TCOs). A large component of the measured variation within single recombinant classes is fly-to-fly.

Twelve of the $+f\,m\,cv$ individuals from series 2 confirmed karyotypically to be $w^{m51bL}\ w^{m4R}$ had a drosopterin mean of $66{\cdot}7 \pm 6{\cdot}7$, representative of their SCO-1 recombination class, and double the mean for the NCO ($w^{m4L}\ w^{m4R}$) class. The 41 individuals of the reciprocal class, $y+++$, confirmed karyotypically to be $w^{m4L}\ w^{m51bR}$, had a drosopterin mean of $61{\cdot}0 \pm 2{\cdot}8$, again representative for their SCO-1 recombination class but little more than half the mean for the NCO ($w^{m51bL}\ w^{m51bR}$) class.

Stringent variance analyses certified that the individual recombinant classes could be pooled into

(1) all $y^+\ cv$ single crossovers (SCOs) ($F = 1{\cdot}2$, D.F. = 2, 224),

(2) all $y\ cv^+$ SCOs ($F < 1$, D.F. = 2, 314),

(3) all $y\ cv$ double crossovers (DCOs) ($F < 1$, D.F. = 2, 11),

(4) all $++$DCOs ($F < 1$, D.F. = 2, 16).

whose means and standard errors are also included in Table 1. The pooled frequency distributions of non-crossover (NCO) and SCO pigment phenotypes are shown in Fig. 2. The level of eye drosopterins was increased – i.e. w^{m4} variegation was suppressed – when the indicated *NO*-bearing segment was added distally and the level was decreased – i.e. w^{m51b} variegation was enhanced – when the same segment was subtracted. The amount of the increase in the one case was very similar to the amount of the decrease in the other.

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4. Discussion

(i) *Delimitation of responsible* trans-*acting PEV suppressors*

The following conclusions emerge directly from these results:

1. Double crossovers assume the phenotype characteristic for the tip and base of the chromosome – *y cv* DCOs can be pooled with the w^{m4} NCOs and + + DCOs with the w^{m51b} NCOs.

2. Variegated inactivation of the *white* locus is more extreme in w^{m4R}, marked by *cv*, than in w^{m51bR}, either because of a local difference in the nucleotide base sequence broken near *white* or because of a difference in their new 'heterochromatic' neighbours.

3. The *white* locus is inactivated in fewer cells when the tip includes the larger, *NO*-containing Xh^L, w^{m51bL}, marked by y^+.

Thus, only one chromosome region, that lying between the heterochromatic breakpoints of w^{m4R} and w^{m51bR}, systematically affects the level of variegation in these crosses and thus demonstrably contains *trans*-acting PEV suppressors.

There may be many extraneous features of the total genotypes of the w^{m4} and w^{m51b} stocks that materially affect the PEV phenotype. The crossing protocol assured that any additional genetic factors would be (1) uniform, since all had a Y from the same source; (2) assorted independently, so that the large numbers of measurements made in each X chromosomal class should have randomly sampled the same array of autosomal genotypes; or (3) assessable, since crossovers in different marked intervals along the X could be compared. Any crossing-over between *cv* and w^m would blur rather than sharpen the phenotypic distinction between the two w^ms. Congruence of DCOs and NCOs showed that there was no net difference in the entire marked interval between the two chromosomes. Congruence of the different single crossover classes sharing the same tip and base combination ruled out compensatory differences between chromosomes in the different marked intervals. The crossover frequencies themselves were consistent with results obtained by others for structurally homozygous w^{m4}, and thus consonant with colinearity of the euchromatic portions of the two inversions.

(ii) *The nucleolus organizer as a variegation suppressor*

Most agents suspected of influencing PEV have been shown to affect w^{m4}: low temperature (e.g. Koliantz & Hartmann-Goldstein, 1984*a*), butyrate, supposed to hyperacetylate histones (Mottus, Reeves & Grigliatti, 1980), deletions of the histone loci (Khesin & Leibovitch, 1978; Moore *et al.* 1983), and allelic substitution at a number of specific loci (Spofford, 1969; Reuter, Hoffman & Wolff, 1983; Sinclair *et al.* 1983). It is more extreme in *XO* than in *XY*, and in *XY* than in *XYY* males (e.g. Koliantz & Hartmann-Goldstein, 1984*a*; and author's observations).

Fewer PEV modifying agents have been tested on w^{m51b}. Its response is similar to that of w^{m4} for change in number of Y chromosomes (Dimitri & Pisano, 1989; authors' unpublished observations). Its variegation is suppressed by allopurinol, as are many other variegating systems (Spofford, 1982), and by *Su(var)3-41*. 3^S (manuscript in preparation).

The demonstration that the *NO* itself can suppress variegation complements Hilliker & Appel's (1982) finding that the extent of w^{m51b} variegation corresponds to the extent of distal *Xh* deleted along with $su(f)^+$; deficiencies removing part of *NO* enhance variegation more extensively than deficiencies leaving *NO* intact. In the experiments reported here the most obvious candidate for the variegation-modifier is the *NO* itself, even though the *NO*-distal parts of *Xh* exchanged between w^{m51bL} and w^{m4L} may also differ in molecular detail.

How heterochromatic the portion of *Xh* that lies between the proximal breaks of these two inversions is, is open to question. Various lengths of 'spacer' may separate rDNA genes into clusters of unknown size. The nature of the largest spacers is quite unknown. Classical cytological preparations show prometaphase (heterochromatin) condensation of much of the X and Y that calculation suggests to contain rDNA cistrons. The secondary constriction associated with *NO*, proximal in w^{m4R} and distal in w^{m51bL}, may well be the relict of recent rRNA transcription, separated from the variegating breakpoints by more tightly condensed rDNA cistrons in neuroblast cells.

Both rearrangements lead to inactivation of the white locus comparatively late in development since the size of affected areas (or, of pigmented areas in extreme cases of w^{m4}) can be as small as a single pigment cell. Transcription of the *white* locus begins in the third-larval-instar eye disk at approximately the same time as the final pigment-cell division (Fjose *et al.* 1984; Ready, Hanson & Benzer, 1976; Golic & Lindquist, 1989) and terminates before the midpupal stage (Stellar & Pirrotta, 1985). The 'pepper-and-salt' variegation pattern many reflect transformation of the state of activity of adjacent *NO* DNA only a few cell divisions before pigment cell differentation. The final 'decision' whether w^+ is to be expressed or not appears to be a stochastic process, whose probability may be influenced as early as at cellular blastoderm formation, given the size of clonal areas differing markedly in percentage of pigmented cells (see fig. 2 in Tartof *et al.* 1984).

Thus, the developmental time at which additional doses of the *NO* region suppress PEV might be as early as the flocculent heterochromatization of the proximal X in embryonic cycle 10 interphase (Foe & Alberts, 1985) or not until after mitosis ceases in the

late third-larval-instar eye disk. Certain genetic variegation suppressors that have quite striking maternal (hence, early) effects on some rearrangements, such as the *Su(var)3-41·3* locus on $Dp(1;3)w^{m264\cdot58}$ (Spofford, 1967), have strong zygotic but weak maternal effects on the w^{m4} phenotype (Spofford, 1969). $Dp(1;3)w^{m264\cdot58}$ shows no phenotypic effect of the substitution of either $In(1)sc^{4L}\ sc^{8R}$ (nullo-*NO*) or $In(1)sc^{8L}\ sc^{4R}$ (2-*NO*) for a normal X in the maternal genotype (unpublished observations). The direct test for a maternal *NO* dose effect on w^{m4} or w^{m51b} phenotype has not been conducted, nor has a direct test for a zygotic *NO* dose effect on $Dp(1;3)w^{m264\cdot58}$ phenotype.

(iii) *Explanatory models*

The *Xh* interval under study has been shown to include both sequences that have a PEV-evoking *cis* effect when broken and sequences that have a PEV-suppressing *trans* effect when intact. Some of the current hypotheses defensible for at least some examples of PEV would attribute the *cis* and *trans* effects to the same set of sequences. These hypotheses can be grouped under four headings: (*a*) chromatin structure, (*b*) nuclear geometry, (*c*) mobile element transposition, and (*d*) heterochromatin-promoted-transcript interference with normal transcription (Frankham, 1988). The results described here are compatible with more than one of these hypotheses.

(*a*) *The chromatin structure hypothesis.* Gene inactivation in some cells by inclusion in a self-assembling complex of DNA, histones and specific non-histone heterochromatin proteins, nucleated by specific base sequences within the heterochromatin (Brutlag, 1980; Tartof *et al.* 1984). Interspersed repetitive and unique sequences at the euchromatin-heterochromatin boundary (Miklos *et al.* 1984, 1988; Devlin, Bingham & Wakimoto, 1990) may buffer the remainder of the euchromatin from 'heterochromatization' in normal chromosomes. In a rearrangement, the heterochromatic conformation may overrun the gene whose expression is being monitored, to an extent presumed partly dependent on the supply of specific heterochromatic constituents (Locke, Kotarski & Tartof, 1988). Both w^{m4} and w^{m51b} have a very limited 'spreading effect', though survival of $w^{m51b}/0$ males themselves depends on the presence of an autosomal variegation-suppressor allele, *Su(var)3-41·3*^{S} (manuscript in preparation), suggesting spread of inactivation to the *rst* complex locus when variegation is extreme.

Under this hypothesis, supernumerary heterochromatic segments would suppress PEV by titrating a limiting constituent shared by the PEV-suppressing and the PEV-evoking heterochromatic regions. The heterochromatic sequence interrupted in w^{m4R} is likely to be present intact in w^{m51bL}, able to compete for molecular constituents. Perhaps also, the heterochromatic sequence interrupted in w^{m51bR} is also represented in w^{m51bL}. If so, hypoploidy (in w^{m4L}) would allow heterochromatization to extend to the *white* gene in w^{m51bR}.

(*b*) *The nuclear geometry hypothesis.* Inaccessibility of requisite transcription factors for a variegating gene in the part of the nucleus nearest centromeric heterochromatin. Anisotropic cytological localizations of programming molecules (MacDonald & Struhl, 1988; Berleth *et al.* 1988) may occur as early as when cycle 10 interphase nuclei approach the overlying cortical cytoskeleton which is then reorganized (Karr & Alberts, 1986). It is also possible that certain genes, as w^{+}, must have access to a particular nuclear domain at the time of tissue-specific transcription (Bingham & Zochar, 1985).

On the simplest form of this model, extra heterochromatin would suppress variegation by 'crowding' the pericentromeric nuclear space, 'squeezing' the affected gene out into a more normal nuclear location.

Such a simple nuclear geometry model is inappropriate here. One would expect w^{m51bR}, closer to the centromere, to be more extremely variegated. One would not expect distal heterochromatic segments in peritelomeric domains (Lifschytz & Hareven, 1982; Foe & Alberts, 1985) to be effective suppressors of variegation of proximally located genes. On the other hand, a more elaborate partitioning of function within nuclear space can be envisioned as compatible with the results presented here.

(*c*) *Mobile element transposition model.* Small w^{-} deficiencies generated somatically by incomplete mobilization of a breakpoint element through the process described by Shapiro (1979). Both of the inversions examined and also $In(1)w^{mMc}$ have an *Xh* break in or beside middle repetitive elements suspected of mobility (Tartof *et al.* 1984). However, a mobile-element PEV model would have to postulate elements that caused deletions more often when in heterochromatin than when in euchromatin and in somatic rather than germinal tissue – the reverse of the P-element situation (McElwain, 1986).

Additional copies of the same kind of mobile element in additional heterochromatin would suppress PEV by titrating transposase, triggering harmless deletion of adjacent satellite sequences.

(*d*) *Heterochromatin promoter activation model.* The resulting transcript repressing gene expression. Frankham (1988) has proposed that heterochromatin contains potentially highly active promoters normally inaccessible to transcriptases. A variegating rearrangement replaces the adjacent heterochromatin, assumed to repress transcription, by euchromatin. The resulting read-through transcripts are anti-sense for genes oriented 3′ toward the break, and can block expression by RNA–RNA hybridization. Heavy transcription from the derepressed heterochromatic promoter is proposed to occlude the (downstream) promoter of genes oriented 5′ toward the break.

It would be especially easy to explain *NO*-evoked PEV as driven by DNA polymerase I promoters, except for the orientation of the disrupted rDNA cistron in w^{mMc} (Tartof *et al.* 1984). Additional *NO*s would reduce the frequency of transcript overruns by providing additional polymerase I-binding sites. Even a single rDNA cistron severely depresses the function of an adjacent reporter gene on the same construct whether monitored in a transient expression assay or by the ability of the construct to produce germ-line transformants (Karpen, 1987), although it may itself be heavily transcribed and organizes a nucleolus (Karpen, Schaefer & Laird, 1988). However, neither promoter occlusion nor anti-sense RNA could produce the inactivation observed with the 5′ end of the rDNA toward the 3′ end of the reporter gene.

PEV suppression by y^+ *Y mal*^{106} can be interpreted by any of the four classes of models. It includes Type I insert-like repeated sequences from the X heterochromatin just distal to the *NO* (Hilliker & Appels, 1982) and some rDNA cistrons from the X *NO* (diCicco & Glover, 1983). It was derived from Y^+ *Y mal*$^+$, which retains the *Xh ABO* genetic function, probably distal to the Type-I-rich region (Pimpinelli *et al.* 1985). The enhanced PEV suppressivity of this Y may be associated with one or more of these additional *Xh* sites.

It would be most interesting if regional regulation of rDNA cistron transcription should prove to be related to the level of white mottling in these two inversions – the distal cistrons for w^{m4}, the proximal for w^{m51b}.

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