Genet. Res., Camb. (1988), **52**, *pp.* 119–123 *With* 1 *text-figure* *Printed in Great Britain*

A correlation between development time and variegated position effect in *Drosophila melanogaster*

JOHN MICHAILIDIS, NEIL D. MURRAY* AND JENNIFER A. MARSHALL GRAVES
Department of Genetics and Human Variation, La Trobe University, Bundoora, Victoria 3083, Australia

(*Received 3 December 1987 and in revised form 10 March 1988*)

Summary

Position-effect variegation is a phenomenon in which cell-autonomous genes, normally expressed in all cells of a tissue, are expressed in some cells but not in others, leading to a mosaic tissue. Variegation occurs when a normally euchromatic gene is re-positioned close to heterochromatin by chromosome rearrangement. The extent of variegation is known to be influenced by a number of environmental and genetic factors. In the courss of investigations of the influence of the pH of larval medium on the extent of eye-colour variegation in In(1)w^{m4} *Drosophila melanogaster*, we have found that the extent of variegation depends on development time. Flies reared at pH 2·6 develop slowly and show more extreme variegation than those reared at higher pH. This effect, as well as variations within the pH treatments, can be accounted for by differences in development time. The observed regression relationship between variegation and development time also appears to accommodate the influences of temperature on both variables. We suggest that development time may account causally for the reported influences of a number of environmental agents (temperature, crowding, chemicals) on variegation. Ways in which this might occur are discussed in the context of models of the molecular basis of differential gene activity.

1. Introduction

Position-effect variegation is a consequence of the juxtaposition of a wild-type gene next to heterochromatin. This results in a heritable somatic inactivation of the gene in cell clones within a tissue, visible as mosaic expression. The phenomenon has been most extensively documented in *Drosophila melanogaster* (Baker, 1968; Spofford, 1976). There is a *cis-trans* relationship between the variegating gene and the breakpoint in the chromosomal rearrangement at which the euchromatin and heterochromatin are joined; *cis*-dominance is the basis for proofs of the position effect nature of variegation (Spofford, 1976), and, in fact, the variegating allele can be rescued in a normal sequence chromosome by recombination between the locus and the breakpoint (Demerec & Slizynska, 1973; Judd, 1955).

Genetic and environmental factors can modify the expression of variegation. Higher temperatures usually suppress (relieve) the variegated phenotype toward wild type, while lower temperatures enhance it (Gowen & Gay, 1934). Crowding of larvae during early development also produces a more extreme effect (Hinton, 1949). Extra heterochromatin, elsewhere in the genome, also affects the expression of the variegating gene; the presence of a *Y* chromosome, or other heterochromatic elements, suppresses variegation, while deletion of these elements enchances it (Gowen & Gay, 1934). Thus, *XXY* females and *XYY* males are almost fully revertant to wild type, while *XO* males show more extreme variegation than *XY* males (Spofford, 1976). Deletion of histone genes has also been reported to suppress variegation (Moore *et al.* 1979; Moore *et al.* 1983; Sinclair *et al.* 1983). Alleles of certain genes also modify the expression of the variegating phenotype, either by enchancing or suppressing it (Reuter *et al.* 1982).

In the few cases studied, position-effect variegation exhibits a polarized spreading of inactivation, such that the loci further from the breakpoint are inactivated less frequently than those closer to it (Spofford, 1976). Inactivation of genes may be accompanied by heterochromatinization of the normally euchromatic segment on which they are located; this may be cytologically visible in the polytene chromosomes of the salivary glands, extending up to 65–80 bands from the break point (Hartman-Goldstein, 1967; Spofford, 1976).

The molecular basis of position-effect variegation is

* Author to whom reprint requests should be addressed.

still unclear. Earlier proposals involving changes in gene number (Schultz, 1956; Procunier & Tartof, 1978), have not been supported by experimental test (Henikoff, 1979; Rushlow *et al.* 1984). Transcriptional inactivation of the variegating gene has now been demonstrated in two systems (Henikoff, 1981; Rushlow *et al.* 1984), but it is not known how this is accomplished. The suggestion has been made, on the basis of butyrate-induced suppression of variegation, and of the effects of deletions and duplications of histone genes, that histone deacetylation is involved (Mottus *et al.* 1979; Moore *et al.* 1979, 1983); however, since butyrate has since been found to affect gene expression in several other ways (e.g. Boffa *et al.* 1981; Christman *et al.* 1980), and since DMSO (an agent with no known effect on histone modification) as well as butyrate can also suppress variegation (Michailidis *et al.* in preparation), other interpretations should be considered.

During the course of investigations on the effect of inducing agents on variegation, in which the effects of medium pH were tested as controls, it was observed that flies which emerged later showed more extreme variegation than those emerging earlier within the same treatment regime, suggesting that development time plays an important role in the expression of variegation. We therefore examined expression of a variegating gene in flies emerging at different times from incubation at different temperatures, and at different pH. For these experiments, we have used a strain homozygous for the inversion In(1)w^{m4}, which relocates the *white eye* gene w^+ from its normal position (in the distal arm of the *X*) near to the proximal heterochromatin (Cooper, 1959). Expression of the w^+ gene is cell-autonomous, so that clones of ommatidia are either fully pigmented or colourless, and the eye is a mosaic of wild-type and mutant patches. The percentage pigmented ommatidia is therefore a direct measure of w^+ variegation (Shoup, 1966). Our finding that the percentage pigmented ommatidia decreases regularly as development time is increased, by different agents, suggests that these and other agents have their effect on variegation through their effect on development time.

2. Materials and methods

(i) *Drosophila strains and culture*

Strains of *D. melanogaster* used were a wild type originally collected in Melbourne, white eye (w^1), and a strain homozygous for the *X* chromosome inversion In(1)w^{m4}. Stocks were routinely maintained at 25 °C on an agar-sugar-dried yeast medium containing Nipagin to inhibit mould. The pH of the medium was controlled in all experiments by replacing the water in the medium with a citrate–orthosphosphate buffer (Perrin & Dempsey, 1974) monitored before medium preparation with an Orion Research Digital Ionalyzer 501, and checked, after medium had solidified, with an Owens Illinois pH Combination 2000 solid phase pH probe. Monitoring during the experiment showed that the pH of this buffered medium did not alter. Males and females were allowed to mate for 4 days after emergence at 25 °C, then females were transferred to the experimental conditions for 2 days for egg laying. The pH experiments were carried out at 18 °C, and five females per vial were used to minimize crowding amongst the larvae.

(ii) *Scoring*

All flies emerging at 1 day intervals were scored at a magnification of 30×, using a Wild M7 Stereomicroscope. Males and females were segregated, and the left eye of each fly was scored visually by assessing the percentage pigmented ommatidia, and placing into one of four classes: 0–24%, 25–49%, 50–74%, and 75–100%. The mean percentage pigmented ommatidia (PPO) was calculated for each treatment from

$$\text{PPO} = \frac{\sum(\text{no. flies enclosed per class} \times \text{midpoint of class})}{\sum(\text{no. flies per class})} \times 100$$

Given that this score is based on midpoints of classes, the maximum and minimum PPO are 88·0 and 12·5, respectively. PPO was calibrated against spectrophotometric measurements by extracting pigment from the heads of 10 male and 10 female flies in each class, homogenizing in several drops of solvent (20 parts n-butanol, 3 parts acetic acid, 7 parts dH_20) making up to 1 ml with solvent, then centrifuging. The OD at 480 nm of the supernatants were measured using a Beckman model 26 Spectrophotometer. Extracts of wild-type and w^1 mutant flies were measured to provide, respectively, values for 100% and 0% pigmented ommatidia. The OD at 480 nm was found to be positively correlated with PPO ($r = +0{\cdot}90$ for males, $0{\cdot}05 > P > 0{\cdot}01$; $r = +0{\cdot}87$ for females, $0{\cdot}05 > P > 0{\cdot}01$).

Development time was measured as the mean number of days between oviposition and eclosion for each group of flies.

3. Results

Variegation in the eye of In(1)w^{m4} flies was observed as patches of pigmented (red) and unpigmented (white) ommatidia. Expression varied from a fully pigmented eye to an eye with one or a few small red spots.

Table 1 shows the effect of pH treatment on both the mean percentage pigmented ommatidia (PPO) and development time. Males possessed higher levels of pigment than females for any treatment, but, within each sex, PPO was found to be homogeneous over a pH range of 3·0–5·0 (heterogeneity $\chi^2_{15} = 23{\cdot}912$ and 5·590 for males and females). The mean development time of both males and females was not significantly

Table 1. *Effect of medium pH on the mean percent pigmented ommatidia (PPO)* ± S.E. *and on mean development time (DT)* ± S.E. *of male and female In(1)w^{m4} flies*

pH	Sample size ♂	PPO ♂	DT ♂	Sample size ♀	PPO ♀	DT ♀
2·6	48	46·0 ± 6·7	27·9 ± 0·3	45	14·2 ± 1·2	27·5 ± 0·3
3·0	39	69·9 ± 4·4	23·1 ± 0·3	56	43·5 ± 4·3	22·7 ± 0·3
3·4	27	80·5 ± 4·0	22·0 ± 0·3	30	54·3 ± 5·6	21·2 ± 0·4
3·8	34	75·4 ± 4·0	22·5 ± 0·3	38	49·5 ± 5·6	21·9 ± 0·3
4·2	29	72·3 ± 5·1	23·0 ± 0·4	23	47·5 ± 7·2	23·0 ± 0·4
4·6	41	66·4 ± 3·8	22·2 ± 0·3	54	48·8 ± 4·6	22·1 ± 0·3
5·0	9	76·8 ± 8·5	23·0 ± 0·8	9	46·0 ± 11·9	23·0 ± 0·8

Table 2. *The relationship between development time (DT) and mean percent pigmented ommatidia (PPO)* ± S.E. *for males and females from pooled pH treatments*

DT	Sample size ♂	PPO ♂	Sample size ♀	PPO ♀
19·5	15	82·9 ± 3·7	37	75·3 ± 3·1
21·5	86	77·1 ± 2·3	95	51·6 ± 8·9
23·5	51	68·2 ± 3·8	53	29·6 ± 3·7
25·5	40	61·5 ± 4·0	33	18·6 ± 3·1
27·5	12	39·7 ± 9·0	16	14·1 ± 1·6
29·5	14	50·1 ± 7·8	16	14·1 ± 1·6
32·0	9	20·9 ± 9·4	4	12·5 ± 0·0

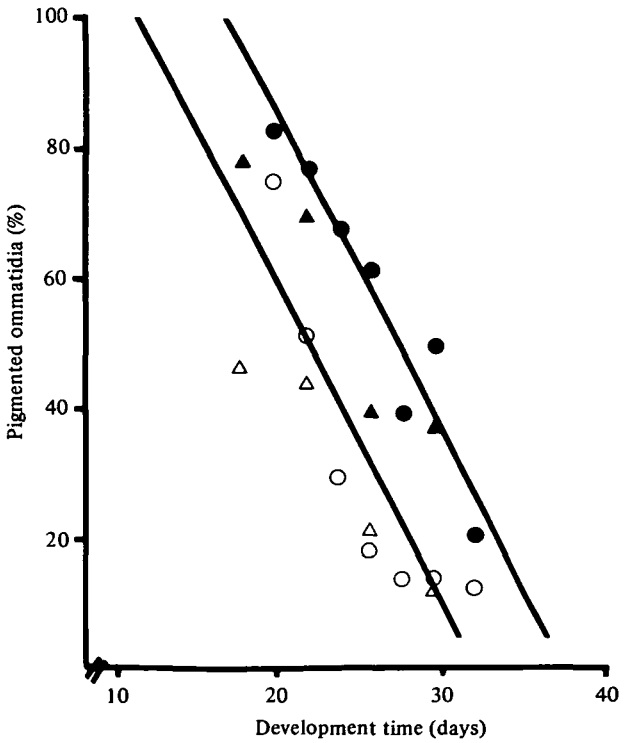

Fig. 1. The relation between development time (days), and mean percentage pigmented ommatidia (PPO) for flies reared on media at different pH (circles) and at different temperature (triangles). Data from males are represented by closed symbols, females by open symbols. The two regression lines are drawn from the pH data. The equation for males is PPO = 177·3 − 4·7 DT and for females PPO = 153·6 − 4·8 DT.

different over the pH range 3·0 to 5·0, but a highly significant delay was observed for flies reared at pH 2·6 compared to those reared at pH 3·0 ($t_{85} = 11{\cdot}3661$ for males, $t_{99} = 11{\cdot}2421$ for females, with $P < 0{\cdot}001$ for both sexes). Therefore, although very acid medium significantly enhances variegation, this effect could equally well be attributed to the delay in development time at this low pH.

The hypothesis that the extent of variegation is related to development time is supported by an inverse correlation observed between pigment expression and development time *within* each pH treatment group. The data from all pH's were pooled, then grouped according to development time; PPO was calculated for flies reaching eclosion at two-day intervals (Table 2), and plotted against development time (points marked as circles in Fig. 1). There is a linear relationship between PPO and development time. Linear regression coefficients were calculated as −4·70 and −4·80 for males and females respectively, and analysis of variance showed that these were significantly different from zero ($F_{1,5} = 49{\cdot}42$ and 18·41 respectively, both $P < 0{\cdot}01$). The regression lines are shown in Figure 1.

In a separate experiment, In(1)w^{m4} flies were grown in medium (pH 3·4) at three different temperatures (18, 20, 22 °C). Flies reared at higher temperatures developed more rapidly, and expressed more pigment than flies reared at lower temperatures. PPO was plotted against mean development time for temper-

ature data, pooled as described for pH experiments. These points (triangles in Fig. 1), appear to conform to the regression relationships obtained for the pooled pH data, although there was lower overall expression of pigment in these experiments.

4. Discussion

We have observed a negative correlation between expression of the variegating w^+ gene and development time for In(1)w^{m4} flies reared under a range of environmental conditions (specifically pH and temperature). This correlation could mean either that the extent of variegation is influenced by development time, or that flies having more extreme variegation develop more slowly. The observation that development of wild-type flies is also delayed by low temperatures suggests that developmental delay is the primary effect. A third possibility, that variegation and development time are independently influenced by environmental factors, is rendered unlikely by our observation that the correlation between the variables holds within, as well as between treatments.

We suggest that developmental delay, induced by a variety of environmental stresses, enhances position-effect variegation. A number of agents which modify position-effect variegation, described in the literature, may prove to have their primary influence on development time. For instance, larval crowding enhances variegation (Hinton, 1949), and also prolongs development; it seems likely that the primary effect of crowding is to retard development because of nutrient stress. Nutrient stress may also account for the developmental delay in flies raised on very acid medium; we observed that medium buffered at pH 2·6 had a fine, gel-like consistency, which might restrict nutrient uptake.

Other treatments which enhance position-effect variegation may also have their primary effect on development time. For instance, some agents which interfere with DNA synthesis have been found to enhance variegation (Schultz, 1956); while these results were interpreted in terms of an effect of heterochromatin on DNA synthesis, it seems likely that the primary effect of these inhibitors is to retard development.

Since suppression or enhancement of variegation by chemicals, or by genetic changes at other loci, may be confounded by effects on development, it is obviously important in such studies to show that changes in gene expression are independent of development time. For example, altered dosage of histone genes affects development time (Rushlow *et al.* 1984) as well as variegation (Moore *et al.* 1979, 1983). It is difficult to design appropriate controls for such experiments; perhaps the most satisfactory approach is to make comparisons of phenotype with reference to an observed relationship between variegation and development time, such as has been established for the flies used in these experiments (Fig. 1).

There are, however, agents whose effects are separable from their effects on development time; for instance butyrate and propionate relieve variegation in In(1)w^{m4} flies, although they significantly prolong development (Mottus *et al.* 1980; Rushlow *et al.* 1984; Michailidis *et al.* in preparation). A major factor whose effect on variegation is clearly independent of development time is sex. In our experiments, both males and females showed an inverse correlation between development time and pigment expression (Fig. 1). The regression lines have nearly identical slope, but expression in males is consistently higher than in females at any one development time. We attribute this difference to the presence of the largely heterochromatic *Y* chromosome, in accordance with the interpretations of Gowen & Gay (1934) and of Spofford (1976). The effects of supernumerary heterochromatic elements also must be independent of their effects on development time, since their presence relieves variegation without accelerating development.

Our hypothesis, that the extent of variegation depends on development time, requires a time-dependent step in the control of expression of the variegating gene, perhaps in the control of the spread of inactivation from the heterochromatin into euchromatin. How can such a time-dependent process be accommodated within a molecular model? Assuming that variable expression of the w^+ gene reflects variable transcription (Henikoff, 1981; Rushlow *et al.* 1984), it is necessary to propose that some *time-dependent* (rather than developmental stage-dependent) process affects the probability that the gene will be transcribed in a clone of cells. For instance, in terms of the model proposed by Mottus *et al.* (1980) in which active transcription is associated with histone acetylation in the heterochromatin adjacent to the variegating genes, histone accumulation and/or acetylation would need to be a function of the absolute duration of development. Indeed, any molecular model of variegation can accommodate a development time effect, provided that one or more processes are time-dependent; that is, are governed by a clock, in the same way as are circadian rhythms, well known in *Drosophila* (Saunders, 1982).

It would ultimately be very satisfying to incorporate observations on position-effect variegation into a generally applicable model of the effect on gene expression of clock-dependent processes. Mampell (1965) reported a relationship between development time and the degree of expression of a number of mutant genes, including the variegating sc^4 gene in *D. melanogaster*. Further experiments on the relationship between development time and gene expression (including variegation), involving agents that extend larval development in different ways, and mutants which prolong development or disturb circadian rhythms, may provide an avenue for such a synthesis.

Time-dependent changes in *Drosophila* gene expression may also have parallels to age-related changes in mammalian *X* chromosome inactivation. It has been observed that genes inserted into the mouse inactive *X* (Cattanach, 1974), or borne on the *X* at maximal distance from the inactivation centre (Wareham *et al.* 1986) are reactivated with age. This suggests that the spreading of inactivation from the centre becomes progressively reduced, perhaps as the result of a decreased efficiency of maintenance methylation as proposed by Wareham *et al.* (1987) and discussed by Holliday (1987); alternatively it may reflect a mitigation of chromatin contraction, which has been proposed to act as a second step in a complex inactivation mechanism (Gartler *et al.* 1985). There is conflicting evidence of DNA methylation in *Drosophila* (Urieli-Shovel *et al.* 1983; Achwal *et al.* 1984; Patel & Gopinathan, 1987); however, the time-dependent repression of the relocated w^+ gene in *Drosophila* that we observed could be explained by an increase in condensation of heterochromatin and bordering euchromatin. Changes in chromatin conformation may therefore be responsible for abnormal, and perhaps also normal time- or age-dependent changes in gene expression in eukaryotes.

We thank Dr J. Sved (University of Sydney) for providing the In(1)w^{m4} and Garry Spence for providing wild-type and w^1 stocks. We thank the following colleagues for stimulating discussion and/or critical review of the manuscript; Dr M. Goldman and Dr S. Henikoff (University of Washington, Seattle), Dr T. Grigliatti (University of British Columbia), Dr R. Reeves (Washington State University, Pullman).

References

Achwal, C. W., Ganguly, P. & Chandra, H. S. (1984). Estimation of the amount of 5-methylcytosine in *Drosophilia melanogaster* DNA by amplified ELISA and photoacoustic spectroscopy. *EMBOJ.* **3**, 263–266.

Baker, W. K. (1968). Position-effect variegation. *Advances in Genetics* **14**, 133–169.

Boffa, L. C., Gruss, R. J. & Allfrey, V. A. (1981). Manifold effects of sodium butyrate on nuclear functions. *Journal of Biological Chemistry* **256**, 9612–9621.

Cattanach, B. M. (1974). Position-effect variegation in the mouse. *Genetical Research* **23**, 291–306.

Christman, J. K., Weich, N., Schoenbrun, B., Schneiderman, N. K. & Acs, A. (1980). Hypomethylation of DNA during differentiation of Friend erythroleukemia cells. *Journal of Cell Biology* **86**, 366–370.

Cooper, K. W. (1959). Cytogenetic analysis of major heterochromatic elements (especially Xh and Y) in *Drosophila melanogaster* and the theory of 'heterochromatin'. *Chromosoma* **10**, 535–588.

Demerec, M. & Slizynska, H. (1973). Mottled white 258-18 of *Drosophila melanogaster*. *Genetics* **25**, 641–649.

Gartler, S. M., Dyer, K. A., Graves, J. A. M. & Rocchi, M. (1985). A two-step model for mammalian X chromosome inactivation. In *Chemistry, Biochemistry and Biology of DNA Methylation* (ed. G. L. Cantoni and A. Razin) pp. 223–235. New York; Alan R. Liss.

Gowen, J. W. & Gay, E. H. (1934). Chromosome constitution and behaviour in eversporting and mottling in *Drosophila melanogaster*. *Genetics* **19**, 189–208.

Hartmann-Goldstein, I. J. (1967). On the relationship between heterochromatinization and variegation in *Drosophila* with special reference to temperature-sensitive periods. *Genetical Research* **10**, 143–159.

Henikoff, S. (1981). Position-effect variegation and chromosome structure of a heat shock puff in *Drosophila*. *Chromosoma* **83**, 381–393.

Hinton, T. (1949). A correlation of phenotypic changes and chromosomal rearrangements at the two ends of an inversion. *Genetics* **35**, 188–205.

Holliday, R. (1987). X chromosome reactivation. *Nature* **327**, 661–662.

Judd, B. H. (1955). Direct proof of a variegated-type position effect at the white locus in *Drosophila melanogaster*. *Genetics* **40**, 739–744.

Mampell, K. (1965). Gene expression and development rate. *Genetica* **36**, 135–146.

Moore, G. D., Procunier, J. D., Cross, D. P. & Grigliatti, T. A. (1979). Histone gene deficiencies and position-effect variegation in *Drosophila*. *Nature* **282**, 312–314.

Moore, G. D., Sinclair, D. A. & Grigliatti, T. A. (1983). Histone gene multiplicity and position-effect variegation in *Drosophila melanogaster*. *Genetics* **105**, 327–344.

Mottus, R., Reeves, R. & Grigliatti, T. A. (1980). Butyrate suppression of position-effect variegation in *Drosophila melanogaster*. *Molecular and General Genetics* **78**, 465–469.

Patel, C. V. & Gopinathan, K. P. (1987). Determination of trace amounts of 5-methylcytosine in DNA by reverse-phase high-performance liquid chromatography. *Analytical Biochemistry* **164**, 164–169.

Perrin, D. D. & Dempsey, B. (1974). *Buffers for pH and Metal Ion Control*, p. 176. London: Chapman and Hall.

Procunier, J. D. & Tartof, K. D. (1978). A genetic locus having *trans* and contiguous *cis* functions that control the disproportionate replication of ribosomal RNA genes in *Drosophila melanogaster*. *Genetics* **88**, 67–79.

Reuter, G., Werner, W. & Hoffman, H. J. (1982). Mutants affecting position-effect heterochromatinization in *Drosophila melanogaster*. *Chromosoma* **85**, 539–551.

Rushlow, C. A., Bender, W. & Chovnick, A. (1984). Studies on the mechanism of heterochromatic position effect at the *rosy* locus of *Drosophila melanogaster*. *Genetics* **108**, 603–615.

Saunders, D. S. (1982). *Insect Clocks*. 2nd Edn. New York: Pergamon Press.

Schultz, J. (1956). The relation of the heterochromatic chromosome regions to other nucleic acids of the cell. *Cold Spring Harbor Symposia on Quantitative Biology* **21**, 307–328.

Shoup, J. R. (1966). The development of pigment granules in the eyes of wild-type and mutant *Drosphila melanogaster*. *Journal of Cell Biology* **29**, 223–249.

Sinclair, D. A. R., Mottus, R. & Grigliatti, T. A. (1983). Genes which suppress position-effect variegation in *Drosophila melanogaster* are clustered. *Molecular and General Genetics* **191**, 326–333.

Spofford, J. B. (1976). Position-effect variegation in *Drosophila*. In *The Genetics and Biology of Drosophila*, (ed. M. Ashburner and E. Novitski Vol. 1c, pp. 955–1018. Academic Press.

Urieli-Shoval, S., Gruenbaum, Y., Sedat, J. & Razin, A. (1982). The absence of detectable methylated bases in *Drosophila melanogaster*. *FEBS Letters* **146**, 148–152.

Wareham, K. A. Lyon, M. F., Glenister, P. H. & Williams, E. D. (1987). Age related reactivation of an X-linked gene. *Nature* **327**, 725–727.