Genotype-specific modifiers of transgene methylation and expression in the zebrafish, *Danio rerio*

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Summary

Previous reports involving mammalian systems, particularly mice, have demonstrated the existence of *cis-* and *trans*-acting modifiers of transgene methylation. These modifiers are thought to be important in dominance modification, genome imprinting and cellular expression mosaicism. Their potential role in the penetrance and severity of many complex human diseases could be of even greater significance. In the present investigation we demonstrate that modifiers that act in a similar fashion to those identified in mice also exist in a non-mammalian vertebrate, the zebrafish *Danio rerio*. We also provide evidence that the transgene methylation pattern may be influenced by the sex of the individual and environmental modulators such as temperature and sodium butyrate. These data support the theory that this type of dominance modification is mechanistically similar to position effect variegation in *Drosophila*. Furthermore, these data suggest evolutionary conservation of the modifiers, at least within vertebrates, and imply that they and their actions are important in normal vertebrate development.

1. Introduction

Mammalian DNA modification involves, among other things, the addition of a methyl group to position five of cytosine and occurs most frequently at CpG dinucleotides. This epigenetic modification of DNA by methylation is associated with transcriptional silencing of genetic loci with the result that inactive DNA sequences tend to be more methylated than active sequences. This relationship has led numerous investigators to propose that DNA methylation may play a role in developmental regulation (Monk *et al.* 1987), genomic imprinting (Monk, 1990) and even, possibly, the speciation process (Varmuza, 1993).

The advent of transgenic technology has made it possible to analyse the inheritance of methylation within an individual allele. This is because, by selective breeding, it is possible to maintain a locus in a hemizygous state where only one allele is present. Using this method to analyse a transgenic line of mice McGowan *et al.* (1989) observed various levels of methylation at the transgene locus in different individuals. This transgenic line also displayed mosaic expression of the *lacZ* reporter transgene between cells sharing the same developmental and phenotypic

lineage; *i.e.*, not all apparently identical cells within a tissue expressed the transgene. The degree of mosaic transgene expression was inversely correlated with the level of the transgene's methylation. Mice with hypermethylated transgenes had only a few expressing cells; whereas many expressing cells were evident in mice with a hypomethylated transgene locus. The alterations in the methylation (and expression) of the transgene locus were shown to be affected by strain-specific modifiers of methylation consistent with what has been found for other transgene loci (Sapienza *et al.* 1989; Allen *et al.* 1990; Forejt & Gregorova, 1992). Transcriptional silencing, mosaic expression, strain-specific modifiers and positional specificity associated with transgene loci are very reminiscent of position effect variegation as observed in *Drosophila* in which there is mosaic expression of alleles that can be enhanced or suppressed by unlinked dosage-sensitive modifiers. This similarity lead McGowan *et al.* (1989) and Sapienza (1990*a*) to propose that we are looking at a dominance modification phenomenon analogous to position effect variegation and, therefore, the modifier or Su(var) genes involved in position effect variegation (Henikoff, 1990) may be functionally analogous to the modifiers identified in association with the imprinting of transgenes in mice.

The expression of a rearranged *white* locus as well as many other variegating loci in *Drosophila* has been

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shown to be affected by a variety of factors including environmental temperature, the presence of sodium butyrate, the sex of the individual displaying the variegated phenotype, and the sex of the parent contributing the variegating rearrangement (reviewed in Spofford, 1976). Lower temperatures generally enhance variegation or increase the number of mutant cells while higher temperatures suppress it or decrease the number of mutant cells. The presence of sodium butyrate in the culture media also suppresses variegation (Mottus *et al.* 1980). The proportion of mutant cells is frequently more extensive in female *Drosophila* as compared to males. Probably the most interesting modulator of PEV in relation to vertebrates is the effect of the parental source of the variegating rearrangement in which variegation is enhanced when passage is through the egg and suppressed after passage through the sperm. This gamete-of-origin effect is analogous to the genomic imprinting phenomenon observed in mice.

Some recent observations on the expression of a transgene in fish suggested the possibility of similar modifiers being present in this non-mammalian vertebrate. Stuart *et al.* (1990) produced several stable transgenic lines of zebrafish containing the plasmid pUSVCAT, the reporter gene chloramphenicol acetyltransferase (CAT) positioned downstream of the Rous sarcoma virus LTR. With the use of immunohistochemical staining, they observed a mosaic pattern of CAT expression in the skin epithelium of the caudal fin. The similarity between the mosaic transgene expression in zebrafish and the previously described mosaic transgene expression in mice (McGowan *et al.* 1989) suggested the possibility of a similar modification system operating in fish.

In a previous report we showed that genome imprinting is evident in a line of transgenic zebrafish (*Danio rerio*) containing the plasmid pUSVCAT (Martin & McGowan, 1994). In the present report we demonstrate that strain-specific modifiers of methylation are also evident in the same transgenic line. The identification of genetic modifiers in zebrafish that are similar to those identified in mice, suggests evolutionary conservation of this modification system among vertebrates at least.

We also present evidence in support of the PEV-like models of McGowan *et al.* (1989) and Sapienza (1990*a*) which were used to explain the mosaic expression of genomically imprinted murine transgenes. Because of the similarities between the modifiers previously identified in mice and those we have now identified in fish it will be possible to use the zebrafish as an alternative model system for the future analysis of the modification phenomenon. External fertilization and the high reproductive potential of these fish provides a model system that may be superior to that provided by the mouse, particularly for studies of early developmental events.

2. Methods

Zebrafish

Transgenic zebrafish, *Danio rerio*, containing the plasmid pUSVCAT and derived from the AB wild-type line of the University of Oregon (CATfish IV) were generously supplied by Monte Westerfield (Stuart *et al.* 1990). A lab stock of wildtype fish (small inbreeding population) were also generously supplied by Hans Laale (Dept. Zoology, University of Manitoba). Wildtype, longtail zebrafish, and leopard danios (*Danio rerio frankei*) were obtained from a local pet supply. Fish were maintained in 10–20 gallon aquaria at 28 °C on a 14 h light/10 h dark cycle. Prior to breeding, individuals were placed in 1 gallon aquaria for 1 week. On the morning of egg collection a single male and female were placed in a spawning tank containing a floor of glass bars which served to protect the eggs from being eaten by the adults. Eggs were collected 2–3 h after the initiation of spawning, which occurs spontaneously at the onset of the light cycle. Embryos and larvae were maintained in Plexiglas holding tubes with fine mesh bottoms which were suspended in a 20 gallon aquarium. Larval fish were fed live *Paramecia*, finely ground flake food, and baby brine shrimp. General zebrafish care was according to Westerfield (1989).

Southern hybridization

DNA samples were prepared (as previously described by Sapienza *et al.* 1987) from whole fish and pooled samples of small fish from a single breeding. DNA was cleaved with the restriction enconuclease *Hpa* II or *Hpa* II/*Bam*H I as suggested by the manufacturer (BRL). Complete digestion was tested by the addition of control plasmid DNA to a sample of the experimental digest. Restriction digestion was considered complete when the internal control digest gave a digest pattern identical to the pattern obtained with test plasmid and the same enzyme alone. Gel electrophoresis, transfer of DNA to nylon membrane (Hybond N$^+$, Amersham), prehybridization, production of ^{32}P-labelled probes, hybridizations and autoradiography were performed according to Sapienza *et al.* (1987). Blots were probed with a 1·6 Kb *Hin*d III fragment of pUSVCAT containing the gene encoding the enzyme chloramphenicol acetyltransferase.

Immunohistochemistry

The chloramphenicol acetyltransferase antigen was visualized in the epithelial cells of individual scales using a rabbit anti-CAT antibody (5′-3′ Inc.) and a VECTASTAIN ABC-alkaline phosphatase kit (Vector Laboratories Inc., Burlingame, CA). Fish were

anaesthetized with 2-phenoxyethanol. Individual scales dorsal to the lateral line and immediately posterior to the dorsal fin were removed with fine forceps. Scales were rinsed in PBS, pH 7·3 (0·07 M-Na_2HPO_4, 0·03 M-NaH_2PO_4, 0·15 M-NaCl) for 15 min and fixed for 30–45 min in 4% paraformaldehyde in PBS pH 7·3 at room temperature. Following fixation, scales were washed in PBS for 30 min and incubated overnight in blocking buffer containing 10% normal goat serum, 0·1% crystalline BSA, and 0·01% Triton X-100 in PBS pH 7·3. Overnight steps were performed at 4 °C. Endogenous biotin was blocked by applying avidin (3 h) and d-biotin (3 h) (Zymed Laboratories). Washes in buffer containing 0·1% BSA and 0·05% Triton X-100 in PBS pH 7·3 were performed for 30 min between all steps. The primary antibody was diluted 1/200 in blocking buffer and incubated overnight. A secondary biotinylated goat anti-rabbit antibody was diluted 1/200 in washing buffer and also incubated overnight. The avidin-alkaline phosphatase complex was produced according to manufacturer and applied for 1·5 h. The substrates and buffer containing 1 mM levamisol were prepared and incubated for 15–30 min. The staining reaction was stopped by washing specimens in distilled water for 30 min. Scales were temporarily mounted in glycerol and viewed using a compound microscope.

3. Results

To identify *trans*-acting modifiers similar to those found by McGowan *et al.* (1989) for the Tg4 transgenic mouse line and by Sapienza *et al.* (1987) for 379 tropinin I transgenic mice, individual homozygous transgenic zebrafish were bred to a variety of non-transgenic mates which included two different wildtype populations, a single population displaying a longtail phenotype, and the leopard danio *Danio rerio frankei* (Fig. 1). These particular breedings were used on the assumption that different strains represent different genotypes. The single homozygous, transgenic parent shown in Fig. 1 displayed a relatively high degree of transgene methylation, *i.e.*, was hypermethylated. However, this fish carried two alleles at this locus and we do not know if they were methylated differently. Methylation sensitive, *Hpa* II restriction digests were used to analyse the transgene locus in F1 progeny from the crosses illustrated in Fig. 1 and revealed that methylation differences do exist that are, presumably, due to the genotype of the non-transgenic parent. Breeding the same transgenic fish to four different non-transgenic mates produced different degrees of transgene methylation in individual offspring (Fig. 1) *and samples of pooled offspring (not shown). These results strongly suggest the presence of *trans*-acting modifiers in the zebrafish that behave similarly to those previously identified in the mouse, *i.e.*, that produce changes in the transgene methylation pattern.
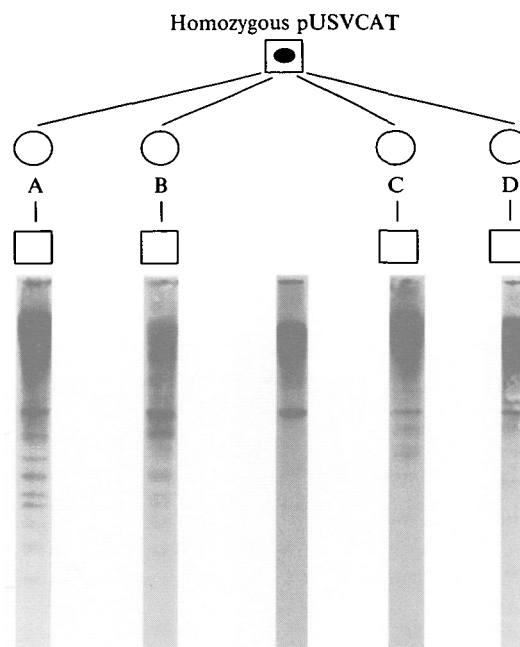

Fig. 1. (Top) A pedigree is presented and shows the mating of a single homozygous pUSVCAT transgenic male (circle in square) to different wildtype strain females (circles): (A) commercial wildtype, (B) longtail phenotype, (C) lab stock wildtype, (D) *Danio rerio frankei.* *Hpa* II-cleaved DNAs from individual offspring hybridized with a 1·6 Kb transgene-specific sequence are shown beneath the pedigree and demonstrate that the same male can give rise to progeny with different transgene methylation phenotypes, depending on the genotype of the non-transgenic female. A *Hpa* II digest of the homozygous, transgenic male used in the above breedings is shown under the symbol representing that individual.

In some transgenic mice previously studied (McGowan *et al.* 1989) changes in transgene methylation were associated with changes in the numbers of transgene-expressing cells producing different degrees of mosaicism. Immunohistochemical analysis of CAT transgene expression in epithelial cells of our transgenic zebrafish clearly demonstrates a mosaic expression phenotype (Fig. 2) as was reported by Stuart *et al.* (1990). These stained scale epithelia also suggest that expression of the transgene is cell autonomous. Clusters and individual expressing cells are often entirely surrounded by cells with undetectable levels of expression.

A comparison of the methylation of the transgene in whole fish DNA and the transgene expression in the scale epithelia indicates that the mosaicism can be inversely correlated with the degree of transgene methylation (Fig. 2). Epithelial cells from female fish consistently produced more non-specific staining than epithelial cells from males. The reason for the sex-specific difference in the background is not yet clear to us, but because of it only males were used for immuno-staining. Precise quantification of the number of staining cells was difficult, but relative comparisons between scales could be made. Like the mice, fish with

CAT expression in scale epithelial layer

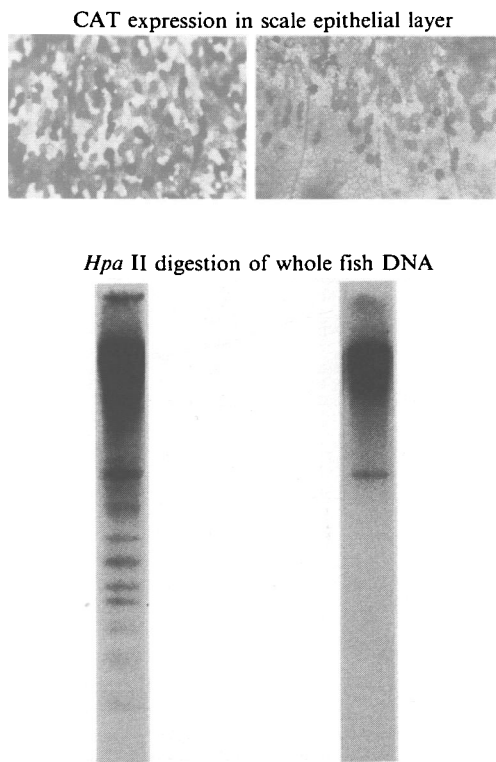Hpa II digestion of whole fish DNA

Fig. 2. Chloramphenicol acetyltransferase expression in scale epithelial tissues and restriction endonuclease digestions of DNAs from male F1 hemizygous pUSVCAT transgenic zebrafish from different breedings. At top is shown the variation in the number of CAT expressing cells visualized by immunohistochemical staining. Beneath each stained tissue is Hpa II-cleaved DNAs from the same individual. Restriction digests were probed with a 1·6 Kb sequence representing the CAT gene. Individuals having fewer expressing cells carry a hypermethylated transgene, where individuals with more expressing cells carry a hypomethylated transgene.

Fig. 3. The effect of developmental temperature on the zebrafish transgene methylation phenotype. Hpa II cleaved DNAs from transgenic sibling offspring raised at 20 °C and 30 °C until hatching. The level of methylation is increased in the 20 °C fish as compared to their siblings raised at a higher temperature (some bands with altered intensities shown with arrows).

a relatively high level of transgene methylation in somatic DNA displayed a relatively low number of CAT expressing cells in their epithelial layer and the opposite was also true. Low methylation correlated with high numbers of expressing cells (Fig. 2). These findings suggest that (1) the proportion of expressing cells can vary and (2) it is inversely related to the transgene methylation pattern. By extrapolation, the differences in methylation observed when the transgene is passed onto different genetic backgrounds are the result of changes in the relative proportion of hypo- and hypermethylated cells in the population.

The mosaic expression and dominance modification observed in zebrafish are virtually identical to those seen in mice. However, because mammals are internal developers, it has been difficult to experimentally manipulate the developmental environment of early embryonic stages when at least some of this epigenetic modification occurs. Zebrafish use external fertilization, and can be directly manipulated prior to first cleavage and throughout early development. The zebrafish, therefore, provides a marvellous oppor-
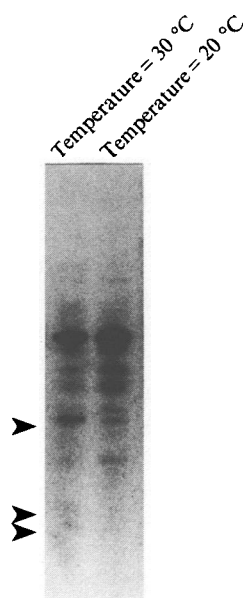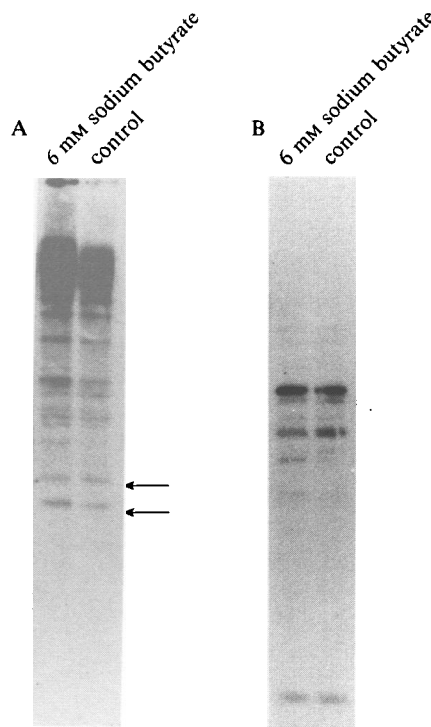

Fig. 4. The effect of sodium butyrate on the zebrafish transgene methylation. Hpa II (A) and Hpa II/BamH I (B) cleaved DNAs from transgenic sibling offspring raised in a 6 mM sodium butyrate solution and the control group raised in normal aquarium water. The treated group shows a lower transgene methylation pattern than the control group. The directionality of the transgene methylation differences between treatment and control groups is best illustrated by comparing the relative intensity of the indicated fragments (arrows).
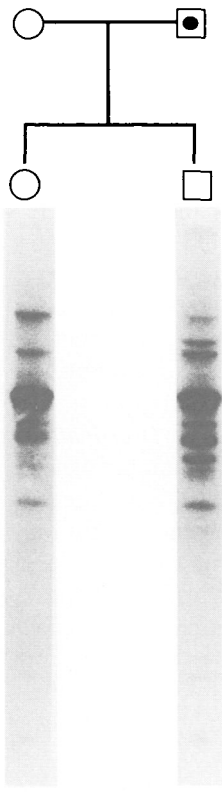
Fig. 5. *Hpa* II/*Bam*H I cleaved DNAs from female and male transgenic sibling offspring. In this particular cross, there is an apparent sex based difference in the transgene methylation pattern in which females have a higher degree of transgene methylation than sibling males.

tunity to test predictions of the position effect variegation model. Figure 3 shows the methylation pattern of two different spawns, of which half of each spawn was raised at 20 °C and the other half raised at 30 °C. The colder temperature fish displayed a higher transgene methylation pattern than the higher temperature fish. Similarly, fish raised in 6 mM sodium butyrate displayed a lower methylation pattern than their siblings raised in normal aquarium water (Fig. 4). Because fry were analysed shortly after hatching, the effect of these environmental factors on expression of the CAT gene could not be determined. However, the link between transgene methylation and CAT expression suggests that expression of the transgene may also be altered (Fig. 2). The similarity between the effects of environmental modulators on transgene methylation in the zebrafish and PEV in *Drosophila* supports the idea that the genetic modifiers involved in both processes are acting through a similar mechanism.

Most crosses between transgenic and non-transgenic parents resulted in all offspring within a spawn having virtually identical transgene methylation phenotypes (tested on individual and pooled samples). Certain genotypic combinations, however, produced a subtle but distinct difference in the methylation pattern between male and female siblings (Fig. 5). In these

crosses, the males displayed a lower degree of methylation than the females. These data suggest, therefore, that sex differences are possible in these fish in the extent of transgene methylation and, possibly, the mosaic expression of the locus.

4. Discussion

The existence of genotype-specific loci that influence the expression of other loci was proposed by Fisher (1928) and his contemporaries in the early part of this century. This phenomenon was called dominance modification. The modification of dominance which produces incompletely penetrant genetic traits is now recognized as a common genetic phenomenon and results from allelic variation at loci that interact with other genetic loci. Dominance modifiers that are presumably of this type have been identified in mice at the level of the methylation and expression of transgene loci (Sapienza *et al.* 1987; McGowan *et al.* 1989; Allen *et al.* 1990).

Genetic modifiers apparently analogous to those found in mice can be demonstrated in zebrafish by passage of a transgene onto different genetic backgrounds. Breeding the same transgenic fish to different non-transgenic mates resulted in different transgene methylation phenotypes. Clearly, loci must exist in the non-transgenic mates that effect the methylation of the transgene locus and that can: (a) act in *trans* and; (b) show genetic variability. The presence of modifier loci, similar to those identified in mice, in a non-mammalian vertebrate suggests evolutionary conservation of these factors. It stands to reason, therefore, that the action of these modifiers must be important in the biology and/or development of these animals and, potentially, other or even all sexually reproducing organisms. Whether those actions simply maintain the sexual process, ensure production of germline or are more complex and fundamental to animal development remains to be determined.

In several of the murine transgenic lines examined, the expression of the transgene loci was observed to be inversely correlated with the extent of their methylation (Swain *et al.* 1987; McGowan *et al.* 1989). Highly methylated transgene loci exhibited low to non-existent expression, whereas less methylated loci exhibited high levels of expression. This accords with previous findings on the relationship between methylation and expression (Cedar, 1988). McGowan and his co-workers (1989) also demonstrated that the changes in methylation could be correlated with changes in the proportion of expressing and non-expressing cells.

In the zebrafish expression of the transgene was also inversely correlated with its level of methylation and, like the murine transgenes, was found in a mosaic pattern. The effect of changing the genotypic background on both the methylation and, by extrapolation,

the expression of the transgene suggests that the modifiers that we have identified in these fish are acting (at least partially) by changing the number of affected cells present in a similar manner to that seen previously in transgenic mice. Therefore, at least some of the genotype-specific modifiers appear to affect the transgene indirectly by altering the proportion of differently methylated cell types present rather than altering the extent of transgene methylation itself.

The similarities between the variegated expression observed with transgenes in mice (McGowan et al. 1989) and position effect variegation in Drosophila have led to the creation of models to explain transgene modifications based on the action of the Drosophila Su(Var) genes on heterochromatin formation (Sapienza, 1990a). Though Drosophila actually have little or no DNA methylation this does not mean that position effect variegation cannot be used as a model. In mammals the centromeric heterochromatin is found to be enriched by 5-methyl-cytosine (Miller et al. 1974), and its role in the production of heterochromatin can be demonstrated by the use of methylation inhibitors which leads to the under-condensation of the same centromeric heterochromatin (Schmid et al. 1984). The methylation being monitored in mice, therefore, may just be reflecting the formation of heterochromatin rather than being the inactivator itself.

Models based on the idea of dosage-sensitive modifiers have been applied to the study of several diseases including Huntington's disease (Sapienza, 1990a; Laird, 1990) and heritable cancers (Scable et al. 1989; Sapienza, 1990b) and are capable of successfully explaining all of the disparate information available about these diseases. These models have also generated predictions which have been proven to be correct (Scrable et al. 1989; Sapienza, 1990b).

However, with the mouse, it has not been possible to test the PEV model for dominance modification by direct manipulation of the developing organism as was possible in Drosophila. In the zebrafish, on the other hand, external fertilization has allowed us to make and test predictions based on PEV in Drosophila. The increase in transgene methylation as a result of lower developmental temperature is analogous to the effect observed in Drosophila PEV where lower temperatures enhance variegation or increase the number of mutant cells (Gowen & Gay, 1934). Very little is known about the mechanism for this temperature effect but it is thought to affect protein–protein interaction and the formation of protein multimeric complexes and protein-DNA interactions (Spofford, 1976).

Similarly, the effect of sodium butyrate on transgene methylation in the zebrafish was also shown to fulfill predictions based on its effects on Drosophila PEV. In variegating mutants of Drosophila, sodium butyrate suppresses the variegation or decreases the number of mutant cells. The spreading of heterochromatin, by histone-mediated chromatin condensation, into euchromatic regions of the chromosome is thought to be responsible for the PEV phenomenon. Transcriptionally active chromatin is associated with hyper-acetylated histones, particularly H4 (Levy-Wilson et al. 1979). Sodium butyrate works to prevent histone-deacetylation in both Drosophila and mammalian cells (Mottus et al. 1980; Peter et al. 1978). Therefore, it suppresses variegation by blocking the formation of heterochromatin. In our transgenic zebrafish, sodium butyrate caused a decrease in the transgene's methylation level suggesting that here also sodium butyrate may be blocking heterochromatin formation.

Sex-specific suppression of PEV in Drosophila is probably due to the presence of the highly heterochromatic Y-chromosome. The additional heterochromatic regions on that chromosome are thought to sequester heterochromatin forming proteins and, therefore, act to suppress PEV. This effect of the Y-chromosome has been well documented in Drosophila. The basal heterochromatin of the X-chromosome centromeric regions has also been shown to suppress PEV to a nearly equal extent as the Y-chromosome (reviewed in Spofford, 1976). The sequestering of DNA binding proteins by the sex chromosomes, therefore, can produce a distinct sex effect in the extent of allelic variegation on certain genetic backgrounds. Typically, in Drosophila PEV suppression is greatest in males. An identical effect which is probably occurring via a similar mechanism was observed in the zebrafish where, in some crosses, the male offspring have a distinctly lower transgene methylation phenotype than females. However, no information exists with respect to the mechanism of sex determination in zebrafish (i.e. heterogametic sex in zebrafish) or the extent of heterochromatic regions in the zebrafish sex chromosomes. Potentially though, the inactivation of a sex chromosome in either sex in order to achieve dosage compensation (which does not occur in Drosophila) may soak up considerable heterochromatin factors and produce the observed effect. The types and origins of the modifiers producing this effect in fish, however, are difficult to interpret in the absence of information on the mechanism of sex determination in that organism.

The inactivation of loci by heterochromatin formation may not just be a genetic anomaly of Drosophila. The Drosophila HP1 gene (which is allelic to the suppressor of variegation Suvar (2)5) encodes a product shown to be a structural component of heterochromatin (James & Elgin, 1986). A region of the HP1 gene, termed the chromobox, has been shown to be highly conserved across a wide range of phyla (Singh et al. 1991) and has been used to isolate the murine M31 and M32 and the human HSM1 counterparts which have now been shown to be involved in the packing of mammalian chromosomal DNA into constitutive heterochromatin (Wreggett et al. 1994). Further, the similar homology between HP1

and the repressor of homeotic genes *polycomb* (Paro & Hogness, 1991) and the relationship between heterochromatin and yeast mating-type silencing (*SIR* genes) (Braunstein *et al.* 1993; Lee & Gross, 1993) argue that this type of gene control may be widespread and developmentally very significant.

An understanding of dominance modification in mammals is important, particularly in terms of human disease. However, the identification of a similar phenomenon operating in a non-mammalian vertebrate is equally significant. It suggests that the process is evolutionarily conserved and, therefore, probably important in the biology of animals. Furthermore, there is considerably more potential using the zebrafish as a model system instead of the mouse, particularly in a developmental sense, because of the obvious experimental benefits of external fertilization, large reproductive potential and optically clear embryos. These fish also represent a considerably more natural population then inbred mice which have been selected for unusual traits (such as, for example, coat colour or cancer susceptibility) and, therefore, should be more useful in trying to determine the biological relevance of this phenomenon.

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