Mechanisms for the construction and developmental control of heterochromatin formation and imprinted chromosome domains

KENNETH D. TARTOF and MARILYN BREMER
Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA 19111, USA

Summary

The study of variegating position effects in Drosophila provides a model system to explore the mechanism and material basis for the construction and developmental control of heterochromatin domains and the imprinted genomic structures that they may create. The results of our experiments in this regard have implications for a diverse assortment of long-range chromosome phenomena related to gene and chromosome inactivation. Specifically, as a consequence of our studies on position effect variegation, we propose a simple mechanism of X-chromosome inactivation, suggest a purpose for genomic imprinting, and postulate a general means for regulating the time in development at which certain genes become heterochromatically repressed.

Key words: Drosophila, position effect variegation, X-inactivation, chromosome imprinting, heterochromatin, mass action, macromolecular assembly, gene dosage effects.

Introduction

By following chromosomes through their division cycles, Heitz (1928, 1929) demonstrated that the densely staining heterochromatic structures in the nucleus were actually parts of chromosomes that failed to unravel at the end of mitosis, a behavior that contrasts with the lighter staining euchromatic regions that unfold as soon as cells enter interphase. The centromeric heterochromatin that Heitz studied is sometimes referred to as constitutive heterochromatin because such domains remain compacted in most cells, most of the time. Facultative heterochromatin, on the other hand, is defined as a chromosome (or chromosome domain) for which being heterochromatic is optional rather than obligatory. The heterochromatic inactivation of just one of the two X chromosomes present in the cells of female mammals is an example of this type.

We are interested in heterochromatic domains because of their conspicuous involvement in a wide variety of long-range chromosome effects. These include X-chromosome inactivation in female mammals (Lyon, 1961; Lyon, 1972), the precocious inactivation of the X chromosome during spermatogenesis in male-heterogametic organisms (Lifschytz and Lindsley, 1972; Lindsley and Tokuyasu, 1980; Monesi, 1971), the fragile site syndrome in humans (Laird, 1987), the possible loss of functional heterozygosity in malignant disease (Scrable et al. 1989), and position effect variegation in mice and fruit flies (Eicher, 1970; Spofford, 1976). Of these examples, variegating position effects are of particular significance to us. We know from studies in mice that X-autosome translocations result in variegation of those autosomal genes now located adjacent to the X when the translocated X is inactivated. This behavior is quite similar to, and probably identical with, the phenomenon of position effect variegation that occurs in Drosophila as first described by Muller in 1930. In this case, variegating position effects result from chromosome rearrangements that place wild-type genes adjacent to centric heterochromatin. The fact that heterochromatin formed either during X-inactivation or constructed as part of a centromeric domain can variegate neighboring euchromatic loci indicates that constitutive and facultative heterochromatic classes may be functionally and structurally quite similar, if not identical, being derived for the very ancient purpose of repressing large extents of the genome.

The formation of facultative heterochromatin also appears to be intimately connected with the phenomenon of chromosome imprinting as demonstrated in both insects (Brown and Nur, 1964; Crouse, 1960; Gerbi, 1986; Nur et al. 1988) and mammals (Gartler and Riggs, 1983). As a consequence of her studies of Sciara, Crouse (1960) coined the term 'imprinting' to refer to the situation in which chromosomes passing through the male germ line acquire an imprint that results in a behavior exactly opposite to the imprint conferred on the same chromosome by the female germ line. In Sciara, for example, one or both of the paternally inherited X chromosomes become heterochromatic and eliminated during the early zygotic divisions in the
somatic tissue of female or male embryos, respectively. In marsupials and placentals mammals it is the paternally derived X that is heterochromatically inactivated in somatic or extraembryonic cells, respectively. All of the above biological curiosities share the common ability to pass the imprint of heterochromatic inactivation from one cell generation to the next in a remarkably stable manner. X-inactivation demonstrates this point quite convincingly. The packaging of selected regions of the genome into heterochromatin is a type of regulatory mechanism not commonly available to prokaryotes. It is a chromosome phenomenon. The significant feature of this apparently unique eukaryotic form of gene regulation is that stable gene states are stored directly in a somatically inherited chromatin structure rather than in a feedback loop of regulator proteins that diffuse from place to place (Alberts et al. 1983).

Our studies of position effect variegation in Drosophila have allowed us to address two important questions regarding the initiation and perpetuation of the heterochromatically repressed state. First, what sorts of genes control heterochromatin formation and what do they tell us about the mechanism that governs its construction? Second, what controls the time in development at which a variegating gene becomes heterochromatically repressed? The answers to these questions have implications for our understanding of the general process by which imprinted (i.e. inactivated) chromatin domains may be created and maintained.

Results and discussion

Position effect variegation in Drosophila

In Drosophila melanogaster, the euchromatin occurs as approximately 5000 bands observed in the giant chromosomes of polytene nuclei. About 85% of euchromatin is composed of unique sequences interspersed with middle repetitive elements to create a genome characterized by rather long stretches of unique sequence interrupted only by an occasional transposon or other moderately repeated sequence. Heterochromatin, on the other hand, may be divided into α, β and intercalary domains. α-heterochromatin consists primarily of highly redundant sequences such as satellites and middle repetitive elements that are located adjacent to the centromere. Cytologically, α-heterochromatin is recognizable in mitotic chromosomes as densely staining regions around the centromere whereas in polytene chromosomes this same region undergoes fewer rounds of replication. β-heterochromatin is morphologically defined as the less distinctly banded material adjacent to the centric regions of most of the polytene chromosome arms (Heitz, 1934). Intercalary heterochromatin, a term first used by Kaufmann (1942), is found at the constrictions or ‘weak points’ along the euchromatic arms of polytene chromosomes. It is characterized as regions having a large target size for X-ray-induced breaks and undergoing ectopic pairing with each other or the centromere. Of the three forms of heterochromatin, only α-heterochromatin is known to regularly induce variegated position effects.

The classic example of position effect variegation in Drosophila is illustrated by the w<sup>1118</sup> (white mottled 4) mutation in which the white gene, which is normally found near the distal end of the X chromosome, is now located next to a block of centromeric α-heterochromatin as a result of a chromosome inversion (Fig. 1). In this context, the expression of white is dramatically altered (Muller, 1930; Schultz, 1936). Rather than displaying the usual wild-type red-eye phenotype, the ommatidia now exhibit a variegated or mottled appearance. In such rearrangements, it appears that white is inactivated in some cells, but not others, thereby giving rise to an eye that contains patches of mutant and wild-type tissue. Since it is also possible to revert a variegating gene simply by relocating it away from heterochromatin to another euchromatic site in the genome, it is the placement next to heterochromatin per se, and not a mutation in the affected gene, that is the cause of this altered form of gene expression (Dubinin and Sidorov, 1935; Judd, 1955; Kaufmann, 1942; Panshin, 1935).

The most reasonable hypothesis to explain position effect variegation requires that the compacted structure of heterochromatin be propagated into the adjoining euchromatic sequences thereby inactivating neighboring loci. Several facts are consistent with this mechanism. Schultz (1936) was the first to demonstrate that variegating position effects arise as a consequence of placing euchromatic genes adjacent to compacted centromeric heterochromatin. Demerec (1940) and Hartmann-Goldstein (1967) showed, for example, that variegation can spread to include loci 50 bands away from the euchromatic–heterochromatic breakpoint. This spreading effect has the characteristic that genes closest to heterochromatin variegate more frequently than genes located further away. There is also a cytogenetic correlate of variegation. In polytene chromosomes, variegating genes tend to lose their typical banded conformation and appear to merge with the adjacent α-heterochromatin (Cole and Sutton, 1941; Prokofyeva-Belgovskaya, 1939; Schultz and Casspersson, 1939). More recent studies of variegating heat-shock and rosy loci suggest that transcription from these genes is also impaired (Henikoff, 1981; Rushlow et al. 1984).

Examination of the size and shape of the patches of mutant and wild-type tissue in mottled phenotypes can be quite instructive. For example, the w<sup>1118</sup> mutation frequently produces eyes containing large sectors of wild-type and white ommatidia as illustrated in Fig. 1. Becker (1966) and Janning (1970) have shown that strikingly similar sectored patterns may also be produced when mitotic recombination is induced during embryonic development. From these observations these investigators have suggested that, in the case of w<sup>1118</sup>, the decision to inactivate the white gene is made early in the development of the eye, and that once
Fig. 1. The \( w^{m4} \) mutation and its phenotype. \( w^{m4} \) is an inversion of the X chromosome such that the \( w^+ \) gene is relocated next to the centric heterochromatin. The dark blocks indicate heterochromatic regions and the open blocks define the location of the 200 copies of the ribosomal RNA genes. Below, representative eye phenotypes of flies bearing the following genotypes are depicted (from left to right): wild type, \( w^{m4} \), and \( w^{m4}/Y; E(var)8/+ \). \( E(var)8 \) is a trans-active dominant Enhancer of variegation.
where $K_{eq}$ is the equilibrium constant, and $k$ and $k'$ are the forward and reverse rate constants, respectively.

In the general case, for a single value of $n$, a multimeric assembly reaction is given by the equation,

$$K_{eq} = \frac{k}{k'} = \frac{\langle (\text{PIP2P3P4}...\text{Px})_n \rangle}{\langle \text{PI}\rangle^n\langle \text{P2}\rangle^n\langle \text{P3}\rangle^n...\langle \text{Px}\rangle^n} \quad (4)$$

The exponential character of the proportionality expressed in equation (3) has important consequences for the accumulation of product as shown in Fig. 2. When $n=1$, the amount of multimer formed (heterochromatin) is directly proportional to the concentration of each of P1 to P4. Thus, if [P1], or any other reactant, is reduced to 0.5 (such as is the case with a deficiency), so too is the concentration of heterochromatin. Alternatively, an increase by 1.5 (a duplication) would increase the concentration of heterochromatin by 1.5. In this case, the difference in the amount of heterochromatin formed between deficiency and duplication is threefold. However, at $n=4$ where four molecules of each protein are needed, the multimer concentration would decrease to one-sixteenth $(1/2^4)$ with a deficiency or increase by 5.06 times $(1.5^4)$ with a duplication. Here the difference in the amount of heterochromatin formed between deficiency and duplication is 80-fold. Thus, at increasing values of $n$, the exponential character of the $[\text{Px}]^n$ function in the equation permits large changes in the amount of assembled heterochromatin from relatively minor changes in the concentration of any single protein component.

Equation (3) also explains how changes in gene dosage at any one of several independent loci can act in a dominant fashion to influence the extent of heterochromatin spreading and produce similar phenotypic consequences. Because all the dominant suppressor-enhancer loci affect variegation, and are probably part of the same dosage-sensitive system, the phenotypic effects of combining enhancers and suppressors should, in some manner, be cumulative and not epistatic. This prediction is supported by our experiments in which we found that increasing the dosage of a Class I enhancer from 2 to 3 to 4 copies continues to enhance the variegating mutant phenotype (Locke et al. 1988). Similarly, we have also demonstrated that two different enhancer loci may be combined to further enhance variegation (Locke et al. 1988). Equation (3) also predicts that the dosage-dependent nature of all modifiers of variegation will be interrelated, such that the effect of a suppressor at one locus may be offset by an enhancer at another locus (or vice versa). However, within a heterochromatin multimer the value of $n$ may not be the same for all protein components so the numbers of molecules needed for each type of protein can be different. This would suggest that there may be suppressors (or enhancers) of different strengths, depending on the value of $n$. Nevertheless, the essential feature of our model holds that the construction of heterochromatic domains proceeds by the simultaneous interaction of all subunits involved in the assembly reaction, that the concentration of any one subunit can influence the amount of product formed and that the value of $n$ is $\geq 2$ for most, if not all, of the subunits.

The Class II modifiers of variegation behave in reciprocal fashion to those of Class I. In the case of Class II loci, deficiencies are enhancers of variegation. That is, the reduction or removal of a Class II gene product increases the spread of a heterochromatic domain. This implies that the wild-type function of such loci negatively regulate the propagation of heterochromatin. Thus, Class II genes may code for proteins that: (1) interact with, or affect indirectly. Class I products to inhibit their assembly into heterochromatin; (2) may bind to hypothetical termination sites that define euchromatic–heterochromatic boundaries (Tartof et al. 1984) and thereby limit the spread of heterochromatin; or (3) promote euchromatin formation. Regardless of their exact function, their apparent rarity implies a more specialized and perhaps pivotal role in heterochromatin formation.

Given that there may be some 20 genes capable of modifying variegation and assuming that they code for heterochromatine structural proteins, then heterochromatin domains may be constructed in any of three general ways. An ‘inclusive model’ requires that all domains contain the same set of proteins. In this case, all heterochromatic regions are structurally and, therefore, functionally equivalent. An ‘exclusive model’ holds that each domain be composed of a subset of the heterochromatin-forming proteins such that no two blocks of heterochromatin share the same protein. Alternatively, a ‘combinatorial model’ stipulates that each domain is composed of different combinations of proteins, some of which may be commonly shared. We have shown that the four enhancer loci we have discussed are general enhancers of variegation in that they increase the extent of the mutant phenotype for three different variegating mutations. Yet, the same set of enhancers act with different relative strengths on different variegating rearrangements. It has also been noted that different blocks of heterochromatin vary in their ability to induce variegation (Spofford 1976). These observations lead us to propose that heterochromatic domains are constructed in a combinatorial manner.

Recently, two enhancer–suppressor of variegation genes have been cloned (Eisenberg, 1989; James and Elgin, 1986; Reuter et al. 1990). Both have been shown to code for proteins containing polyacidic stretches that may interact with histones, and one of these also possesses five widely spaced zinc-fingers that possibly facilitate binding to stretches of DNA at distant sites. Significantly, flies transformed with these loci behave in precisely the manner predicted by our dosage-dependent mass action model for enhancers and suppressors of variegation.

**Gene dosage effects and biological ‘switches’**

There are several reports in Drosophila of loci that produce antipodal phenotypes as a result of increases or decreases in the dosage of a particular gene. This may be a general feature of most eukaryotic genomes and, if
equilibrium versus the gene dosage for any one of the phenotype. The latter consists of a periodic anti-runt subunits where $P_x = P_1, P_2, P_3$ or $P_4$ according to the so, it has important implications for our understanding example, duplication of the of a variety of developmentally significant loci. For dependent on the value of $n$, where $n$ is the number of expressed in equation (3). The shape of each curve is proportionality, $[P_1]^n[P_2]^n[P_3]^n[P_4]^n = \alpha [P_1P_2P_3P_4]^n$ subunits of a specific type that are present in the multimer. n is the number of subunits of a specific type that are present in the multimer.

Notch Schultz (1941) showed that duplications of the mutation. runt of the runt locus cause an excessive wing vein phenotype known as notched wing (Demerec et al. 1987) have demonstrated that there are four regions or loci in the Drosophila genome that either enhance or suppress, in a reciprocally acting dosage-sensitive manner, the mutant phenotype of various members of the Polycomb family.

While the antipodal behavior of these loci may be explained in terms of substrate balance in a branched metabolic pathway (Ashburner, 1982), we believe that this is probably not the case for many of these examples. Rather, we suggest that these antipodal phenotypes are the result of a mass action effect on the construction of multimeric structures similar to those that we have proposed for heterochromatin. We note that the suppressors and enhancers of variegation, and presumably the Polycomb family modifiers, encode chromosomal proteins. It is, therefore, quite reasonable to expect that such proteins might have multiple contacts with other molecules in order to produce higher order complexes for regulating gene expression. In addition, protein components employed in the construction of other cellular compartments, such as the Notch locus, also illustrate this concept. This gene codes for a transmembrane protein with an extracellular domain composed of an array of epidermal growth factor repeats (Artavanis-Tsakonas, 1988). The dosage-sensitive antipodal behavior of this gene is most simply explained by proposing that the Notch protein is involved in the construction of a multimeric complex where $n \geq 2$.

Dosage-sensitive mutations have been reported in humans as well (McKusick, 1988). Inherited diseases such as Down's syndrome are caused by the presence of an extra copy of a gene (or genes) in band q22 of chromosome 21, cri du chat by a deficiency in the short arm of chromosome 5, and the Piebald trait results from an interstitial deletion of the long arm of chromosome 4 in band q13. Among somatic cells, one of the most striking features of malignant transformation is their aneuploid or heteroploid karyotypes. Holliday (1989) has recently suggested that the maintenance of the normal diploid karyotype depends on the presence of two copies of certain genes and that a change in the dosage of one or more of them may trigger the destabilization of chromosome number. While dosage-sensitive phenotypes could be the result of enzyme or metabolic imbalances, it is also possible that dosage-sensitive loci may exert their effect on the phenotype by coding for structural protein subunits involved in the assembly of multimeric complexes. In these cases, the impact of such dosage-sensitive loci on the phenotype will critically depend on the value of $n$ as seen in Fig. 2.

The important principle illustrated by these examples that may involve dosage-dependent assembly reactions, is their special ability to constitute biological 'switches'. As the law of mass action dictates, multimeric complexes necessarily becomes exquisitely sensitive to the concentration of their constituents as the value of $n$ increases. If one or more components of the assembly reaction is present in increased or decreased amounts as the result of mutation or gene regulation, then the amount of final product formed will be exponentially affected as a function of the power of $n$. By extension, if a component of an assembly reaction is distributed as a concentration gradient, then a boundary will arise at which one of two antipodal states (say 'on' or 'off') may be imposed. In this way, such switches may ultimately create differentiated states such as parasegments and compartments.

A dosage assembly model for X-inactivation

There are several situations in which it is necessary to count, in a rather precise way, the number of macromolecular entities present within a cell. Examples include the measurement of the X:autosome ratio required for sex determination in diverse organisms from fruit flies and nematodes to man, and the ability of mammalian cells to permit but a single X chromosome to remain active, independent of the number of Xs initially present in an otherwise diploid genome. As might be noted from Fig. 2, the exponential relationship between the dosage of a constituent subunit and construction.
encoded by the X chromosome while the others (PA) are capable of distinguishing small differences in the constitutions of the heterochromatin complex. One X chromosome will "nucleate" and inactivate itself only a single X remains active. Since the assembly of Px into a multimeric complex is necessarily highly cooperative, one X chromosome will 'nucleate' and inactivate itself before the other. Subsequent methylation may stabilize or reinforce the repression of the chromosome initiated by the construction of the heterochromatin complex.

The amount of complex it participates in forming also constitutes a highly discriminating counting device capable of distinguishing small differences in the abundance (i.e. dosage) or a given cellular component. A simple model for how this principle might be applied to X-inactivation is discussed below.

Our 'dosage assembly' model for the control of random X-inactivation as occurs in the embryonic tissues of placental mammals is illustrated in Fig. 3. It is different from previous conjectures on this issue (Gartler and Riggs, 1983) and requires two assumptions. First, we assume that of the several Class I proteins participating in the assembly of X-inactivating heterochromatin, the X chromosome codes for at least one of these (Px) with the others being specified by the autosomes (PA). Second, we stipulate that the value of n for Px, and most PA, is \( \geq 2 \). As the Px gene on the inactivated X is repressed, the cellular concentration of this protein falls to half its previous value. Consequently, the ability to form further complex falls exponentially so that only a single X remains active. Since the assembly of Px into a multimeric complex is necessarily highly cooperative, one X chromosome will 'nucleate' and inactivate itself before the other. Subsequent methylation may stabilize or reinforce the repression of the chromosome initiated by the construction of the heterochromatin complex.

The dosage assembly model as proposed here for X-inactivation makes certain direct and testable predictions. If heterochromatic inactivation in mammals is a gene-dosage-sensitive process then it should be possible to identify Class I loci whose duplication or deletion will either enhance or suppress X inactivation, respectively. While we predict that at least one of these genes will reside on the X, there is no \textit{a priori} reason to expect that all loci responsible for X-chromosome heterochromatin production will be. Therefore, it may be possible to discern which autosomes harbor such genes by examining the karyotypes of spontaneous human abortuses to determine if haplo-deficiency for a particular chromosome is associated with the failure of X-inactivation. Alternatively, appropriate crosses utilizing Robertsonian translocations in the mouse may be similarly effective for systematically reducing the dosage of specific auto-
somes (or regions of autosomes) in zygotes which may then be examined for possible effects of X-inactivation. Finally, if the Drosophila examples of Class I and Class II modifiers of variegation can be extended to mammals, then examining variegating phenotypes in the mouse in conjunction with varying chromosome dosage should also uncover such genes.

Why chromosome imprinting?
Chromosome imprinting is a term currently used to refer to a parental source effect in which chromosomes, or certain genes they contain, are differentially expressed in the zygote depending on whether they are maternally or paternally derived. Instances of imprinting in humans and in the mouse are particularly revealing.

The Prader-Willi syndrome in humans is characterized by obesity, hypogonadism and mental retardation. About 60% of these individuals possess a small paternally inherited deletion of chromosome 15g11g13. Angelman syndrome, on the other hand, results in children given to unusual and frequent laughter, mental retardation and ataxic movement. About half of these patients also exhibit deletions of 15g11g13 similar to those observed for the Prader-Willi condition. However, Angelman syndrome is observed when the deficiency is maternally inherited. Recently, Nicholls et al. (1989) have shown that for several non-deletion examples of Prader-Willi both chromosomes 15 had been maternally inherited. These results strongly suggest two important conclusions. First, normal zygotic development requires that one copy a gene (or genes) in 15g11g13 be inherited from each parent. Second, the fact that a paternally inherited 15g11g13 deletion and maternal uniparental disomy result in virtually identical Prader-Willi phenotypes implies that imprinting serves to regulate the expressed dosage of a gene(s) in this region.

Eight or nine segments of the mouse genome demonstrate parental source or chromosome imprinting effects (see Cattanach, this volume). Some of these exhibit striking antipodal phenotypes depending on which parent contributes a given region of the genome. For example, Cattanach and Kirk (1985) have described two situations in which anomalous and contrasting phenotypic effects are produced by certain portions of chromosomes 11 and 2 according to their parental origin. They showed that when both copies of the proximal portion of chromosome 11 are of paternal origin, fetuses and adults are produced that are larger than their normal littermates whereas if the chromosomes 11 are of maternal origin they are smaller. In another instance, when the distal portions of chromosome 2 are maternally derived, the newborns have long flat-sided bodies, are almost totally inactive within a few hours after birth, and rarely survive for more than 24 h. However, if the distal portions of chromosome 2 are of paternal origin, the newborns have short square bodies with broad flat backs, are notably hyperkinetic, and usually survive for several days but fail to grow normally.

These anomalous phenotypes most likely result from the differential functioning of maternally and paternally derived alleles, a condition determined by the parental origin of each chromosome. While the mechanism by which imprinting occurs is unknown, it seems likely that certain genes are repressed specifically in the gametes of one parent or the other, so that only a single allele is functional and capable of expression in the fertilized zygote. The opposing nature of the phenotypes observed in the examples of imprinting for mouse chromosomes 2 and 11 is reminiscent of the dosage-sensitive antipodal phenotypes previously discussed for Drosophila, a phenomenon that we explain as the result of such loci coding for polypeptides involved in the assembly of multimeric proteins. We propose, therefore, that imprinted genes are loci that will be found to encode products involved in assembly driven reactions where n≥2 as described by equations (1) through (4). Stated another way, the necessity of imprinting is a consequence of the involvement of the products of imprinted genes in assembly reactions where one, rather than two, doses of a given gene is required to achieve the appropriate amount of a multimeric product. We suppose, but it has not been demonstrated, that sub-chromosome regions exhibiting imprinting effects are repressed by heterochromatinization in a manner similar to what takes place in the imprinting of an entire chromosome.

Temporal control of heterochromatic gene repression
What is perhaps most striking about heterochromatic inactivation is that it is temporally regulated in a very precise way so as to occur within a restricted period of development. For example, in mice, X-inactivation begins in the trophectoderm of the early blastocyst (Gartler and Riggs, 1983); in coccids, heterochromatinization of the paternal set of chromosomes occurs during early embryonic development (Brown and Nur, 1964); and, in Drosophila, the inactivation of w+/wmd individuals begins around the time of eye disc formation (Becker, 1966). Yet the mechanism responsible for the temporal regulation in these and other cases of gene and chromosome inactivation remains elusive.

We have examined the temporal control of heterochromatin-induced gene repression by studying a variegating mutation known as y<sup>3P</sup> (yellow-3 of Patterson). This mutation is an inversion of the X chromosome that places the yellow gene, normally located at the distal end of the X, adjacent to the proximal heterochromatin (Fig. 4). In this situation, the expression of yellow is variegated and produces mosaic individuals that display black (wild-type) and yellow (mutant) bristles on the body of the adult fly and especially along the anterior margin of the wing. The linear array of the approximately 80 anterior wing margin bristles is particularly useful for this study because, as Garcia-Bellido and Merriam (1971) have shown, neighboring bristles are clonally related to each other as evidenced by the fact that the patch size of contiguousy marked bristles (labeled by X-ray induced somatic crossing over) is a function of the time in
Temperature shift 28° to 18°

![Graph A](image)

Temperature shift 18° to 28°

![Graph B](image)

Fig. 5. Determination of the temperature-sensitive period (TSP) of the $\gamma^{3P}$ mutation. In panels A and B, embryos were collected and maintained at 28° or 18° for the indicated number of days and then shifted to 18° or 28° for the duration of development, respectively. The percent of yellow bristles on the anterior margin of about 20 adult wings (± standard error of the mean) is plotted as a function of the number of days at which the animals were reared at the indicated temperature before being shifted to the alternate temperature. The arrow at the bottom of each graph signifies the time of pupariation.

Since virtually all variegating position effects are cold sensitive, the mutant phenotype being more pronounced at lower temperatures, it is possible by temperature-shift experiments to determine the temperature-sensitive period (TSP) of the inactivating event. As shown in Fig. 5, both shift up and shift down regimens reveal that the TSP of $\gamma^{3P}$ occurs during the pupal period, very late in developmental terms. Garcia-Bellido and Merriam (1971) have demonstrated that this late TSP corresponds to a time at which all of the single bristle-forming cells of the anterior wing margin have been determined.

An answer to this question comes from an examination of the effect of various Enhancers of variegation on $\gamma^{3P}$. As we have observed, Enhancers increase the penetrance of the mutant phenotype of variegating loci in a rather striking fashion. But how do they do it? In general, there are two ways that these loci may exert their effect. One model requires that Enhancers cause the inactivating decision to be made at an earlier time in development so as to produce more mitotic descendants of the progenitor cell expressing that decision. Alternatively, Enhancers could simply increase the frequency with which the inactivating event occurs, without affecting its temporal regulation. It is possible to decide between these models as follows.

In either $\gamma^{3P}/Y;+/+$ and $\gamma^{3P}/Y; E(var)/+$ flies, the presence of two or more adjacent yellow bristles may be due either to an early inactivating event in a pre-mother cell whose clonal products eventually give rise to the cluster, or result from late single non-clonal inactivating events that by chance happen to lie adjacent to each other. It is possible to distinguish between these alternatives with the aid of a computer to simulate the linear arrangement of yellow (variegated) and black (wild type) bristles along the anterior wing margin that would be obtained by chance, that is by the occurrence of late single inactivating events, given only the total number of yellow ($\gamma$) and wild-type (+) bristles present. This provides us with a distribution of the number of times two or more contiguous yellow bristles would be expected to arise at random. These data are then compared (using a chi-square test) to the experimentally obtained distribution for the same number of $\gamma$ and + bristles.

To simulate the expected number of contiguous yellow bristles that might arise as a result of single late inactivating events, an interactive algorithm was devised that begins by entering the number of $\gamma$ and + bristles and the number of trials desired. The computer first constructs a linear array of + 'cells' equal to the sum of $\gamma$ and + bristles and then randomly changes a given number of these to $\gamma$ as specified by the input. The number of clusters containing 1, 2, 3... etc. contiguous $\gamma$ cells are tabulated and the process is repeated for a selected number of trials. At the end of the simulation, the number of clusters containing 1 to 7 $\gamma$ bristles is calculated. These results are then compared to the data actually obtained from $\gamma^{3P}$ wings in the presence of various Enhancers.

$\gamma^{3P}$ females were mated to wild-type (+/+) or $E(var)/Balancer$ males and the male progeny of the appropriate genotype were collected and scored. 20 wings, or about 1600 bristles, from each of the $\gamma^{3P}/Y;+/+$ and $\gamma^{3P}/Y; E(var)/+$ male progeny were examined and the number of clusters containing from 1
to 7 contiguous yellow bristles per wing was obtained. As the results in Table 1 show, in y\textsuperscript{P}/Y;+:+ males, there is a modest level (8.8%) of yellow variegation which is dramatically increased by three different Enhancers of variegation. The data also reveal that the observed distribution of yellow bristle clusters along the anterior wing margin bristles in both y\textsuperscript{P}/Y;+:+ and y\textsuperscript{P}/Y; E(var)\textsubscript{8} or 66/+ flies is in excellent agreement with what would be expected from late single inactivating events as predicted by computer simulation. This conclusion is also consistent with our experiments that define the TSP of y\textsuperscript{P} variegation in the anterior wing margin bristles to the pupal period when all of the bristle-forming cells have been determined. Furthermore, we note that the three Enhancers of variegation studied cause a 2- to 4-fold increase in the number of yellow anterior wing margin bristles. If a shift in the timing of the inactivating event is solely responsible for this increase in yellow bristle variegation, the initiation of repression must occur at least one or two divisions before mother cell differentiation takes place. If any of these Enhancers caused such a shift in the temporal period of inactivation, even by one cell division, it would be readily observed as a striking increase in the number of clones containing two or more yellow bristles. Nevertheless, despite a 4-fold increase in yellow bristle variegation in y\textsuperscript{P}/Y; E(var)\textsubscript{8} or 66/+ flies, the distribution of the number of contiguous yellow bristles per clone is virtually identical to that expected for late single random inactivation events. Thus, the Enhancers studied here increase variegation by increasing the frequency at which heterochromatic repression occurs at a given time in development and not by adjusting the time of inactivation to an earlier developmental stage.

What mechanism might account for the rigid developmental control of the heterochromatic gene repression as described here? It could be argued that temporal regulation of yellow is positively regulated by initiating the construction of a specific block of heterochromatin late in development. However, one of the Enhancers that we have examined (E(var)\textsubscript{19}) is known to be expressed early, at about the tenth nuclear division of the preblastoderm stage (James et al. 1989). Moreover, it is well recognized that the centric heterochromatin domains of all chromosomes are conspicuously apparent at the blastoderm stage of early embryonic development. Together, these observations suggest that the late inactivation of yellow is not the result of initiating a new block of heterochromatin during pupal development.

Alternatively, the time of heterochromatic inactivation may be negatively regulated and depend on whether or not the adjoining euchromatic sequences will permit the spread of a neighboring heterochromatic domain. That is, temporal regulation of the spreading of heterochromatin is negatively controlled by the state (permissive or nonpermissive) of the adjoining euchromatin as illustrated in Fig. 6. Functions such as the commitment to gene expression or chromosome replication may control the characteristic time at which the adjoining (eu)chromatin establishes its permissive or nonpermissive state. We postulate that factors involved in gene expression or replication located in or near the y\textsuperscript{+} locus block the advancement of an adjoining heterochromatin complex into downstream sequences. Removal of such proteins at specific times in development may occur because they are no longer synthesized, reflect their inherent instability or turnover, or result from a change in the time in S-phase when the gene is replicated. In any case, once these components are dislocated, the adjoining heterochromatin now invades and represses downstream genes. Thus, like a coiled spring, heterochromatin is poised to take advantage of circumstances in neighboring sequences whenever possible to extend its repressive effects. Support for this negative control hypothesis comes from the observation that the important feature determining 'sectored' (early inactivation) versus 'peppered' (late inactivation) eye color patterns in Drosophila appears to be the location of the euchromatic breakpoint near the white locus rather than the type of flanking heterochromatin to which it is adjoined (Tartof et al. 1984). In a wider context, the negative regulation of heterochromatic gene repression as proposed here may also be

### Table 1. The effect of various Enhancers of variegation on the clone size of yellow anterior wing margin bristles in y\textsuperscript{P} flies

<table>
<thead>
<tr>
<th>Genotype or computer simulation</th>
<th>Yellow bristles per clone</th>
<th>Significance *</th>
<th>Average number of bristles per wing</th>
<th>Per cent variegation</th>
</tr>
</thead>
<tbody>
<tr>
<td>y\textsuperscript{P}/Y;+:+</td>
<td>20 119 11 0 0 0 0 0 0</td>
<td>0.92</td>
<td>Wild type 72.9 7.0 79.9 8.8</td>
<td></td>
</tr>
<tr>
<td>computer simulation</td>
<td>20 135 11 1 0 0 0 0 0</td>
<td>73.0</td>
<td>Yellow 70.0 5.0 80.0 7.0</td>
<td></td>
</tr>
<tr>
<td>y\textsuperscript{P}/Y; E(var)\textsubscript{8} or 66/+</td>
<td>20 227 72 24 8 6 1 1 0</td>
<td>0.49</td>
<td>Sum 60.7 18.4 79.1 23.3</td>
<td></td>
</tr>
<tr>
<td>computer simulation</td>
<td>20 255 71 29 5 2 1 0 0</td>
<td>0.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>y\textsuperscript{P}/Y; E(var)\textsubscript{19}/+</td>
<td>20 199 51 16 1 0 1 0 0</td>
<td>0.82</td>
<td>48.1 29.7 77.8 38.2</td>
<td></td>
</tr>
<tr>
<td>computer simulation</td>
<td>20 208 55 14 0 0 0 0 0</td>
<td>61.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>y\textsuperscript{P}/Y; E(var)\textsubscript{66}/+</td>
<td>20 202 74 33 19 10 2 1 1</td>
<td>0.82</td>
<td>48.0 30.0 78.0 38.0</td>
<td></td>
</tr>
</tbody>
</table>

*P is the probability that the equidistribution of yellow bristle clone sizes observed in anterior wing margins and computer simulations is significantly different from each other (if P<0.05) as determined by the chi-square generalized likelihood ratio test.
the spreading of heterochromatin is proposed to be heterochromatic gene repression. Temporal regulation of development may occur because they are no longer nonpermissive) of the adjoining euchromatin. Factors negatively controlled by the state (permissive or nonpermissive) of the adjoining euchromatin. Factors involved in gene expression or replication located in or synthesized, reflect their inherent instability or turnover, or dislocated the adjoining heterochromatin now invades to the y+ locus may block the advancement of an adjoining heterochromatin complex (dark arrow) in the y+\textsuperscript{hp} mutation. Removal of such proteins at specific times in development may occur because they are no longer synthesized, reflect their inherent instability or turnover, or result from a change in the time in S-phase when the gene is replicated. In any case, once these components are dislocated the adjoining heterochromatin now invades to repress downstream genes.

At present, the mechanism responsible for stably transmitting the repressed state produced by heterochromatin is unknown. However, the creation of order in biological systems appears to proceed through molecular recognition processes involving structural complementarity as illustrated by the paradigm of nucleic acid base pairing receptor–ligand complexes. Precisely how this complementarity principle applies to the initiation and construction of heterochromatin domains, and the imprinted structures they may produce, is yet to be addressed.

We thank Dr Sam Litwin for devising the computer simulation described here and for statistical consultation. This work was supported by Public Health Service Grants CA-06927, RR-05539 and GM-19194 from the National Institutes of Health, an appropriation from the Commonwealth of Pennsylvania.

**References**


**James, T. C. and Elgin, S. C. R.** (1986). Identification of a nonhistone chromosomal protein associated with...


