A GENETIC AND MOLECULAR ANALYSIS OF HISTONE DEACETYLASE

ONE IN *DROSOPHILA MELANOGASTER*: SPECIFIC MISSENSE

MUTATIONS SUPPRESS POSITION EFFECT VARIEGATION

by

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Abstract

Essentially all higher organisms are made up of two or more types of tissues. The specific identity of those tissues is dependent on the genes that are expressed within the cells of the particular tissue type. The correct set of genes must be expressed and genes, that are not part of the set specific to that tissue, must be kept silenced. In addition, in most cells, the decision whether a gene will be active or not is made early in development and therefore must be passed on to daughter cells. The focus of this thesis is an investigation into the mechanism or mechanisms employed by eukaryotes to silence genes and to maintain that silenced state throughout development. The model system our laboratory has been using to investigate silencing is position effect variegation (PEV) in *D. melanogaster*. In PEV a gene is silenced in a certain proportion of the cells of a tissue in which it is normally expressed due to its proximity to an heterochromatic breakpoint. The decision whether a gene will be active or inactive is made early in development and that decision is passed on to daughter cells with reasonable fidelity. Thus PEV mimics normal development in many ways. This has led our lab, and several others, to try to dissect the mechanisms underlying PEV with the hope they will shed some light on the more general silencing mechanisms that occur during normal development.

In Chapter 2 of this thesis I describe the cloning and characterization of a gene identified in a screen for dominant suppressors of the variegation associated with PEV [Su(var)s]. The gene encodes HDAC1, an histone deacetylase homologous to HDAC1 from mammals and Rpd3 from *S. cerevisiae*.

Specific mis-sense mutations in *HDAC1* cause strong dominant suppression of PEV while null or hypomorphic mutations have no effect on the variegating phenotype. I present a model proposing that the mis-sense mutations are acting as anti-morphic mutations that "poison" the deacetylase complex.

The level of variegation of a gene subject to PEV is very sensitive to a wide variety of factors, some, which may be acting directly and some, which may be acting indirectly. HDAC1 localizes to a large number of sites on the polytene chromosomes of *D. melanogaster* (Pile and Wasserman, 2000) and therefore appears to regulate a large number of genes. Thus it is a possibility that the Su(var) mutations in *HDAC1* are affecting PEV indirectly. In Chapter 3 I present data from chromatin immuno-precipitation experiments (X-ChIP) that provides compelling evidence that HDAC1 is acting directly on the euchromatic region subject to silencing in PEV. I propose a model linking the histone deacetylase activity of HDAC1 to the function of other proteins known to be involved in the silencing associated with PEV.

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Literature Review

Eukaryotes are complex organisms that must precisely regulate their genes to survive. As an example, in a multicellular organism, with many different tissue types, the identity of a tissue is determined by the combination of genes that are active. Thus mechanisms must exist to activate the correct genes in a particular tissue and then remember that pattern through subsequent cell divisions. On the other hand, it would be deleterious or lethal, if genes that were supposed to be inactive, were expressed, and therefore complementary mechanisms must exist to inactivate specific genes and maintain that inactive state throughout subsequent cell divisions. This thesis will be concerned with the latter problem: what is the mechanism or mechanisms that eukaryotes employ to silence genes and then to pass this decision on to daughter cells? The model system our lab has used to address this question is position effect variegation in *Drosophila melanogaster*.

Gene Silencing and Position-Effect Variegation (PEV)

Classical PEV occurs when a chromosomal rearrangement abuts a normally euchromatic region of a chromosome to an heterochromatic breakpoint. Genes located in the euchromatin, immediately adjacent to the breakpoint, often display a mosaic phenotype in the tissues in which they are normally expressed. In some cells the gene or genes are fully active and the cells appear normal, however, in others, the gene(s) is silenced, and the tissue appears mutant. This silencing phenomenon mimics normal development in that the decision as to whether a gene will be active or silenced appears to be made early and then is passed on to daughter cells. Since the genes in

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euchromatin are not mutated (see below) but are silenced due to the proximity of the heterochromatic breakpoint, PEV has been employed as a model system for dissecting the silencing effects of chromatin structure, in particular heterochromatin. More details on the nature of PEV will be provided below. For now suffice it to say this approach has been fruitful and the dissection of PEV has provided tremendous insights into the structure of heterochromatin and some of the mechanisms of silencing employed by eukaryotes to control gene expression. One feature of gene expression that has become apparent in recent years is that regulation of gene expression, including silencing, is dependent on chromatin structure.

This chapter will provide a brief review of chromatin structure and its role in gene regulation followed by a review of the phenomenon of PEV and the role of some of the more well characterized chromatin proteins that have been isolated as modifiers of PEV. The focus of the thesis will be histone deacetylase one (HDAC1), a protein that was identified in a screen for mutations that modify PEV.

Chromatin Structure

Much is now known about the basic structure of chromatin. Chromatin is a dynamic assemblage consisting of approximately 50% DNA and 50% protein. About one-half of the proteins are histones, a group of five basic proteins, which are among the most highly conserved proteins in all eukaryotes. Histones H2A, H2B, H3 and H4 are known as the core histones and they associate to form an octamer. Initially, H3 and H4 form heterodimers that associate to form a tetramer. The tetramer then associates with two

heterodimers of H2A and H2B to form an octamer that is shaped like a flattened sphere. Approximately 146 base pairs of DNA wind about 1.7 times around the outside of the sphere in a left-hand supercoil. This structure, the nucleosome, forms the basic building block of all eukaryotic chromatin. The fifth histone, histone H1, also called the "linker histone", binds to nucleosomes and protects approximately 20 additional base pairs of DNA outside the nucleosome (Horn and Peterson, 2002; Luger et al., 1997; Turner, 2002; Workman and Kingston, 1998).

X-ray crystallographic analysis of the nucleosome at a resolution of 2.8 A has been completed and provides the following picture of nucleosome structure (Luger et al., 1997). The core histones contain two functionally separable regions, the central "histone fold" domain, and the amino- and carboxy-terminal tail domains. The histone fold domains of the histones are involved in histone/histone interactions that stabilize the nucleosome and also make contact with the DNA as it winds around the nucleosome. Where the DNA makes contact with the nucleosome, its structure is highly ordered. Those regions of the histones not in contact with DNA, the amino- and carboxy-terminal tails, appear to be much more flexible and extend out and between the DNA gyres.

The apparently simple structure of the nucleosome led early investigators to assume it was a passive structure that formed a scaffold for DNA architecture but did not play a role in regulating genetic activity. However, the first indications that this was far from accurate were reported almost 40 years ago when Allfrey et al. (1964) observed a correlation between acetylation of histone residues and active transcription of genes. With the

emergence of a possible role for histone modification in the regulation of gene activity, the search for further histone modifications revealed that histones are subject to a surprising number of post-translational modifications. These modifications include lysine acetylation, lysine and arginine methylation, serine phosphorylation and ubiquination of H2A (Spotswood and Turner, 2002). As noted above, the amino- and carboxy-terminal tail domains of all core histones extend out from the core nucleosome particle and it was initially assumed that all modifications would occur in these domains. However very recent experiments have revealed modifications occur to the globular, histone fold domain, as well (Briggs et al., 2002). The existence of further modifications cannot be ruled out. All of the modifications have the potential to alter the structure of the nucleosome and/or the structure of the chromatin fiber.

It has been suggested the pattern of modification on a nucleosome forms a "histone code" which not only regulates whether a gene is expressed or not, but also modulates expression levels of active genes. In addition, the histone code could provide the epigenetic mark that allows a cell to pass expression patterns on from one generation to the next (Strahl and Allis, 2000). The large number of potential modifications combined with the wide variety of sites available for modification creates the opportunity for an extremely complex code to be created on a nucleosome; in fact, several thousand different patterns are possible (Turner, 2002). Histone H3 alone can be acetylated at six lysines, methylated at five lysines (and this can be mono-, di- or trimethylation), methylated at one arginine and phosphorylated at serine 10. A similar complexity of modifications exists on H4, slightly less on H2B and still less on H2A. In addition, since the nucleosome is an octamer containing two of each of

the core histones, it is possible that each histone maybe modified independently.

Finally, there is the potential for, and the likelihood of, inter-nucleosomal interactions that are also affected by histone modifications. Complete elucidation of the histone code, if indeed it does exist, will require that many genes be probed for every possible modification and the pattern observed must be correlated with transcriptional activity, or the potential for activity, and transcription levels. The pattern observed at one locus must then be compared to that of other genes to determine similarities and differences. However, this is only the first level of analysis. The pattern may also control the positioning or location of a gene within specific compartments of the nucleus (see below) or play a role in heritability of the expression pattern and thus cell lineages will also have to be examined before a complete picture will be generated. While this task may appear daunting, this area of research is one of the most active in molecular biology and some consensus, regarding the effect of histone modifications, is emerging. For example methylation of histone H3 lysine 4 appears to be a widely used mark for gene activity, while methylation of lysine 9 on H3 appears to mark a gene for repression (see below).

The nucleosome is only the first level of chromatin packaging. A typical cell may contain about one meter of DNA that must be packaged into an organelle as small as the nucleus and thus chromatin can not exist as a string of nucleosomes. Exactly what the higher order structure of chromatin is has been the subject of intensive study and remains contentious (Horn and Peterson, 2002). *In vitro* chromatin reconstitution studies suggest that, as the divalent cation concentration is increased to physiological levels, the nucleosome first

condenses into a 30 nm fiber and then into higher order irregular aggregates. The formation of these higher order aggregates *in vitro* requires the histone tails to be present on the core histones (Hansen, 2002; Luger et al., 1997). The higher order irregular aggregates were made more regular, stabilized and compacted by the addition of a linker histone, such as H5 (Carruthers and Hansen, 2000). *In vivo,* studies that followed decondensation of the chromosomes following mitosis suggest they first decondense to a 100 - 130 nm fiber that may further decondense to a 60 - 80 nm fiber for short intervals. Only occasional, very short, stretches of a 30 nm fiber were observed (Belmont and Bruce, 1994). This observation suggests that the basic structural unit of chromatin is a 100 nm fiber (Tumbar et al., 1999) that may present a considerable obstacle to factors that must modify the histones on a nucleosome to regulate gene expression.

How then, do the factors that regulate transcription gain access to a gene? This is accomplished with two types of cellular complexes, one which relies on adenosine triphosphate (ATP) to provide the energy to remodel chromatin and one which modifies the histones to stabilize an active or inactive state (Narlikar et al., 2002). Three types of ATP dependent remodeling complexes have been classified based on the kind of ATPase employed by the complex: the SWI2/SNF2 family; the ISW1 family; and, the Mi-2 family. Each family may have several different complexes that differ in the proteins associated with the ATPase. Although each family increases the accessibility of nucleosomal DNA, they appear to do so by slightly different mechanisms that may reflect the different chromosomal context in which the genes they regulate are located (Narlikar et al., 2002).

ATP dependent remodeling complexes work in conjunction with complexes that, on one hand, contain histone acetyltransferases (HATs) that hyperacetylate histone tails creating a chromatin structure correlated with transcriptional activation (Reid et al., 2000; Vogelauer et al., 2000). On the other, they either contain, as in the case of the NuRD complex, or work with complexes that contain, histone deacetylases (HDACs) that hypoacetylate histones creating a structure correlated with repression of gene activity and heterochromatin (Xue et al., 1998; Zhang et al., 1998). Exactly how these complexes are targeted to a specific site is not known for certain but evidence is accumulating that at least some sequence specific factors can recognize their cognate sequences in the context of the chromatin fiber and recruit both an ATP dependent remodeling complex and a histone modifying complex (Narlikar et al., 2002).

Heterochromatin and Euchromatin

Early cytologists studying eukaryotic cells identified two types of chromatin: heterochromatin and euchromatin. Euchromatin, which becomes diffuse and lightly staining in the interphase cell, contains most, but not all, of the active genes. Heterochromatin, on the other hand, remains darkly staining throughout the cell cycle, contains relatively few genes and is found primarily associated with the centromeres and telomeres.

Subsequent work has shown that heterochromatin in characterized by a number of structural features (Henikoff, 2000; Richards and Elgin, 2002). Heterochromatin contains an abundance of repetitive DNA sequences, including satellites sequences, derivatives of viruses and transposons. It is

often characterized as being genetically inert, but it is not entirely devoid of genes. Approximately 40 to 50 genes have been identified in the pericentric heterochromatin of *D. melanogaster* (Eberl et al., 1993; Weiler and Wakimoto, 1995). These genes are also subject to PEV but react in a manner complementary to euchromatic loci (see below). Heterochromatin displays low levels of meiotic recombination and replicates late in S phase.

Heterochromatin has also been characterized, to a limited extent, biochemically. It has a lowered accessibility to nucleases, suggesting it has an altered, closed, form of packaging. Analysis of euchromatin suggests the nucleosomal arrays are irregular and contain nucleosome-free, nuclease hypersensitive sites associated with active genes. In contrast, the nucleosomes in heterochromatin are regularly spaced over large regions and a higher proportion of the DNA is associated with the nucleosomal core (Grewal and Elgin, 2002; Sun et al., 2001). The histones of heterochromatic regions are hypoacetylated relative to those found in euchromatin. Interestingly, when a gene is inactivated in euchromatin, inactivity is often associated with hypoacetylation of the nucleosomes at or near the promoter. More recently it has been discovered that another histone modification is strongly associated with the heterochromatic state. The methylation of lysine 9 on histone H3 (H3 mK9) is found in the pericentric heterochromatin of *Drosophila* and on the largely heterochromatic fourth chromosome (Jacobs et al., 2001). Chromatin immuno-precipitation experiments in *Schizosaccharomyces pombe* show the silent mating locus is enriched in H3 mK9 while flanking euchromatic regions contain little or no H3 mK9 (Noma et al., 2001). The methylation H3 K9 is also found on facultative heterochromatin, such as the inactive X chromosome in

mammalian females (Boggs et al., 2002; Heard et al., 2001; Peters et al., 2002). However, this modification is not restricted to heterochromatin. It has also been associated with the silencing of genes in euchromatin (Hwang et al., 2001) but unlike in heterochromatin, where H3 mK9 is widely dispersed, in euchromatin it occurs at a single nucleosome at the promoter of the inactivated gene (Nielsen et al., 2001).

Another biochemical marker frequently found in heterochromatin is the presence of methylation of cytosine residues in the DNA. It is only found at very low levels in *Drosophila* but in most other higher organisms it is the most common form of DNA modification. The modification is also found at some silenced loci located in euchromatin. Not only is it important for the stability of the pericentric heterochromatin (Bachman et al., 2001; Okano et al., 1999; Xu et al., 1999) but it also plays a role in maintaining the epigenetic expression pattern both in heterochromatin and euchromatin (Jones and Takai, 2001; Martienssen and Henikoff, 1999).

History of Position-Effect Variegation

Position-effect variegation (PEV) was first documented over 70 years ago when Muller (1930) observed mosaic expression in the colour of eyes of *D. melanogaster* that had been exposed to X-rays. Normally the eyes of this species are bright red, but in these mutant lines, the eyes were a patchwork of normal red eye cells and colourless eye cells. Mosaic gene expression, as a result of PEV, has since been observed in vertebrates (Cattanach, 1974), lower eukaryotes (Clutterbuck and Spathas, 1984; Ekwall et al., 1997) and plants (Catcheside, 1947). Thus it occurs in representatives from all eukaryotic kingdoms.

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However, since it has been most extensively studied in *Drosophila* (reviewed in (Baker, 1968; Grigliatti, 1991; Henikoff, 1994; Spofford, 1976; Spradling and Karpen, 1990), this review will focus on the work done in fruit flies. Accordingly, unless otherwise specified, all studies of PEV described herein employed *D. melanogaster* as the experimental organism.

PEV typically occurs when, as the result of chromosome breakage, a normally euchromatic region of the chromosome is rejoined to a heterochromatic breakpoint. A gene or, in some cases several genes, in the euchromatin immediately adjacent to the heterochromatic breakpoint, often display a mosaic phenotype in the tissues in which they are normally expressed. In some cells the gene is on, and the tissue appears wild-type, while in others, the gene is off, and the cells display a mutant phenotype. Virtually all genes that display a cell autonomous phenotype and have been tested, can be made subject to PEV. However, exceptions have been noted: *ebony* (Brosseau, 1970) and the bithorax complex (E.B. Lewis, cited in Henikoff, 1990).

Classical PEV involves the association of a euchromatic gene with heterochromatin but the complementary situation also occurs. Variegation for genes located in β-heterochromatin also occurs when chromosomal rearrangements juxtapose them to euchromatic DNA. The *light*[+] gene, located in the β-heterochromatin at the base of the left arm of chromosome 2, and the *cubitus interruptus*[+] gene, located on the largely heterochromatic fourth chromosome, will variegate when moved to a euchromatic environment (Hearn et al., 1991; Hessler, 1958; Stern and Kodani, 1955).

There is now overwhelming evidence that the mosaic expression observed in PEV is not the result of mutation of the variegating gene, but is the result of the new association between the euchromatic region and heterochromatin located at the illicit breakpoint. The first of these studies demonstrated that the variegating gene was still intact by moving it away from the heterochromatic breakpoint, either by recombination (Judd, 1955) or by further chromosomal rearrangement (Hinton and Goodsmith, 1950). These studies confirmed that the variegating gene had not been mutated and could be restored to full expression by removing it from the vicinity of heterochromatin.

Further evidence that it was proximity to the heterochromatic breakpoint that was causing mosaic expression came from studies involving rearrangements in which the expression pattern of more than one gene could be observed. It was noted that the gene closest to the breakpoint was inactivated at a higher frequency than genes located further away. This was particularly evident in the $T(1;4)w^{258-21}$ strain, which variegates for $white^+$, a gene required for the normal red eye colour of D. *melanogaster* and for $roughest^+$, a gene which causes disorganized eye facets when silenced. These genes are very tightly linked and in this strain the translocation places $roughest^+$ closer to the breakpoint than $white^+$. Careful analysis of the eye revealed that $white^+$ clones were always smaller and completely contained within *roughest* clones. There were no examples reported of clones in which $white^+$ was silenced but $roughest^+$ was active. This suggests that inactivation is spreading out from the heterochromatic breakpoint. (Demeric and Slizynska, 1937; cited in (Cohen, 1962)). Spreading appears to occur at heterochromatic genes as well since a

similar spreading effect has been reported for such loci in *Drosophila hydei* (Hess, 1970).

Cytological observations also seem to support the model that silencing is a polar phenomenon that emanates from the heterochromatin at the breakpoint. In polytene chromosome preparations from variegating strains, the euchromatic region adjacent to the breakpoint often adopts a morphology similar to β-heterochromatin, darkly staining and unbanded (Hartmann-Goldstein, 1967). The translocation $T(1;4)wm^{258-21}$ variegates for *white*[+], located at band 3C2 and for *notch*[+], located at 3C7. In this strain the band containing *white* is closer to the breakpoint than the band containing *notch*[+]. In polytene preparations from this strain, one either sees both bands, the band at 3C7 or neither band. The band at 3C2 was never visible when the band at 3C7 was not (Schultz, 1936).

This notion has been recently challenged by a careful analysis of several X chromosome inversions that variegate for *white*[+] and *roughest*[+] (Talbert and Henikoff, 2000). The authors found that in two strains, $In(1)w^{m51b}$ and $In(1)w^{mMc}$, patches of eye cells could be found that were mutant for *roughest*[+] gene but normally pigmented suggesting that the *white*[+] gene was fully active. In these strains *white*[+] is closer to the heterochromatic breakpoint than *roughest*[+], and therefore this appears to be an exception to the notion that silencing occurs in a polar fashion extending out from the heterochromatic breakpoint. However, unknown to these authors, there is evidence that, over time, these two strains have acquired modifiers that radically affect expression of the *white*[+] gene. In lab stocks of these strains the eyes are very red, almost indistinguishable from wild-type eyes, and therefore the *white*[+] gene is not silenced in very many cells.

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V. Lloyd (personal communication) outcrossed the lab stocks for five generations and found that the eye colour became almost completely white, indicating the lab stocks had acquired modifiers that either prevented silencing of the *white*⁺ gene or reactivated it in most cells after it had been silenced by PEV. If the latter is the case then an alternative explanation for the observations of Talbert and Henikoff is that the modifiers reactivate *white*⁺ in some cases without reactivating *roughest*⁺. Alternatively, it may simply be the case that the factors that silence genes do spread out from the heterochromatin at the breakpoint, but do not necessarily silence every gene they encounter. A particular promoter may be strong enough to overcome the repression. Finally, it is possible the silencing process may occasionally skip regions in the euchromatin and therefore genes in that region will escape repression.

The polarity displayed by neighbouring genes with respect to the breakpoint led Schultz (1939) to propose that an inactivation process spreads out from the heterochromatic breakpoint causing PEV. In some cases the inactivation process spreads far enough to silence a gene while in other cells the process does not spread as far as the gene and it retains its normal function. This theory, the "Spreading Model", has endured for over 60 years, but other models have been proposed.

An alternative theory, which initially generated a lot of attention, posits that it is the position in the nucleus that determines whether a variegating gene will be on or off. This theory is based on the observation that the heterochromatic regions of chromosomes occupy distinct regions or compartments in the nucleus. The chromosomes adopt a conformation with the heterochromatic regions surrounding the centromere and at the telomeres

occupying the periphery of the nucleus while euchromatin, containing most of the active genes, occupies the interior region (Ferreira et al., 1997; Rabl, 1885; Sadoni et al., 1999). A locus subject to PEV can be located in a euchromatic compartment, which contains all the factors required for transcription, where it is expressed normally. However, due the to nearby heterochromatin it can also be mis-located to a heterochromatic compartment on the nuclear periphery. In this compartment the factors necessary for transcription are either absent or in very short supply and therefore the variegating gene is not expressed (Sass and Henikoff, 1999).

The evidence for this model is, for the most part, circumstantial. It is based primarily on the correlation between the nuclear localization of a variegating gene and its level of expression (Sass and Henikoff, 1999). However, studies on the location of developmentally regulated genes in B and T lymphocytes also demonstrated an expression dependent location in the nucleus; when the genes were inactive they were associated with heterochromatin at the nuclear periphery (Brown et al., 1999; Brown et al., 1997). However, subsequent studies indicate that, even though the silenced gene was associated with the pericentric heterochromatin, it was not packaged as heterochromatin (Sabbattini et al., 2001). Thus, while it may indeed be the case that a silenced gene is located in a specific compartment at the nuclear periphery, there is no evidence to distinguish whether this is the cause of silencing or the result of silencing.

In any event these models are not mutually exclusive and PEV may in fact be the result of contributions from both models. For example, as putative silencing factors begin to spread out from the heterochromatic breakpoint they

might increase the likelihood the surrounding region will be localized to the nuclear periphery where the concentration of silencing factors is increased and the concentration of transcriptional activators is decreased. This in turn could increase the likelihood that the variegating gene is silenced. Failure to relocate to the nuclear periphery would reduce the spread of "heterochromatic factors" and increase the likelihood the gene was expressed.

Timing of PEV

Careful examination of the mosaic phenotype associated with PEV suggests that the initial decision as to whether a variegating locus will be on or off is made early in development and the decision, once made, is then passed on to daughter cells with reasonable fidelity (Janning, 1970). In some rearrangements, the fields of cells that are either mutant or wild-type were large and roughly followed boundaries similar to cell lineages. Several other studies indicate the on/off decision is made early. The strain, *In(1)sc^{s1}*, variegates for the ribosomal DNA genes. In *X/0* males, newly hatched larvae already had levels of rDNA 14% lower than their wild-type counterparts indicating that silencing had occurred prior to hatching (Puckett and Snyder, cited in Spofford, 1976). Similar results were found for the variegating *peach$^+$* gene in *Drosophila virilis* (Baker, 1967). Evidence from variegation of the *yellow$^+$* gene and *white$^+$* gene in *D. melanogaster* suggest the initial determinative event occurs at the time of blastoderm formation (Baker, 1967; Janning, 1970).

PEV is sensitive to temperature (see below) and this sensitivity has been employed to determine the temperature sensitive period (tsp) of the variegating phenotype. Temperature shift studies reveal two main temperature sensitive

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periods: one during early embryogenesis and a second during pupation (Spofford, 1976). The early tsp was the most sensitive, again pointing to early embryogenesis as the time for the initial determinative decision (Spofford, 1976).

Finally, flies reared on Na-butyrate or propionate show strong suppression of PEV (Mottus, 1979; Mottus, 1983) and see below). Studies in which embryos, larvae and pupae were reared on media containing these compounds for defined developmental periods also showed definite times during development that were sensitive to the effects of these chemicals. Again, the most sensitive period was in embryogenesis, although developing flies were also sensitive at other periods during their development that roughly followed the cell division patterns in the eye imaginal disk (Mottus, 1983). These data suggest that butyrate and propionate affect both the early determinative decision and the maintenance of that decision, perhaps through a common mechanism.

Is there an event that occurs at blastoderm formation that could be this determinative event? After fertilization *Drosophila* embryos undergo 13 rounds of rapid nuclear division without cell division (Lawrence, 1992). The embryonic genes are not expressed and the chromosomes are uniformly staining and do not appear to be packaged as heterochromatin. However, at about the time of blastoderm formation, the embryonic genome begins to function, the chromosomes undergo a change in morphology and distinct regions of euchromatin and heterochromatin appear. Since PEV is obviously closely linked to heterochromatin, this suggests the formation of heterochromatin as a likely candidate for the early determinative event. If this

is the case, then one would expect factors that affect the formation of heterochromatin would modify PEV.

While this initial decision is passed on with reasonable fidelity in most rearrangements, in others one can readily observe cells or patches of cells of wild-type tissue within a large clone of mutant cells (unpublished observations). Thus it appears the early decision can be unstable and the variegating locus may be subject to reactivation. In addition, when variegating strains are kept in lab stocks without selection for long periods of time, the number of cells in which the gene is silenced becomes reduced, sometimes markedly. Outcrossing the strain for several generations returns the level of silencing to initial levels (V. Lloyd, personal communication), suggesting that the strains have acquired modifiers that decrease the likelihood a gene will be silenced. This phenomenon has not been well characterized and it is not clear whether the modifiers are acting on the initial determinative event(s), are affecting the maintenance of that decision or both.

Factors that modify PEV

Over the last 60 years many factors have been identified that modify PEV. Some, apparently disparate modifiers, appear to act through a common route, developmental rate. It appears, broadly speaking, that most factors that slow development cause enhancement of PEV, as seen by an increase in the number of cells in which the variegating gene is silenced.

Temperature was one of the first modifiers of PEV identified. Rearing flies at high temperature suppresses PEV, that is, decreases silencing, while low temperature has the opposite effect and enhances PEV (Gowen and Gay, 1934).

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Fruit flies, like all insects, are cold-blooded and therefore at higher temperature develop at a much faster rate than at cold temperatures. For example, flies reared at 25°C develop from egg to adult in approximately 12 days, while flies reared at 18°C require 21 days.

Hinton (1949) first noted that flies reared in crowded cultures showed enhanced variegation relative to non-crowded cultures. Competition for nutrients in crowded cultures slows development.

Many chemicals have been tested for their effect on PEV. Predictably, most, if not all, chemicals that are added to growing cultures slow development. For example a number of DNA synthesis inhibitors caused delayed development and enhanced PEV (Schultz, 1956) and rearing flies in acidic culture conditions of pH 2.6 also delayed development and enhanced PEV (Michailidis et al., 1988).

However, one exception occurred when flies were reared on Na-butyrate or propionate. These chemicals were tested for their effects on PEV because it was suspected that histone proteins, the basic building blocks of chromatin, might be involved in the mechanism of PEV. It had been shown that exposure of Friend leukemia cells to butyrate, which was associated with an increase in the level of acetylated histones in the cell, caused them to differentiate (Reeves and Cserjesi, 1979). In addition, deletion of the histone gene cluster caused suppression PEV. In spite of the fact both chemicals caused prolonged development, they strongly suppressed PEV (Mottus, 1983; Mottus et al., 1980; Rushlow et al., 1984). It was suggested that butyrate's effects were the result of inhibition of an, as yet unidentified, histone deacetylase (Candido et al., 1978; Mottus et al., 1980). However, exposure to butyrate was shown to have a large

number of effects on other cellular processes (Boffa et al., 1981; Christman et al., 1980) and it might have been butyrate's effect on these that caused suppression of PEV. More recent work has confirmed that butyrate does affect chromatin structure (Annunziato et al., 1988) and confirmed it is a potent inhibitor of certain classes of histone deacetylases (Barlow et al., 2001; Emiliani et al., 1998).

Why would an increase in development time cause an increase in the number of cells in which a gene is silent? Zuckerkandl (1974) proposed that the silencing observed in PEV is dependent upon the formation of macromolecular complexes. Delayed development allows more time for these complexes for form and thereby increases silencing. Surprisingly, this hypothesis, or perhaps a slightly more sophisticated version of it, still seems plausible today.

Several genetic factors have also been shown to modify the variegating phenotype including the amount, and perhaps the kind, of heterochromatin. The Y chromosome in *Drosophila* is almost completely heterochromatic. An extra Y chromosome, for example in XYY males, suppresses PEV (Gowen and Gay, 1934) while loss of the Y chromosome (XO males) enhances PEV. It has been reported the strength of this effect is proportional to the amount of Y chromosome material (Dimitri and Pisano, 1989) however there is some evidence from more detailed studies, employing small fragments of the Y chromosome, that some regions of the Y exert a stronger effect on PEV than others (T. Grigliatti, personal communication). That this effect is due to the heterochromatin of the Y chromosome is supported by studies that show duplications and deficiencies of autosomal heterochromatin modify variegation in a similar manner (Spofford, 1976).

Histones are one of the basic building blocks of chromatin, and since PEV appears to be related to chromatin structure, this led some groups to ask whether modifying the dosage of the histone genes would have any effect on PEV. Two groups reported that deficiencies for the histone gene cluster caused strong suppression of the *white*$^+$ gene in the strain *In(1)w*^{m4} (Khesin and Leibovitch, 1978; Moore et al., 1979). It was originally proposed that haploidy for the histone cluster would cause a reduction in the cellular histone pool. Since heterochromatin replicates late in the cell cycle, at a time when histone proteins might be limiting, their short supply would impede the formation of the silencing structure (Moore et al., 1979). Surprisingly, however, more recent work has shown that a deficiency for the histone cluster actually increases transcription from the remaining genes and the cell accumulates more histone mRNAs than normal (Ner et al., 2002). This increased transcription of the histones may lead to a slight increase in protein levels and this may favour the formation of euchromatin at the variegating locus thereby suppressing PEV. Alternatively, mis-regulation of histone metabolism caused by deficiencies of the histone cluster may be acting indirectly on PEV to suppress variegation (Ner et al., 2002).

The Polycomb Group (PcG) of proteins maintain silencing of HOX genes in multicellular organisms (Breiling et al., 2001). The founding member of that group, POLYCOMB, and a known suppressor of PEV, HP1, have a domain in common, the chromo domain (see below). The observation that both POLYCOMB and HP1 contain chromo domains coupled with the known function of the PcG suggested the PcG might also be involved in silencing at variegating loci. However, most PcG proteins do not have a marked effect on

PEV (Sinclair et al., 1998) with only *Enhancer of Polycomb* showing strong suppression and *Additional sex combs* showing strong enhancement of PEV. Thus it appears that, while there may be some very limited interaction between these silencing phenomena, for the most part, they represent two distinct silencing mechanisms in the cell.

Isolation and Characterization of Dominant Mutations Affecting PEV

During the course of investigating PEV over the years, several spontaneous mutations were identified that dominantly modified the variegating phenotype (Spofford, 1976). The fact that single site modifiers of PEV could be isolated, coupled with the relationship between PEV and chromatin structure, led several labs to undertake large scale genetic screens to isolate and identify dominant mutations that either suppress, Su(var)s or enhance, E(var)s, the variegating phenotype. The hope was that such screens would identify factors involved in chromatin structure and shed light, not only on the mechanism underlying PEV but provide some insight into gene regulation generally. A large number of single site modifiers have been described (Locke et al., 1988; Mottus, 1983; Reuter and Wolff, 1981; Sinclair et al., 1983) and over 40 E(var)s and more than 140 Su(var)s are currently listed on Flybase (http://flybase.bio.indiana.edu:82/). This number closely matches the number of genes of these classes that were predicted to exist from duplication/deficiencies studies of the *Drosophila* genome (Locke et al., 1988; Reuter et al., 1987; Reuter and Spierer, 1992; Wustmann et al., 1989). Although most have been recombinationally mapped the vast majority of these mutations have not been cloned. This is despite numerous attempts to use conventional P

element gene tagging to clone Su(var)s (Locke et al., 1988; Tschiersch et al., 1994). Only a single gene, *Su(var)3-9* has been cloned using this method. The reasons for this have not been clear. During the course of these screens numerous new Su(var) mutations were recovered, but with the exception of *Su(var)3-9*, none of them contained P elements. Analysis of the newly induced mutations indicated they were duplications or deficiencies (Locke et al., 1988), suggesting that P elements were indeed transposing into genes that were involved in PEV, but it was only when they improperly excised, that a visible mutation was created. The inability to clone these genes has been a major impediment to advancing our understanding of PEV.

Since there are a large number of mutations that modify PEV and only a few have been cloned and characterized, I will limit my review to those that have been cloned and characterized to the extent that their role in silencing has been partly elucidated. In addition, most of the research in this area has concentrated on the Su(var) class of mutations. This was a reflection of opinion about the mechanism of PEV. The evidence overwhelmingly indicates that PEV is a silencing phenomenon related to the new association with heterochromatin. Accordingly, mutations that disrupt the formation of heterochromatin would be expected to decrease silencing and therefore be Su(var)s. The E(var) class of mutations were thought to represent mutations in factors that affect the function or structure of euchromatic loci. This might include a variety of transcription factors (both general and specific) as well as transcription associated regulatory proteins. Obviously these factors are interesting in their own right but would not shed any light directly on the silencing associated with PEV. This view may have been short-sighted. The recent cloning of *E(var)93D*,

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also known as *mod(mdg4)*, is a case in point. It encodes a factor involved in the regulation of numerous genes and is believed to act at euchromatic boundary elements where it functions in maintaining an open chromatin conformation (Gerasimova and Corces, 2001). The failure of this protein to act at a boundary element may allow silencing complexes to spread more efficiently from the heterochromatic breakpoint and thereby enhance PEV. Thus E(var)s and Su(var)s may work antagonistically and thus a complete understanding of PEV will require characterization of both classes of modifiers.

Approximately a dozen Su(var)s have now been cloned but, for most, their involvement in PEV has remained unclear. Accordingly, the remainder of this review will focus on several Su(var) genes that have been cloned and for which a potential role in PEV has been determined. The first Su(var) cloned was *Su(var)3-7*. A small deficiency, that only removed two coding regions, enabled Rueter et al. (1990) to clone the gene using germ-line transformation. It codes for a peptide of 932 amino acids with 7 widely spaced, atypical zinc fingers (Cleard et al., 1995). Domain analysis of SU(VAR)3-7 revealed the protein consists of two complementary domains. The N-terminal domain, that contains the seven atypical zinc fingers, confers DNA binding with a preference for the repeat sequences of satellite DNA located in pericentric heterochromatin (Cleard and Spierer, 2001). The C-terminal portion of the protein promotes dimerization through a BESS motif (Jaquet et al., 2002). Immunoprecipitation studies suggest that SU(VAR)3-7 interacts with heterochromatic protein 1 (HP1, see below), although this interaction is probably indirect (Jaquet et al., 2002) and the domain required for the interaction has not been characterized.

Immunostaining of *Drosophila* polytene chromosomes reveals that SU(VAR)3-7 is primarily associated with pericentric heterochromatin, although a few euchromatic sites are also detected by immunostaining (Delattre et al., 2000). This staining pattern is almost identical to that observed for HP1 (James et al., 1989). The similar staining pattern and the immunoprecipitation studies suggest SU(VAR)3-7 and HP1 may be acting together in the silencing that occurs in PEV. Given the DNA binding preference of SU(VAR)3-7 for pericentric satellite sequences, it is tempting to speculate that SU(VAR)3-7 binds to heterochromatin and recruits other proteins, including HP1, to establish a silencing complex. However, the failure to demonstrate a direct interaction between HP1 and SU(VAR)3-7 (Jaquet et al., 2002) suggests other proteins must exist that bridge the gap between SU(VAR)3-7 and HP1.

The best characterized suppressor of PEV is heterochromatic protein 1, HP1, originally identified in *Drosophila* (Eissenberg and Elgin, 2000; James and Elgin, 1986). *HP1* was cloned by raising antibodies to proteins enriched in heterochromatin and, using reverse genetics, the gene was localized to band 29A, a site where the suppressors, *Su(var)205* (Mottus, 1983; Sinclair et al., 1983) and *Su(var)2-5* (Reuter and Wolff, 1981), had been independently localized. Subsequent analysis demonstrated the mutations were in the same gene and had created single base pair substitutions in HP1 (James and Elgin, 1986). HP1 homologues have been identified in organisms from yeast to humans. Most organisms have three closely related HP1-like proteins coded for by different genes. For example, HP1a, b, and c are found in *Drosophila*, and HP1α, β and γ in humans. *S. pombe*, on the other hand, appears to have only one member, *Swi6* (Eissenberg and Elgin, 2000). All contain an amino-terminal chromo

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domain, a hinge region and a carboxy-terminal chromo shadow domain. The chromo domain is an approximately 44 amino acid region shared by the POLYCOMB protein in *Drosophila* (Paro and Hogness, 1991). Domain swapping studies (Messmer et al., 1992; Platero et al., 1999) implicated the chromo domain in protein-protein interactions, however, more recent studies have shown the chromo domains of Swi6, HP1a and M31 (HP1α from mouse) are capable of recognizing the tail of histone H3 but only when it is methylated at lysine 9 (H3 mK9) (Bannister et al., 2001; Jacobs et al., 2001; Lachner et al., 2001; Nakayama et al., 2001; Nielsen et al., 2001). Interestingly, the protein responsible for methylating H3 K9 is SU(VAR)3-9 (see below) and SU(VAR)3-9 interacts with both HP1 and SU(VAR)3-7 (Schotta et al., 2002).

The hinge and chromo shadow domains appear to be responsible for the chromosomal targeting of the HP1 family (Smothers and Henikoff, 2000) to different locations on the chromosome. Studies in humans and mouse suggest HP1α and β are enriched in heterochromatin while HP1γ is found exclusively in euchromatin (Horsley et al., 1996; Minc et al., 1999). These studies were extended and confirmed by Smothers and Henikoff (2001) who created antibodies specific for each of the family members. They demonstrated that, in *Drosophila*, HP1c is localized exclusively to euchromatin, HP1b is found in both heterochromatin and euchromatin and HP1a, the founding member, localizes primarily to heterochromatin. In domain swapping experiments they went on to show the HP1a hinge and chromo shadow domains can separately target heterochromatin, while the HP1c chromo shadow domain exclusively targets euchromatin. Thus, although the chromo domain is capable of binding to H3

mK9, this binding capacity is not responsible for localizing the HP1 proteins. The targeting function of the HP1 family members is contained in the hinge and chromo shadow domains (Smothers and Henikoff, 2000).

Several groups, employing a wide variety of techniques, have identified more than 40 proteins that interact with HP1. The proteins are from almost all aspects of chromosomal metabolism including: transcriptional regulation/chromatin modifying proteins; DNA replication and repair; nuclear architecture; and, other chromosome-associated proteins (Li et al., 2002). Thus it appears the HP1 family of proteins are involved in a wide variety of processes in the nucleus which may account for the presence of three different *HP1* genes. What then is its function with respect to silencing and PEV? No mutations are available for HP1b and c, however deficiency studies indicate that absence of these HP1 family members does not have any dominant effect on PEV (Greg Doheney, personal communication). In contrast a duplication for HP1a enhances PEV while a deficiency suppresses (Wustmann et al., 1989) and, as noted above, the only mutations that affect PEV have been recovered in HP1a, despite extensive screening, (Locke et al., 1988; Mottus, 1983; Reuter and Wolff, 1981; Sinclair et al., 1983). This suggests that only HP1a is involved in the silencing associated with PEV.

Immunostaining studies with a monoclonal antibody for HP1 demonstrated HP1 is associated with the euchromatin silenced due to PEV. This association was abolished in *HP1* mutants (Belyaeva et al., 1993). As noted above, an euchromatic region subject to silencing due to PEV, displays a reduced accessibility to nucleases. However, in strains bearing an HP1 mutation that suppresses PEV, the region shows increased accessibility to

nuclease attack (Cryderman et al., 1998). These experiments suggest HP1a is acting directly at the variegating locus and participates in creating a heterochromatic environment that is more compact and less accessible to chromosomal proteins, like transcription factors. This has led to the notion that HP1a is a "bifunctional cross-linker, perhaps organizing higher order chromatin structure by linking or anchoring chromatin subunits" (Eissenberg and Elgin, 2000). There is now additional evidence that this may indeed be the case. HP1 interacts with SU(VAR)3-7 and SU(VAR)3-9 (Schotta et al., 2002) and the chromo domain of HP1 binds H3 mK9.

The only Su(var) that has been cloned in a screen for P element-induced mutations is *Su(var)3-9* (Tschiersch et al., 1994). Analysis of the protein revealed that it contained a chromo domain, similar to the ones found in HP1 and POLYCOMB. In addition, it contains a SET domain so called because it was found in SU(VAR)3-9 and two other known chromatin proteins, ENHANCER OF ZESTE and TRITHORAX, (Tschiersch et al., 1994). The SET domain has now been found in a number of chromatin proteins (Jenuwein et al., 1998). SU(VAR)3-9 is highly conserved in homologues from yeast to humans as is its distribution pattern in the nucleus. In all organisms examined SU(VAR)3-9 is associated primarily with heterochromatin. In *Drosophila* it is found at the chromocenter and in a banded pattern at the largely heterochromatic fourth chromosome (Schotta et al., 2002). In *S. pombe* it is associated with the regions flanking the centromere and with the silent mating locus (Hall et al., 2002; Nakayama et al., 2001; Noma et al., 2001) and in mice at the pericentric heterochromatin (Peters et al., 2001). However, its localization is not exclusively heterochromatic and it has been shown to localize to and

regulate euchromatic genes in *Drosophila* (Hwang et al., 2001; Ner et al., 2002) and human cell lines (Nielsen et al., 2001).

It has been shown in a number of organisms that SU(VAR)3-9, and its homologues, are the primary enzymes responsible for the methylation of lysine 9 on histone H3 (H3 mK9) (Rea et al., 2000) which creates a site for HP1 binding. *S. pombe* and *Drosophila* have a single gene, *clr4* and *Su(var)3-9* respectively (Noma et al., 2001; Schotta et al., 2002), while in mice and humans there are two very closely related genes, *SUV39H1* and *SUV39H2* (Nielsen et al., 2001; Peters et al., 2001). Single knock-outs of the *S. pombe* gene, and double knock-outs of the mammalian genes, leads to chromosome instability and mitotic defects. Surprisingly, null mutations of *Drosophila Su(var)3-9* are homozygous viable and show no segregation defects (Tschiersch et al., 1994). Perhaps, in *Drosophila*, there may be a second, as yet unidentified, *Su(var)3-9*-like gene. However, in *Su(var)3-9* null mutations, H3 mK9 at the chromocenter is severely reduced suggesting this is the major histone methyltransferase (HMT) specific for H3 K9 (Schotta et al., 2002).

Mutational analysis in *Drosophila* and *S. pombe* has confirmed the SET domain is responsible for SU(VAR)3-9's HMT activity (Nakayama et al., 2001; Schotta et al., 2002) while the chromo domain, the SET domain and the cysteine-rich domain adjacent to the SET domain all participate in heterochromatic targeting to H3 (Rea et al., 2000; Schotta et al., 2002).

As noted above SU(VAR)3-9 associates with HP1 and SU(VAR)3-7. The domain that interacts with both proteins maps to the amino terminus of HP1 in *Drosophila* (Schotta et al., 2002). These interaction studies were done in the yeast dihybrid system suggesting a direct interaction between SU(VAR)3-9,

HP1 and SU(VAR)3-7. However, this result has not been confirmed employing a more direct technique, such as GST pull-down assays. In addition to these associations, immunoprecipitation of SU(VAR)3-9 from embryo extracts in *Drosophila* also precipitated an histone deacetylase activity which proved to be the histone deacetylase, HDAC1. However, SU(VAR)3-9-GST fusions did not precipitate HDAC1 suggesting the association between these proteins is indirect (Czermin et al., 2001). Interestingly, the activity of Clr4, the SU(VAR)3-9 homologue in *S. pombe*, is dependent on the activities of two HDACs, Clr3 and Clr6. Clr6 is the *S. pombe* homologue of HDAC1 and Clr3 is the homologue of the *S. cerevisiae* histone deacetylase, Hda1 (Nakayama et al., 2001). Thus it appears that SU(VAR)3-9 is in a complex with two other suppressors of PEV and with one or perhaps two histone deacetylase enzymes.

While SU(VAR)3-7 has not been shown to have homologues outside *Drosophila*, HP1 and SU(VAR)3-9 are conserved from yeast to humans. In *S. pombe* the roles of and relationships between these proteins has been analyzed in considerable detail. In *S. pombe* the silent mating-type region occupies about twenty kilobases of DNA and has many of the characteristics of heterochromatin. The histone tails are hypoacetylated, the DNA shows reduced accessibility to nucleases, recombination is suppressed and reporter genes inserted into the region are silenced (Nakayama et al., 2001; Noma et al., 2001). The region includes the *mat2* and *mat3* loci and an interval between them known as the *K*-region. Mutational analysis had identified several genes which, when mutated, resulted in expression of a reporter gene that had been silenced because it was inserted in the mating-type region. These genes included HDACs, *clr3* and *clr6*, the *Su(var)3-9* homologue, *clr4*, and the *HP1* homologue,

swi6, (Grewal et al., 1998). Several landmark papers in the past two years have employed mutational studies and X-ChIP analysis to provide evidence for a model that explains, not only how genes are silenced when they are in or near heterochromatin, but offers an explanation about how this on/off decision is established and then passed on by way of an epigenetic mark (Hall et al., 2002; Nakayama et al., 2001; Noma et al., 2001).

The model proposes that a requirement for heterochromatin (and heterochromatic silencing) in *S. Pombe* is that H3 K9 be methylated by Clr4, the SU(VAR)3-9 homologue. However, if, on the H3 tail, K 14 or K 9 is acetylated, H3 K9 methylation is inhibited. Accordingly, a necessary step in the process is deacetylation of K14 by Clr3, the Hda1 homologue and deacetylation of K9 by Clr6, the HDAC1 homologue. At this point, Clr4 is recruited to the *cenH* repeats, located in the *K*-region, and Clr4 methylates H3 K9. Concomitantly, either directly or with other partners, Clr4 recruits Swi6 that binds to H3 mK9 through its chromo domain. This recruitment is reciprocal and localization of Clr4 and Swi6 are mutually dependent. This provides the basics for the heterochromatic structure and it spreads from the *cenh* repeats until it reaches specialized boundary elements that prevent the silencing from spreading into the adjacent euchromatin. The mutual recruitment of Clr4 and Swi6 was demonstrated by removing the boundary elements. When Clr4 was overexpressed, it spread into the adjacent euchromatin as did Swi6. When Swi6 was overexpressed it spread into the neighbouring euchromatin, as did Clr4 (Noma et al., 2001).

While silencing of a reporter gene is complete when it is inserted at most places in the mating-type region, when a reporter gene replaces the *cenH*

repeats in the *K*-region it displays a metastable state, the reporter can be on or off, and this decision is stable through mitosis and meiosis. This may be analogous to the early decision made regarding a variegating locus in PEV (see above). Accordingly, in both cases, there must be a mechanism that passes the decision on to daughter cells. Several groups have suggested this epigenetic mark is simply methylation of K9 on H3 (Czermin et al., 2001; Richards and Elgin, 2002; Schotta et al., 2002; Turner, 2002). However, Hall et al., (2002) provide compelling evidence that both Clr4 and Swi6 are required for accurate maintenance of the decision and suggest the epigenetic mark also requires the function of both proteins.

Hall et al. (2002) then went on to ask how the initial decision was made in *S. Pombe*. Recent work in their lab had shown that the formation of heterochromatin at centromeric repeats required a functional RNA interference (RNAi) system (Volpe et al., 2002). They extended this analysis by examining the effect of deletion of any one of three genes involved in RNAi on silencing of a reporter gene introduced at the mating-type region. Repression of the reporter gene was abolished in the deletion strains and the mating-type region could not recruit and/or maintain Swi6 and methylation at H3 K9. However, if they used genetic crosses to introduce a reporter gene, from a wild-type strain in which silencing had been established, into strains bearing mutations in the RNAi genes, silencing remained intact. Thus the RNAi genes were not required to maintain repression once it had been established but perhaps were required to initiate formation of heterochromatin. To confirm this result they treated strains bearing a reporter inserted in the mating-type region with Trichostatin A (TSA), a potent inhibitor of histone deacetylases. Exposure to TSA had

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previously been shown to erase the silencing of genes repressed by insertion into centromeric heterochromatin (Ekwall et al., 1997) or the mating-type region (Grewal et al., 1998). After exposure to TSA for ten generations, silencing of the reporter in wild-type strains and in strains bearing deletions for the RNAi genes was abolished. They removed the TSA and allowed the cells to grow for another 10 generations. In the wild-type cells, silencing was completely reestablished. However, in the RNAi deletion strains only a few cell were able to reestablish silencing. They speculate that the cen*H* region, a region which can recruit Clr4 in an HDAC dependent manner, produces transcripts that are processed by RNAi. The processed transcripts are required to recruit HDACs and Clr4 to the *mat* locus to initiate the formation of heterochromatin.

How does this model relate to PEV? The parallels are obvious and compelling and several groups have suggested the model outlined above, with minor variations, is applicable to PEV and perhaps to the formation of heterochromatin in general (Czermin et al., 2001; Richards and Elgin, 2002; Schotta et al., 2002; Turner, 2002). I have taken the liberty of extending the model to accommodate more recent work. The unusual zinc fingers of SU(VAR)3-7 bind DNA with a preference for the satellite sequences found in pericentric heterochromatin. Accordingly, the zinc fingers of SU(VAR)3-7 target it, and the complex that contains it, to the centromeric heterochromatin. That complex contains HP1, SU(VAR)3-9 and one or more HDACs. The HDACs deacetylate the histone tails creating the hypoacetylated tails found in heterochromatin and clearing the way for methylation of H3 K9 by SU(VAR)3-9. This creates a binding site for HP1 and some or all members of this complex initiate the formation of heterochromatin and propagate it along the

chromosome into the euchromatin, silencing any gene in its path. The process continues until it reaches some as yet unknown boundary element or until the components of the complex become limiting. This initial spreading occurs in *Drosophila* at blastoderm formation when heterochromatin first forms. Whether initiation involves RNA molecules processed by the RNAi system has yet to be examined in published work. However, preliminary results with a mutation in one of the components of the RNAi system, *argonaute*, indicates it is a strong suppressor of PEV (S. Ner, personal communication). Once the extent of the initial spreading is established at blastoderm, the decision is passed on by an epigenetic mark, either methylation of H3 K9, the presence of SU(VAR)3-9, the presence of HP1 or a combination of some or all of these potential markers.

This model for PEV is highly speculative, but does make some predictions that are testable. One prediction is that one or more HDACs should be involved in establishing and maintaining the silencing associated with PEV. This prediction is also suggested by the observation that PEV is strongly suppressed when flies are grown on media supplemented with butyrate, a potent inhibitor of some HDACs (Mottus, 1979; Mottus, 1983).

This thesis presents the cloning and characterization of the *Drosophila HDAC1* gene and provides evidence that it is directly involved in the silencing associated with PEV. The starting point for this analysis was the cloning and characterization of a set of strong Su(var) mutations that formed a single complementation group that had been identified in our original screen for dominant suppressors of PEV (Mottus, 1983; Sinclair et al., 1983). In order to do so, I developed a new method for cloning essential Su(var) mutations in *Drosophila* which should be of widespread utility. As noted above, the gene I

cloned with this technique was the *Drosophila HDAC1* gene. This data is presented in Chapter 2 and has been published (Mottus et al., 2000).

The HDACs in eukaryotes are a surprisingly large group of proteins. Taunton et al. (1996) were the first to isolate and characterize a mammalian histone deacetylase, HDAC1 and showed that it had a high degree of homology to a well-known transcriptional repressor in *S. cerevisiae*, Rpd3. This discovery paved the way for the identification of a large number of proteins with histone deacetylase activity that have been divided into three classes: Class I HDACs, which include HDAC1, that are homologous to Rpd3; Class II HDACs, that are homologous to the *S. cerevisiae* HDAC, Hda1; and, Class III HDACs, that are homologous to Sir2, an NAD dependent HDAC first identified in *S. cerevisiae*. Class I and Class II HDACs are related in sequence but the Sir2-like HDACs do not show any strong relatedness to the other classes, demonstrating that at least two distinct mechanisms have evolved to deacetylate histones. This leaves open the possibility there may be additional classes of proteins with the ability to deacetylate histones that have yet to be identified.

In most eukaryotes there are multiple members in each class, for example, in humans five Class I and six Class II HDACs are known (Hook et al., 2002). In *D. melanogaster*, three Class I HDACs (HDAC1 aka RPD3, HDAC3 and *CG10899*), two Class II HDACs (HDAC4 and HDAC6), and five Class III HDACs (SIR2, *CG5085, CG11305, CG3187,* and *CG6284*) have been identified based on sequence similarity and listed on Flybase (http://flybase.bio.indiana.edu:82/). An obvious question is why would such a diversity of HDACs have evolved? If a "histone code" does indeed exist, it might require a large number of enzymes that target specific residues or

specific histones. Alternatively, nuclear HDACs are always found in large multi-protein complexes and the HDACs could be promiscuous with targeting directed by other members of the complex. These alternatives are not mutually exclusive and evidence exists that a combination of the two may be at work. In *S. cerevisiae*, X-Chip studies, with antibodies to acetylated lysine residues, have shown that Rpd3 is responsible for deacetylating all four core histones (Kadosh and Struhl, 1998; Rundlett et al., 1998; Suka et al., 2001) indicating it is relatively promiscuous. Similar studies with Hda1 suggest its activity is restricted to histones H3 and H2B (Wu et al., 2001).

The subject of this study, HDAC1, is a Class I HDAC. In most cases, Class I HDACs are 500-600 amino acids in length, share a similar structure and are related by their sequence similarity to *S. cerevisiae*, Rpd3. The amino-terminal 200 - 300 amino acids are highly conserved and similar to Rpd3. This region contains the domain required for histone deacetylation. The carboxy-terminal halves of the proteins do not share a high degree of sequence similarity and it is presumed they are involved in protein/protein interactions with members of the HDAC complexes (Khochbin and Wolffe, 1997).

HDAC1 is a transcriptional repressor used by a wide variety of cellular systems (for more details see Chapter 2) and therefore it is possible that mutations in this gene suppress PEV indirectly. Chapter 3 presents evidence that HDAC1 is not normally associated with the *white⁺* gene of *Drosophila*. However, when the *white⁺* gene is subject to silencing due to PEV, HDAC1 is present in abundance at the *white⁺* gene regulatory regions. This association is abolished in the mutations that suppress PEV. I interpret these results to mean

that HDAC1 is acting directly at the variegating locus and plays an integral role in maintaining the gene silencing observed in PEV.

Chapter 2

Mutational Analysis of a Histone Deacetylase in *Drosophila melanogaster:*

Missense Mutations Suppress Gene Silencing Associated with

Position Effect Variegation

The following Chapter is essentially the same as that published under the same

title:

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Introduction

The basic unit of chromatin is the nucleosome which consists of approximately 146 bp of DNA wrapped around the four core histones arranged in an octamer. The amino terminal tails of the histones, in particular H3 and H4, are highly conserved and contain four lysine (K) residues which can be reversibly acetylated (Felsenfeld, 1996; Workman and Kingston, 1998). It was first noted over 30 years ago that there is a correlation between acetylation of histones and transcriptional activity or the potential for transcriptional activity (Allfrey et al., 1964), but the significance of this observation has only become apparent in recent years.

It has now been demonstrated that some transcriptional activators and members of the transcriptional machinery, including GCN5 (Brownell et al., 1996; Wang et al., 1997), PCAF (Yang et al., 1996b), p300/CBP (Ogryzko et al., 1996) and $TAF_{II}230/250$ (Mizzen et al., 1996) are capable of acetylating H3 and H4 both *in vitro* and *in vivo*. These histone acetyl transferases (HATs) are members of large protein complexes which are targeted to the genes they regulate by members of the complex which have DNA binding activity (Grant et al., 1997).

Conversely, histone hypoacetylation is generally correlated with transcriptional inactivity, telomeric and centromeric heterochromatin and silenced areas of the genome such as the donor mating-type loci in yeast (Turner, 1998; Workman and Kingston, 1998). As is the case with the HATs, histone deacetylases (HDACs) also exist as members of large multi-protein complexes. However, an unexpected finding was that some HDAC complexes,

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in both yeast (Rundlett et al., 1996) and mammals (Hassig et al., 1998), contain more than one deacetylase, suggesting that each deacetylase may have a specific target and that full repression may require the activity of more than one HDAC (Kuo and Allis, 1998). The HDACs isolated thus far do not appear to have any DNA binding activity and therefore targeting of the HDAC complexes to the genes they regulate appears to depend on association with DNA binding co-repressor proteins, such as MAD (Laherty et al., 1997), UME6 (Kadosh and Struhl, 1997), YY1 (Yang et al., 1996a), SMRT (Nagy et al., 1997), N-CoR (Alland et al., 1997; Heinzel et al., 1997) and RB (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998), that have the ability to bind to specific target loci.

It has been suggested that acetylation of the lysines in the N-terminal tails of the histones may function by opening up chromatin structure because it eliminates positive charges which may reduce nucleosome/DNA or nucleosome/nucleosome interactions (Workman and Kingston, 1998). Accordingly, one would predict that mutations in HATs or members of their complexes, should result in reduced histone acetylation, and thus impair gene activation (Grunstein, 1997). This prediction appears to be true. GCN5 was first identified as a transcriptional activator before its HAT function was elucidated because mutations in the gene reduce activation of target loci. Conversely, mutations in an HDAC or members of its complex, should impair deacetylation of the histones at target genes, and thus result in de-repression of the targets. This also appeared to be the case. Mutational analysis in *S. cerevisiae* identified RPD3 as a global repressor before its function in histone deacetylation was known.

However, a number of unexpected observations have been documented in *HDAC* null mutations. In *RPD3* deletion lines of *S. cerevisiae*, a small subset of genes were more strongly repressed rather than activated. In addition, careful analysis of the genes normally subject to regulation by *RPD3* demonstrated that when they were activated in the *RPD3* null strains, the level of transcription of target genes was lower than in wild-type strains (Vidal and Gaber, 1991). Finally, the straightforward prediction that mutations in *HDACs* would result in de-repression of silenced genes was confounded when it was reported, in both *S. cerevisiae* and *Drosophila*, that mutations which reduced or eliminated a histone deacetylase resulted in transcriptional silencing of genes subject to telomeric and heterochromatic position effect variegation (De Rubertis et al., 1996).

Position effect variegation (PEV) most often occurs when a chromosomal rearrangement abuts a normally euchromatic region of a chromosome, containing active genes, to a breakpoint in centromeric heterochromatin (Grigliatti, 1991; Henikoff, 1992; Reuter and Spierer, 1992). In tissues where the relocated euchromatic genes are usually active, some cells express the genes normally, whereas in neighboring cells, the genes are transcriptionally silent, resulting in a mosaic pattern of gene expression. An analogous situation is thought to occur in the phenomenon of telomeric position effects (TPEV). This occurs when a reporter gene is inserted in or near to the heterochromatin of the telomeres of *S. cerevisiae* chromosomes (De Rubertis et al., 1996; Grewal et al., 1998; Grunstein, 1998). In some cells the reporter is transcriptionally silent while in others the gene is transcribed normally. In both systems there is a correlation between position relative to the heterochromatic material and

silencing. In TPEV and PEV the likelihood of silencing is dependent on how close the reporter is to the telomere or centromeric heterochromatin respectively; if inserted closer it is more often silent. Mosaic gene expression in both cases is believed to reflect differences in chromatin structure; when the gene is active, it is packaged normally, however, when the gene is inactive, it is packaged more like heterochromatin and is therefore transcriptionally silent.

Here we report the isolation and characterization of six new mutations in the *HDAC1* gene of *D. melanogaster*. This is the first instance of a mutational analysis of an HDAC in a multi-cellular eukaryotic organism. In contrast to previous findings, we report that specific mis-sense mutations in the structural gene of *HDAC1* suppress silencing and increase the expression of a w^+ gene subject to PEV. We propose that these mis-sense mutations are acting as anti-morphic mutations that poison the deacetylase complex, without eliminating it, and that this in turn causes hyperacetylation of histones and activation of genes normally subject to silencing as a result of PEV. Furthermore, we show that null, or very severe hypomorphic mutations, have no significant effect on PEV. We further propose that the unexpected observations noted above in the *RPD3* deletion strains in *S. cerevisiae*, the P insertion line in *D. melanogaster* and the phenotypes of our mis-sense, hypomorphic and null mutations can be explained by a model based on the observations that HDAC1, and its homologues, are members of a structurally related, multi-domain family of proteins which forms part of a large multi-protein complex. Finally, we argue that this model will be relevant in a wide variety of biological applications and as such suggests a need for the isolation and characterization of dominant mutations.

MATERIAL AND METHODS

Fly Stocks

Flies were reared on standard Drosophila media at 22°. Genetic markers used here are described in the text or can be found in Lindsley and Zimm (1992). The putative histone deacetylase described herein has a high level of sequence similarity to RPD3 from *S. cerevisiae* and HDAC1 from humans and other mammals (De Rubertis et al., 1996). The initial report and some subsequent reports (for example (Mannervik and Levine, 1999)) regarding the *Drosophila* histone deacetylase relied on the similarity to the yeast gene and called the *Drosophila* homologue an RPD3-like deacetylase or the RPD3 homologue. In yeast, RPD3 (for reduced potassium dependency 3) was named prior to the discovery that it has histone deacetylase activity and describes only one of the phenotypes associated with lesions in the gene (Vidal and Gaber, 1991). For this reason we prefer the mammalian nomenclature: HDAC, for histone deacetylase, followed by a number indicating to which, of the several similar deacetylases that exist in each organism, it is most similar (Taunton et al., 1996). Accordingly, since the Drosophila deacetylase described here has the highest degree of similarity to HDAC1 from mammals we prefer the name Drosophila HDAC1 for Drosophila histone deacetylase one and will use that nomenclature in this manuscript

The HDAC1 mutations that suppress PEV (hereafter called the Su(var) HDAC1s) described here were induced in a previously described ethyl methane sulfonate screen for dominant suppressors of PEV (Sinclair et al., 1983). The mutations are maintained in stocks balanced over *TM3 Sb Ser* or *TM6 Tb*. *Tb*

was employed because it allows one to readily identify homozygous mutant larvae by the morphology of their spiracles. Late third instar homozygous mutant larvae were selected from cultures and used to obtain the DNA sequence of *HDAC1* in the various mutant strains.

P element insertion strains were obtained from the Bloomington Stock Center and were screened for lethality with the Su(var) HDAC1s. One insert line, 1(3)04556 (hereafter called P-UTR), was almost completely lethal under normal culture conditions with all member of the Su(var) HDAC1 group. However, significant numbers of male and female adults could be reared to adulthood if the fly cultures were uncrowded and the media supplemented with live yeast but survivors are sterile and die within a few days.

The Su(var) HDAC1 group was originally localized because all members failed to complement a small deficiency, *Df(3L)GN24*, Since, in addition to many other loci, this deficiency completely removes the *HDAC1* gene it was employed in the lethal phase analysis as a null allele. Males of the constitution w^{m4} / Y ; *Df(3L)GN24* / + were generated by crossing w^{m4} / w^{m4} ; + /+ females to + / Y ; *Df(3L)GN24* / *TM3 Sb Ser* males. F1 males bearing the deficiency chromosome were collected and crossed to 5-7 day old virgin females of each of the various mutant HDAC1 strains and allowed to lay eggs on petri plates overlaid with an agar, vinegar and ethanol mixture supplemented with live yeast. Eggs were collected by washing with dH_2O, batches of approximately 100 eggs were counted out on construction paper and placed in shell vials. A minimum of five shell vials was set up for each mutant strain. The construction paper was removed after three days and the number of

unhatched eggs counted. Unhatched eggs that failed to darken were considered unfertilized and subtracted from the total number of eggs. Eggs that darkened, but failed to hatch, were scored as embryonic lethals. The number of animals reaching pupation and adulthood were counted and the lethality at each developmental stage determined from the totals. In all cases the results of each group were pooled. In these crosses, the only animals expected to die were those that carried the mutant HDAC1 allele and Df(3L)GN24. All other genotypes were expected to survive. We did not observe any flies that survived and bore a mutant HDAC1 allele and DF(3L)GN24. These flies would have been readily identifiable because of the suite of defects observed in homozygous HDAC1 mutant lines (see Results).

In the recombination experiment in which we tried to separate the lethal lesion in *HDAC1* in the HDAC1^{328} strain from a possible second site suppressor of PEV, the female parents were produced by crossing w^{m4} / w^{m4} ; +/+ females to w^{m4} / Y ; HDAC1^{328} / TM3 Sb Ser males. Virgin F1 females of the constitution, w^{m4} / w^{m4} ; HDAC1^{328} / + , were collected and crossed to w^{m4} / Y ; P-UTR / TM3 Sb Ser males. All flies that displayed suppression of w^{m4} variegation were progeny tested to determine whether they were recombinants or rare surviving HDAC1^{328} / P-UTR flies.

Determination of the level of variegation

To determine the levels of variegated gene expression in the w^{m4} and bw^{vDe2} strains, eye pigment assays were performed employing previously published techniques (Sinclair et al., 1983) and the amount of eye pigment

observed in the variegating strain expressed as a percentage of the amount observed in the wild-type strain, Oregon-R. The level of variegation in the Sb^V strain was determined by assaying the percentage of fourteen bristles displaying a Sb phenotype as previously described (Sinclair et al., 1983).

Remobilization of the P element

The P element in the P-UTR strain carries the ry^+ gene and therefore excision of all or part of the P element can monitored by loss of ry^+. The P element in the P-UTR strain was remobilized by crossing + / + ; *P-UTR / TM3 Sb Ser* females to w^{m4} / Y ; *Ly / TM3 ry^{RK} Sb e P[ry$^+$ Δ2-3]* males. The *TM3 ry^{RK} Sb e P[ry$^+$ Δ2-3]* chromosome carries a P element transposase source (Δ2-3) which is required to remobilize the defective P element in the P-UTR strain. The F1 + / Y ; *P-UTR / TM3 ry^{RK} Sb e P[ry$^+$ Δ2-3]* males were collected and crossed to + / + ; ry^{506} / ry^{506} females and ry^- F2 males collected and stocks established.

DNA manipulations

All standard DNA manipulations were performed as described in SAMBROOK *et al.* (1989).

Plasmid rescue of the DNA surrounding the insertion of the P element in P-UTR was performed according to previously published techniques (Karpen and Spradling, 1992).

Genomic DNA for sequencing from each of the HDAC1 mutant strains was obtained from cultures in which the HDAC1 mutation is balanced over the

TM6 Tb balancer chromosome (see above). Homozygous mutant late third instar larvae were collected and the DNA isolated by standard protocols. Specific fragments of *HDAC1* were amplified using *Pfu* polymerase and primers which were designed from the published sequence of *HDAC1*. The PCR products were gel purified and sequenced employing dye terminators in an automated sequencing facility (UBC NAPS Unit).

Isolation and Analysis of RNA

Total RNA was isolated from either adult females or adult males of each strain using the TRIzol® Reagent according to manufacturers instructions (Life Technologies). Poly (A) RNA was subsequently isolated employing the Oligotex mRNA Mini Kit produced by Qiagen following the manufacturers instructions. Approximately 1.5 μg of poly (A) RNA for each gender and strain was separated on a formaldehyde agarose denaturing gel prepared according to the protocol provided by Qiagen in the Oligotex Mini Kit. The gels were run at 7 V/cm, transferred to nylon membranes according to the manufacturer's instructions (Amersham) and probed with DNA labeled with [^{32}P]dATP using Boehringer Mannheim's Random Primed DNA labeling kit. The DNA probe for the *HDAC1* mRNA was prepared by PCR employing *Pfu* polymerase and primers for the carboyxl terminal coding regions generated from a cloned cDNA kindly provided to us by Pierre Spierer's laboratory. The relative amounts of poly(A)RNA loaded in each lane was determined by re-probing the Northern blots with a probe for the mRNA for the ribosomal protein, DUb80 (Mottus et al., 1997). Autoradiograms were scanned into a computer and the

amount of poly(A) RNA in each lane was quantified, relative to DUb80, using

NIH Image (data not shown)

RESULTS

Isolation and characterization of mutations in Drosophila _HDAC1_ that suppress PEV

Several groups, including ours, have conducted large genetic screens to isolate Suppressors of position effect variegation or Su(var)s in _D. melanogaster_ . These screens were based on the assumption that these mutations should identify factors involved in the process of chromatin packaging (Locke et al., 1988; Reuter and Wolff, 1981; Sinclair et al., 1983), either structural components of chromatin or factors that modify chromatin structure. Our screen was designed to isolate dominant Su(var)s by selecting progeny from ethyl methane sulfonate (EMS) mutagenized males in which expression of the w^+ gene in the strain, $In(1)w^{m4}$ (w^{m4}), was significantly increased. In the w^{m4} strain, an inversion juxtaposes the w^+ gene to the centromeric heterochromatin of the X chromosome. This causes the w^+ gene to be transcriptionally inactivated in most pigment cells in the fly's eye and since its product is required for deposition of pigment, the eyes of flies in the w^{m4} strain generally have about 5% to 15% of the wild-type levels of eye pigments. Four of the dominant Su(var) mutations isolated comprise a single complementation group (hereafter referred to collectively as Su(var) HDAC1s or individually as $HDAC1^{303}$, $HDAC1^{313}$, $HDAC1^{326}$ and $HDAC1^{328}$). All are strong dominant suppressors of PEV and, in addition to the dominant phenotype, all four alleles are recessive lethal. In w^{m4} strains bearing the Su(var) HDAC1s, pigments in the eyes of
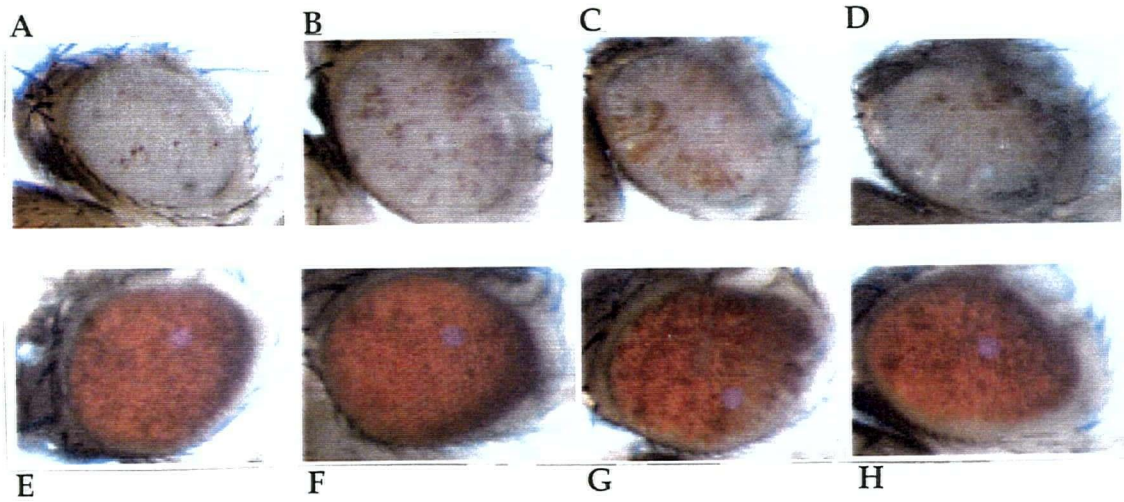
Figure 1 Examples of eyes from male flies bearing the $In(1)w^{m4}$ chromosome and third chromosomes of the following constitutions: (A) $+/+$; (B) $P\text{-}UTR / +$; (C) $HDAC1^{def8} / +$; (D) $HDAC1^{def24} / +$; (E) $HDAC1^{303} / +$; (F) $HDAC^{313}/ +$; (G) $HDAC^{326} / +$ and (H) $HDAC^{328} / +$.

TABLE 1

The effects of selected Su(var) HDAC1 mutations on various genes subject to PEV

Genotype	Sex	w^{m4} [a]	bw^{vDe2} [a]	Sb^v [b]
+ / +	F	8±2	38±24	56±20
	M	13±3	55±15	69±16
$HDAC1^{326}$ / +	F	83±5	55±4	72±22
	M	85±5	55±4	91±9
$HDAC1^{328}$ / +	F	72±8	49±22	72±15
	M	88±6	50±8	93±10

[a] the percentage of eye pigments compared to the amount observed in the wild-type strain, OR-R

[b] the percentage of bristles displaying the Sb phenotype

both males and females are increased from 5-15% to 60-90 % of the pigment levels observed in the wild-type strain, OR-R (Figure 1 and Table 1).

In order to determine whether the effect of these mutations was generally applicable to PEV or specific to the w^+ gene, we monitored the effects of two of the strongest alleles of the Su(var) HDAC1s, $HDAC1^{326}$ and $HDAC1^{328}$, on two other variegating rearrangements: $In(2R)bw^{vDe2}$ (bw^v) which juxtaposes the bw^+ locus to the centromeric heterochromatin of chromosome 2, and; $T(2,3)Sb^v$ (Sb^v) which abuts the dominant third chromosome mutation, Sb^-, to the centromeric heterochromatin of chromosome 2 (Table 1). In females, the mutations caused significant suppression of both bw^v and Sb^v. In males, Sb^v was also strongly suppressed by the mutations, but bw^v was either not affected or somewhat enhanced. Heterogeneity in the response of genes subject to PEV when exposed to suppressor mutations is not uncommon. Each rearrangement abuts the euchromatic variegating gene to a unique region of heterochromatin and therefore a variation in the level of response to trans-acting factors is not unexpected (Lloyd et al., 1997). However, it is clear that although the strength of the suppression of PEV varies, the Su(var) HDAC1s suppress the gene silencing associated with PEV and are not mutations in factors which specifically modify the w^+ gene.

Mapping the Su(var) HDAC1s

We mapped the recessive lethality associated with the Su(var) HDAC1s to 64B17-64C13-15 employing deficiencies and confirmed the Su(var)

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phenotype recombinationally mapped to approximately the same location in all four mutant lines. This placed the complementation group very close to a recently cloned *RPD3*-like *HDAC* (De Rubertis et al., 1996). This Drosophila *HDAC*(hereafter referred to as *HDAC1*) was cloned as a result of a P insertion 1.8 kb 5' to the gene which causes strong dominant enhancement of PEV, but is homozygous viable and fertile. However, complementation analysis with the Su(var) HDAC1s and the P insert line (hereafter referred to as P-1.8) revealed all combinations were viable and fertile (data not shown) suggesting that perhaps P-1.8 and the Su(var) HDAC1s represented two different genes.

We then crossed the Su(var) HDAC1s to a series of recessive lethal, modified P inserts generated by the Berkeley Drosophila Genome Database and localized to the 64B-64C region. The Su(var) HDAC1s were almost completely lethal when heterozygous with the P insert line, l(3)04556 (hereafter called P-UTR). Plasmid rescue of the genomic DNA surrounding the insertion point of the P element revealed it had inserted into the 5' UTR of *HDAC1* (Figure 2). Surprisingly, while P-UTR is homozygous lethal and lethal with the Su(var) HDAC1s, it has no dominant effect on variegation of w^+ in the $In(1)w^{m4}$ strain (Figure 1 and Table 4). Since P-UTR had an insertion into *HDAC1*, but did not have a dominant affect on PEV, this raised the possibility that the P-UTR strain contained a second site mutation that was causing the lethality with the Su(var) HDAC1s. Alternatively, it was possible that the Su(var) HDAC1s, in addition to a recessive lethal lesion in *HDAC1*, carried a second site mutation that was causing the dominant Su(var) phenotype. We addressed these possibilities in two ways.
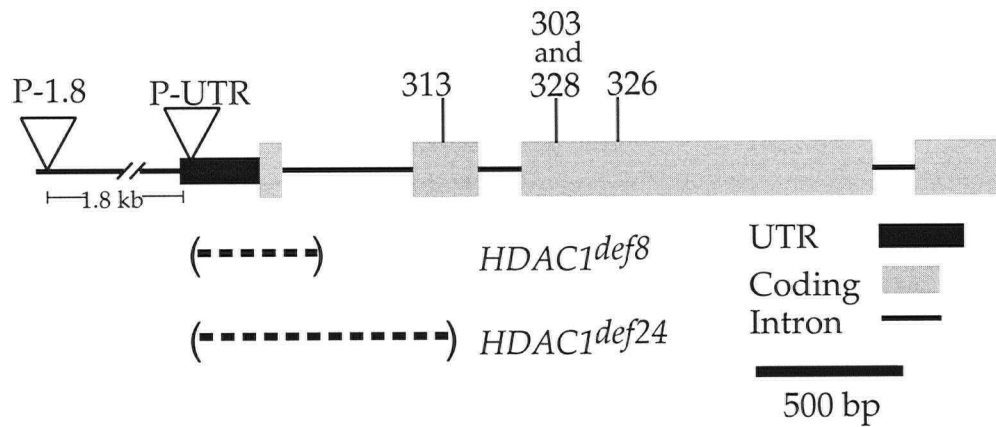
Figure 2 Genomic organization of the *HDAC1* gene. The approximate
location of each of the *Su(var) HDAC1* group mutatations is shown,
the insertion points of the *P* elements in the *P-1.8* and *P-UTR*
strains are indicated by the triangles, and the dashed line indicate
the extent of the deficiencies in the deletions strains.

First, in order to determine whether P-UTR also contained a second site lethal mutation, we generated revertants of P-UTR by remobilizing the P element, which is marked with ry^+, and recovering males that were ry^-. We recovered 25 ry^- revertants. Four of the revertants are homozygous viable, viable as heterozygotes with P-UTR and viable as heterozygotes with all members of the Su(var) HDAC1s. Subsequent analysis by PCR indicated that three of the revertants are precise excisions of the·P element while the fourth retains a small piece of the P element. Since a precise or nearly precise excision of the P element insertion results in a homozygous viable chromosome, the only lethal lesion on the P-UTR chromosome is caused by the insertion of the P element into *HDAC1* and therefore the Su(var) HDAC1s also have a lethal lesion in the *HDAC1* gene.

Second, in order to determine whether the Su(var) HDAC1s, in addition to the lethal lesion in *HDAC1*, carried a dominant second site Su(var) mutation, we tried to separate the lethal phenotype from the Su(var) phenotype by recombination. The cross is outlined in Figure 3 and is based on the observations that: (1) $HDAC1^{328}$ is almost completely lethal when heterozygous with P-UTR, and; (2) P-UTR does not have any dominant effect on PEV. Accordingly, any flies that survive and displayed suppression of w^{m4} variegation would be the result of a recombination event between the lethal lesion in *HDAC1* and the putative second site Su(var). We scored 6125 recombinants but we were unable to separate the lethal phenotype from the Su(var) phenotype. Accordingly, if the lethality and the dominant Su(var) phenotypes are caused by different mutations, then these mutations are less

$$\frac{dHDAC1^{328}}{\text{wild-type}} \;\;♀♀\;\; \text{X} \;\; \frac{\text{P-UTR}}{\text{TM3 Sb Ser}} \;\; ♂♂$$

Figure 3   The cross employed to attempt to generate a recombinant between the lethal lesion in the histone deacetylase gene in the $HDAC1^{328}$ strain and a possible second site suppressor of position effect variegation. In the F_1, $HDAC1^{328}$ / P-UTR is almost completely lethal. All non-Sb Ser flies were examined for suppression of PEV. Suppressed flies are either rare $HDAC^{328}$ / P-UTR survivors or represent potential recombinants between a possible second site suppressor of PEV and $HDAC1^{328}$. Despite examining >6000 recombinant chromosomes, we did not isolate a second site suppressor of PEV

than 1.6×10^{-2} map units apart, a distance representing approximately 4 to 7 kb of DNA in a typical region of the *Drosophila* genome (Lefevre, 1976). Since transcript analysis has shown that there are no other transcripts within approximately 8 kb of the 3' end or 12 kb of the 5' end of *HDAC1* (De Rubertis et al., 1996), it is unlikely that the lethality and the Su(var) phenotype are caused by separate mutations.

Based on the results of the reversion experiments with P-UTR and the failure to separate the lethal lesion and the Su(var) phenotypes by recombination, we conclude that both phenotypes are the result of lesions in *HDAC1*.

Mutant phenotypes associated with lesion in *HDAC1*

During the course of the recombination experiment we observed that some *P-UTR/HDAC1^{328}* adult male flies did eclose but only survived for a few days. These animals displayed very strong suppression of PEV and several other phenotypes. In order to further examine these phenotypes we generated *HDAC1^{303}/P-UTR* flies. In this cross, under carefully maintained culture conditions, adult males eclosed at approximately 40 % of expected and females at approximately 30 % of expected. Both sexes only survived for several days and the females produced a small number of eggs which appeared to be unfertilized. These animals displayed a suite of defects including: very strong suppression of w^{m4}; wings that were severely notched; bristles that were smaller, malformed, often curved and duplicated; alula that were larger than normal; and, a reduction in the number of sex combs on the legs of the males

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from a mean of 10.7± 0.9 to a mean of 7.7± 1.0. This suggests that mutations in the histone deacetylase, *HDAC1*, cause defects in a variety of cellular systems, a phenotype that is consistent with its proposed role as a global transcriptional regulator. It also suggests that the Su(var) HDAC1s retain at least some of their functions, since P-UTR is lethal when homozygous, yet appreciable numbers of adults can be recovered when P-UTR is heterozygous with members of the Su(var) HDAC1s.

Since P-UTR and the Su(var) HDAC1s are recessive lethal it appears that *HDAC1* function is essential for survival in *D. melanogaster*, unlike in *S. cerevisiae*, where null alleles of the *RPD3* gene are viable but display a suite of phenotypes. In order to further characterize the requirements for *HDAC1* we determined the developmental time at which *HDAC1* is required for survival in *D. melanogaster*. Since P-UTR is a very strong hypomorph (Mannervik and Levine, 1999) and we were unable to determine whether or not the Su(var) HDAC1s are complete null alleles of the gene and residual gene activity would mask the earliest requirement for *HDAC1*, we generated null alleles of *HDAC1* (see below for details). The results of our lethal phase analysis are presented in Table 2. Null alleles (*HDAC1^{def8}* and *HDAC1^{def24}*) of *HDAC1* die during larval stage of life. Surprisingly, inspection of the stock cultures revealed that a large percentage of the homozygous mutant larvae survive until very late in third instar. These larvae were readily identifiable because in the stock cultures the mutations are balanced over *TM6Tb*. Larvae bearing the balancer chromosome can be distinguished from larvae homozygous for the *HDAC1* mutations because *Tb* alters the morphology of the larval spiracles. This suggests three

TABLE 2

Lethal phase analysis of mutations in *HDAC1*. Percentage of animals that die at the indicated developmental stage

Mutant Strain Tested	Embryonic Lethality	Larval Lethality	Pupal Lethality	Male Viability[a]
$HDAC1^{def8}$	6.8	24.7	3.9	93
$HDAC1^{def24}$	6.3	25.9	7.6	91
$HDAC1^{303}$	3.9	13.3	17.0	71
$HDAC1^{313}$	3.4	28.4	2.3	51
$HDAC1^{326}$	2.9	30.7	6.5	88
$HDAC1^{328}$	4.1	16.4	16.8	78

[a] viability of males expected to survive as compared to their female siblings

possible scenarios: 1) maternal HDAC1 is perduring until very late in development; 2) HDAC1 is required during embryogenesis and not required again until late in third instar and maternal HDAC1 provides sufficient activity for this early function; or, 3) HDAC1 is not required for the early stages of Drosophila development. Based on a recently published report investigating the phenotypes associated with P-UTR, we favour the second of the above three possibilities. We also conducted lethal phase analyses of the Su(var) HDAC1s. $HDAC1^{313}$ and $HDAC1^{326}$ also died during the larval period. Inspection of the stock cultures revealed a large number of homozygous mutant larvae at the third instar stage and therefore these alleles cause death at approximately the same time as the null alleles. However, only approximately fifty percent of larvae bearing $HDAC1^{303}$ and $HDAC1^{328}$ died during the larval period while about 50% survived into pupation. This is consistent with the sequencing data (see below) which demonstrated these mutations are caused by identical base pair substitutions. Thus, with regard to lethality, it appears that $HDAC1^{313}$ and $HDAC1^{326}$ are indistinguishable from null alleles while $HDAC1^{303}$ and $HDAC1^{328}$ retain some HDAC1 activity.

An unexpected observation from the lethal phase analysis was that the Su(var) HDAC1s appeared to have a dominant semi-lethal affect on males regardless of their genotype. In the lethal phase analysis, three of the four genotypes produced are expected to survive (see Materials and Methods) and one of the classes (+ / TM3) does not carry any chromosomes with a mutation in HDAC1. In the crosses with the null alleles, $HDAC1^{def8}$ and $HDAC1^{def24}$, males and females in the classes expected to live, appear in approximately the

same numbers (Table 2). However, in the Su(var) HDAC1 crosses, males of genotypes expected to survive, including males that have completely wild-type *HDAC1* genes survived at significantly lower rates than expected. For example males in the cross involving $HDAC1^{313}$ only survived at about 50% the level of their genotypically identical female siblings in the same cross. Males in crosses involving the other Su(var) HDAC1s also survived at significantly lower levels than females. Since, in these crosses the mothers carried the Su(var) HDAC1 mutations, one explanation for this observation may be that these mutations may be exerting a dominant maternal effect on the dosage compensation mechanism. In Drosophila, dosage compensation occurs as a result of hyper-transcription of the male X chromosome. The male X chromosome adopts a special conformation which is believed to be necessary for enhanced transcription (Bashaw and Baker, 1996). Accordingly, if histone deacetylation is an essential step in establishing the specialized chromatin structure required in the male, the Su(var) HDAC1s may be defective in this process. Alternatively, although most genes on the male X chromosome are transcribed at double the normal rate, there are loci that are not subject to dosage compensation and therefore need to be silenced or repressed on the specialized male X chromosome (Baker et al., 1994). In the Su(var) HDAC1 strains these loci may escape repression resulting in reduced male viability in the sons of mutant mothers.

Sequence analysis of the Su(var) group

EMS-induced changes in the Su(var) complementation group were identified by sequencing the genomic DNA encoding HDAC1 from the four Su(var) lines and from the chromosome which was originally employed in the screen for Su(var) mutations. The results of this analysis are presented in Figure 2 (Accession Num. AF086715). The genomic organization in our strains is slightly different than that presented in the previously published report (De Rubertis et al., 1996). The coding sequence is interrupted by three introns rather than two and the conceptual translation of the protein yields a product of 521 amino acids rather than 520. The extra amino acid is produced at the additional intron/exon boundary in our sequence. DNA sequencing revealed that there is a single amino acid substitution in each of the four mutant lines that suppress PEV. The locations of the amino acid substitutions are indicated in Figure 2. In two of the strains, $HDAC1^{303}$ and $HDAC1^{328}$, we observed identical base pair substitutions. These mutations were recovered from unrelated bottles in the original EMS screen and therefore most likely represent independent events.

Each single nucleotide substitution resulted in changing an amino acid that is not only perfectly conserved in homologues from Yeast and human, but the substitutions are located in regions of the protein that are almost perfectly conserved in these diverse organisms (Table 3). The functions of these particular residues and the regions in which they occur have not yet been determined. However, evolutionary analysis of the deacetylase proteins and some limited mutational analysis suggest that the amino one-half of the protein

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Table 3

Comparison of amino acid substitutions in the Su(var) HDAC1 group with conserved regions in human and yeast homologues.

	HDAC1^{313}	HDAC1^{303} HDAC1^{328}	HDAC1^{326}
	R30C	C98Y	P204S
D. melanogaster	G H P M K P H R I R M	F N V G E D C P V F D G L	S F H K Y G E Y F P G T G
HDAC1 Mutant Strain	- - - - - - - C - - -	- - - - - - Y - - - - - -	- - - - - - - - - S - - -
H. sapiens HDAC1	- - - - - - - R - - -	- - - - - - C - - - - - -	- - - - - - - - - P - - -
S. cerevisiae RPD3	- - - - - - - R - - -	- - - - D - C - - - - - -	- - - - - - - F - P - - -

"-" indicates identity.

is the domain responsible for catalytic activity (Khochbin and Wolffe, 1997). It is interesting to note that each of the substitutions occurs in the region of the protein thought to be required for deacetylase activity.

Interaction with an P-1.8, an E(var) allele

The *HDAC1* locus has previously been cloned as a dominant enhancer of PEV or E(var) (De Rubertis *et al.* 1996). The phenotype results from the insertion of a P element 1.8 kb 5' to the start site of *HDAC1* which reduces or eliminates transcription of the gene in the eye imaginal disk but not in other imaginal disks from the same animals. Surprisingly, heterozygous flies bearing P-1.8 and any one of members of the Su(var) HDAC1s were viable and fertile. In addition, in these heterozygotes, the eyes of flies bearing w^{m4} show a weak to moderate suppression of PEV (data not shown). Since the eyes appear to be normal in these crosses, with the only apparent phenotype being an effect on PEV, and P-1.8 flies are viable as homozygotes, these observations suggest that either *HDAC1* does not perform any essential function in the eye disk or alternatively, that P-1.8 may be a hypomorph.

Generation of null alleles

Work by Mannervik and Levine (1999) and this study (see below) show that P-UTR produces a message at significantly lower levels than wild-type and thus is likely to be a strong hypomorph. As noted above, we were surprised that P-UTR had no effect on PEV since it is lethal when homozygous. One possible explanation is that, although this mutation is a hypomorph which is

lethal as a homozygote, it produces sufficient activity in a heterozygote such that PEV is not affected. If this were the case then one would predict that a null allele of *HDAC1* would have a dominant effect on PEV. Accordingly, we generated null alleles of *HDAC1* by remobilizing the P element in P-UTR to induce deficiencies of the coding regions of the gene as a result of imperfect excisions of the P element. The P element, which is marked with ry^+, was remobilized by crossing P-UTR to the transposase source, *In(3LR)TM3, Δ2-3 Sb* , and recovering males that were ry^-. From 560 potential excision events we recovered 25 ry^- males, 19 of which were still lethal over P-UTR and the Su(var) HDAC1s and therefore represented potential improper excisions. DNA sequence analysis has shown we generated two deficiencies which begin at the insertion point of P-UTR and remove amino terminal coding regions of *HDAC1*; *HDAC1^{def8}* deletes approximately 440 bp and *HDAC1^{def24}* deletes approximately 870 bp (see Figure 2). Conceptual translations from the first seven AUG codons remaining in *HDAC1^{def8}* and the first three start codons of *HDAC1^{def24}* would produce peptides that bear no similarity to HDAC1, and therefore we believe these represent null alleles of the gene. Surprisingly, we found that null alleles of *HDAC1* have no dominant effect on silencing of the w^+ gene in the w^{m4} strain (Figure 1).

Northern Analysis

Since we had generated a variety of mutations in the *HDAC1* locus it was of considerable interest to determine how the mutations affected the level of transcription. Figure 4 shows Northern blots indicating the levels of

transcription of *HDAC1* in the mutant lines identified in this study. To determine the relative loading in each lane, the Northerns were also challenged with a probe for the ribosomal protein, DUb80 (see Materials and Methods). The transcript levels of *HDAC1*[326] are approximately the same as that observed in the wild-type strains indicating that the Su(var) phenotype is not a result of hyper-transcription of the locus. In the P-UTR strain, as is often the case with P insertions, the level of transcription is reduced relative to wild-type levels and therefore P-UTR is likely a hypomorph. This is in accord with the findings of Mannervik and Levine (1999), who showed the maternal contribution of *HDAC1* in the P-UTR strain was approximately five fold less that wild-type. In a strain heterozygous for the deficiency, *HDAC1*[def8], the message produced from the deleted chromosome is reduced in amount and evident as a widening of the 2.2 kb band produced from the non-deleted homologue. In the strain heterozygous for the deficiency, *HDAC1*[def24], two different sized transcripts are clearly visible indicating both homologues are transcribed, but the amount of the smaller transcript produced from the deleted chromosome is very much reduced. Since the smaller transcripts in both deficiency strains are very unlikely to produce functional proteins, we believe these mutations represent null alleles.
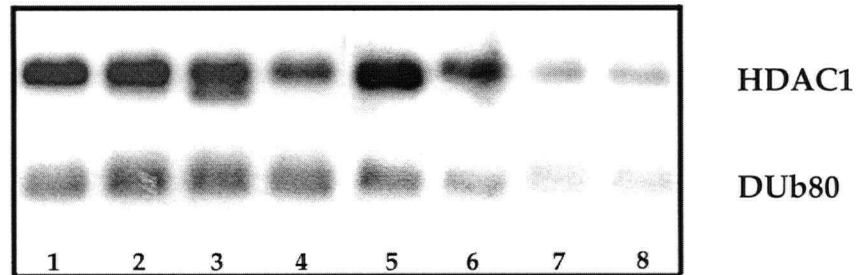
HDAC1

DUb80

1 2 3 4 5 6 7 8

Figure 4 Northern analysis of poly(A) RNA isolated from adults bearing *HDAC1* mutations. Lanes 1 and 6 are from wild-type female controls and lane 7 is from wild-type male controls. Lanes 2-4 are from females of the constitution $HDAC1^{def8}$ / *TM3 Sb Ser*, $HDAC1^{def24}$ / *TM3 Sb Ser* and *P-UTR* / *TM3 Sb Ser*, respectively. The approximate amount of poly(A) RNA loaded in each lane was determined by re-probing the blots with a probe specific for the message for the ribosomal protein DUb80. Loading in lanes 1-4 is approximately equivalent and show that the levels of total HDAC1 message in lanes 1-3 are approximately the same. However, in lane 4 the amount of message is reduced to ~50-60% of Lane 1. Lane 5 contains ~1.6 times the amount of poy(A)RNA as lane 6, and when taken into account, the levels of HDAC1 poly(A)RNA in $HDAC1^{326}$ females (lane 5) and control females (lane 6) are approximately equivalent, as are the amounts in $HDAC1^{326}$ males (lane 8) and control males (lane 7).

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DISCUSSION

In this study we report the isolation and characterization of a number of new mutations in the *D. melanogaster* putative histone deacetylase, *HDAC1*, and test their effects on gene silencing that occurs as a result of PEV. Models of gene regulation, based on the correlation between histone acetylation and gene activity, would predict that mutations in an histone deacetylase gene, which reduce or eliminate histone deacetylase activity, ought to lead to increased levels of histone acetylation which, in turn, would lead to de-repression of silenced genes. Surprisingly, this straightforward prediction was not born out. Instead the effect on gene silencing is dependent upon the nature of the mutation in *HDAC1* (for summary see Table 4).

How then can one explain the apparently contradictory affects on PEV and TPEV of the various kinds of mutations in the histone deacetylase genes in Yeast and Drosophila? It may be that histone deacetylases belong to a growing class of genes which have the following characteristics: 1) they are members of a closely related gene family; 2) they encode multi-domain proteins, and; 3) null mutations have little or no obvious phenotypic effect while point mutants have profound, often dominant effects. One recent example of this class of genes in lower eukaryotes is the *FUS3/KSS1* gene pair of *S. cerevisiae*. Normally, these closely related proteins function in separate pathways. Single deletion strains of either gene are still proficient for mating because when Fus3p is deleted, and only when it is deleted, Kss1p acts as an impostor and replaces Fus3p. However, deletion of both proteins renders the strain sterile (Madhani and Fink, 1998; Madhani et al., 1997). Examples of this class of gene is certainly not

TABLE 4

Summary of the effects of various mutations in *HDAC1* on viability and PEV in *D. melanogaster*

| Mutation[a]	Dominant Effect on PEV	Homozygous Viability	Heterozygous with Su(var) HDAC1s
P-1.8 P element insert 1.8 kb 5' to gene	enhancer	viable	moderate suppression of PEV
P-UTR P element insert into the 5' UTR	no effect	lethal	strong semi-lethal; in rare survivors, PEV strongly suppressed
Su(var) HDAC1s point mutants	strong suppressors	lethal	lethal
HDAC1def8	no effect	lethal	lethal
HDAC1def24 Deletions	no effect	lethal	lethal

[a] for a complete description of mutations see text

limited to lower eukaryotes. For example, gene knockout experiments in mice have revealed a surprising number of genes in which the phenotype of the homozygous null mutation is either not detectable or very minor. A cursory examination of the Mouse Knockout Database (http://www.biomednet.com/db/mkmd) identifies at least 13 such genes. In contrast to the mild phenotypes of knockout alleles, analysis of mutations in some of these genes has shown that point mutations can have very profound, often dominant effects. One example is the SRC oncogene, a member of a closely related family of proteins. The knockout causes only minor dental abnormalities, yet almost all known point mutations have severe phenotypic consequences, including cancer (Lowell and Soriano, 1996).

Recently a model has been proposed to account for the maintenance of closely related gene families during evolution (Gibson and Spring, 1998). By extending this model we believe we can provide an explanation for these apparently contradictory observations regarding relatively benign knockout/null mutations and dominant point mutations which have severe phenotypic consequences. It is now apparent that most, if not all, of the biological activities in the cell are carried out by large, multi-protein complexes. A single type of complex may have multiple targets or functions that are dependent on the specific members of the complex at a particular time during the cell cycle or at a particular location in the cell. If one of the proteins of the complex is absent, as in a null mutation, and that protein is a member of a closely related family, then another member(s) of the family may substitute for the missing protein. Since they are closely related, the impostor can provide partial activity and, as a consequence, a null mutation may have no obvious

phenotype. In contrast, point mutations that only alter a single domain, may allow the aberrant protein to be incorporated into its complex(es). In cases in which the mutation occurs in a domain required for a specific function, the complex would then be completely inactive for that particular function. Accordingly, a point mutation may have a dominant negative effect and display a much more severe phenotype than a null mutation (see Figure 5).

This model may accommodate our observations of the various Drosophila *HDAC1* mutations. In eukaryotes, the HDACs are a closely related family of proteins that form complexes with other proteins including other HDACs. For example in Yeast, two different HDACs, RPD3 and HDA1, have been isolated and characterized, and sequence analysis of the yeast genome suggests there may be at least three additional HDACs. Two large multi-protein complexes, HDA and HDB, containing histone deacetylase activity have been isolated and analysis of HDA has shown that it contains at least two HDACs (Carmen et al., 1996; Rundlett et al., 1996). Similarly, in mammals, five different HDACs have been identified and a complex containing the human RPD3-like deacetylase, HDAC1, also contains HDAC2 (Hassig et al., 1998). In Drosophila, two more HDACs has now been identified, *HDAC2* and *HDAC3* (Johnson et al., 1998; Mannervik and Levine, 1999). It seems likely that more candidate deacetylases will be identified as the genome sequencing projects proceed. Accordingly, the biochemical and sequence analysis of HDACs in Yeast and mammals suggest that HDACs are members of a related gene family and, more importantly to our model, function as members of large protein complexes.

A.
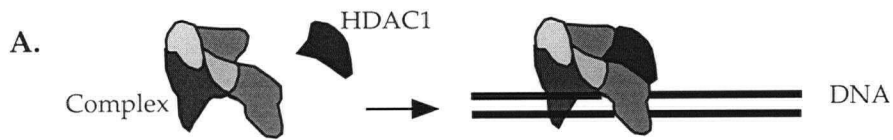
Normally HDAC1 participates in a complex which is targeted to DNA by other members of the complex

B
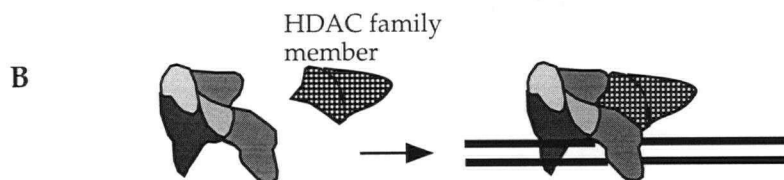
However, when HDAC1 is absent, another member of the HDAC family binds to the complex, is targeted correctly to the DNA but produces an aberrant pattern of histone deacetylation

C
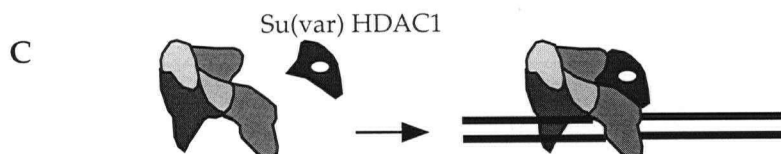
A Su(var) HDAC1 mutation, which has only a single amino acid change, will occupy its normal place in the complex and be targeted correctly, however the Su(var) HDAC1 will be unable to deacetylate its target histone residues leading to increased acetylation and suppression of PEV.

Figure 5 A model to explain the various phenotypes associated with mutations in HDAC1, see text for details.

The foregoing provides the framework for a model that may explain the apparently contradictory results observed with different kinds of mutations in this histone deacetylase and their effects on PEV and TPEV. In the *RPD3* null mutation in yeast, TPEV is enhanced, i.e., the expression of the reporter gene is repressed. We postulate that in the absence of RPD3, other HDACs, with differing specificities, substitute for RPD3 in the multi-protein complex resulting in an incorrect histone deacetylation pattern. The phenotypic consequence of the incorrect deacetylation pattern is enhancement of TPEV, possibly due to excess deacetylation at the site of the reporter gene by the impostor deacetylase. Substitution by other HDACs has also been suggested by other authors to account for the residual repression observed in *RPD3* deletion strains (Kadosh and Struhl, 1998). In Drosophila the only mutation in *HDAC1* that enhances PEV is *P-1.8*, an insertion of a P element 1.8 kb 5' to the coding region. *In situ* hybridization with a probe for the *HDAC1* mRNA demonstrates that, in the eye disk, transcription of *HDAC1* is markedly reduced or absent but in the leg disk the *HDAC1* transcript accumulates to normal levels. One possible explanation for this observation is that the P element has inserted into an eye disk specific enhancer element resulting in little or no transcription in the eye disk. Thus, *HDAC1* may be effectively absent in the eye disk. In its absence, other HDACs could substitute for *HDAC1* producing an incorrect deacetylation pattern, the consequence of which is enhancement of PEV. In contrast, the Su(var) HDAC1s described here are capable of producing a protein with only a single amino acid change in which a specific function has likely been compromised, possibly the deacetylase activity. Since only a single amino acid has been changed, the protein would still associate with its complex, bind

its other components efficiently and be targeted to the correct site. However, the complex would be unable to deacetylate its target histones leading to hyperacetylation and decreased silencing. In this way a point mutation would act as a dominant negative mutation and would suppress PEV. On the other hand, null mutations, such as the deficiencies described here, have no observable affect on PEV because in heterozygotes, wild-type *HDAC1*, produced from the non-deleted homologue, can associate normally with the histone deacetylase complexes. The other HDACs can only substitute for *HDAC1* in its complete absence as is the case with Kss1p and Fus3p in Yeast described above.

This model relies on the supposition that an aberrant form of *HDAC1* is being produced in the Su(var) HDAC1 strains. We believe such a protein is made for the following reasons. First, conceptual translation of the protein produces a full length product with only a single amino acid change. Second, when we crossed the members of the Su(var) HDAC1s to P-1.8, the strain bearing the P element insertion 1.8 kb 5' to the *HDAC1* gene, flies bearing both mutations were viable and fertile and showed a weak to moderate suppression of PEV. Since the P insert line is effectively a null in the eye disk, we interpret the suppression observed in the heterozygotes as evidence that the Su(var) HDAC1s are producing a product. Third, in the complementation and recombination studies, heterozygotes bearing both the P-UTR chromosome and the Su(var) HDAC1s survived at an appreciable frequency. In these flies, PEV in the In(1)w^{m4} strain was very strongly suppressed and the eyes were virtually indistinguishable from wild-type strains. Since P-UTR is lethal as a

homozygote and this lethality is only associated with lesion in *HDAC1*, the observation that such flies survive suggests that the Su(var) HDAC1s are producing a product which retains sufficient activity in the essential function of *HDAC1* to rescue the lethality associated with the P-UTR chromosome. Finally, the observation that the Su(var) HDAC1s displayed a dominant maternal effect reduction in the viability of males, regardless of their phenotype, a reduction which was not observed in crosses with the deficiency strains, implies that the Su(var) HDACs are producing a protein product since this maternal effect observed is not seen in the absence of any product.

The model may also serve to explain other apparently anomalous observations in Yeast strains bearing null mutations in *RPD3*. The gene was first identified as a transcriptional repressor in *S. cerevisiae* because mutations in the gene resulted in de-repression of the majority of genes it regulated. Surprisingly, further analysis of the mutant strains has shown that target genes are also defective in the degree to which they respond to activators and repressors. Regulated genes cannot be activated as fully, nor repressed as completely, as in the wild-type strain (Vidal and Gaber, 1991). Since RPD3 forms part of a histone deacetylase complex, we propose that in the absence of RPD3, other HDACs may fill in resulting in aberrant deacetylation patterns at target genes. Aberrant deacetylation patterns may result in de-repression of most target genes, but would provide less than optimal conditions for transcription in the presence of an activator and would be leaky in the presence of a repressor. Conversely, in some chromosomal contexts, recruitment of the wrong deacetylase may result in an aberrant deacetylation pattern that represses transcription.

Finally, we emphasize that the mutations described here were recovered in a genetic screen for dominant suppressors of PEV. Therefore the single amino acid changes that we recovered may identify domains in the Drosophila HDAC1 that are important for silencing in heterochromatin rather than abolishing all deacetylase activity. In any case, since the domains are conserved in Yeast, site-directed mutagenesis should provide a direct test of the proposed model.

One of the traditional genetic approaches to determining protein function has been to generate null mutations and then examine the organism for phenotypic defects which can be correlated with the null phenotype. In fact, this is the basis for creating the knockout mutations in mice as potential models for human syndromes. It is now apparent that most, if not all biological functions in eukaryotic cells occur as a result of the action of protein complexes and not individual proteins. If the foregoing model is of general applicability then this traditional approach must be applied with caution. If the protein under scrutiny is a member of a gene family then in the absence of that protein, other family member may "fill in" and provide partial, or even complete, rescue (under laboratory conditions) of the functions compromised by the null mutation. In that case, this type of analysis will be compromised and the role of the protein being investigation under appreciated. A more fruitful strategy may be to create dominant mutations, in the best case caused by very small alterations in the protein such as a single amino acid substitution, which will act in a dominant negative fashion and direct our attention to the possible many roles a protein may have because of its membership in one or more multi-protein machines.

Chapter 3

Chromatin Immunoprecipitation Analysis of a Region subject to

Position Effect Variegation in *Drosophila melanogaster*

Introduction

One of the major challenges inherent in any mutational analysis of a gene is determining whether the phenotypes observed are a direct result of the defect in the gene, or whether they are indirect, and a downstream consequence of the mutation altering other metabolic pathways that impinge on and modify the phenotype in question. This is especially important when one is investigating mutations in a gene that is a known, or suspected, general regulator of transcription. HDAC1 falls into the class of known general regulators of transcription and functions as an essential member of several repressor complexes (see Chapter 2). In addition, there is some evidence, and a growing suspicion, that this view is too simplistic. Rather than only being a repressor, HDAC1 may also be an integral part of the system that controls the transcription rate of some active genes (see below, (Breiling et al., 2001)). Since HDAC1 regulates a large number of loci, an obvious and important question is whether the mutations that suppress PEV are acting directly or indirectly to abrogate the silencing normally observed at the variegating locus.

Several methods are currently employed to address this type of question. The oldest method is staining polytene chromosomes with an antibody specific for the subject protein. In the salivary glands of *Drosophila*, and specific tissues of most *Dipterans*, the chromosomes undergo many rounds of endoreduplication. The chromosome arms remain associated and form giant polytene chromosomes with distinct banding patterns that are visible under the light microscope. Thus, by examining well-spread chromosome preparations that have been challenged with an antibody for a particular protein, one can

determine the particular band or interband with which the antibody and hence the protein is associated. This technique has been invaluable in identifying the region or regions where a protein is localized, however, the technique does have limitations. Obviously, if the epitope, to which the antibody reacts, is hidden, which may be the case in some protein/protein or chromatin complexes, the antibody will fail to identify that location of the protein. However, the major limitation of this technique is its resolution. As noted above, one can only determine if an antibody is staining a band or an interband. Only a few studies have attempted to determine how many genes are contained in bands or interbands and have found the number varies considerably, some bands or interbands have high gene densities while others contain only a few genes (Friedman et al., 1991; Hall et al., 1983; Spierer et al., 1983). Thus, one cannot use this technique to determine whether a protein is associated with a specific gene, or whether it is bound at the regulatory or coding regions of that gene.

Pile and Wasserman (2000) used antibody staining of polytene chromosomes to ask where HDAC1 was located in the *D. melanogaster* genome. They found the anti-HDAC1 antibody bound throughout the euchromatic regions of the genome, primarily in the interband, less condensed, regions of euchromatin. Somewhat surprisingly, it did not bind to either $\alpha-$ or $\beta-$ heterochromatin. They also stained the same preparations with an antibody to DNA polymerase II to mark active genes. The pattern of staining did not overlap with anti-HDAC1 staining. These observations led them to conclude that HDAC1's primary function was the repression of genes located in the less DNA dense, interband regions of euchromatin, and that it did not play a role in

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condensing the more compact DNA structures found in the bands or heterochromatic regions of the genome. Some doubts regarding these conclusions have been raised by the application of a newer, more sensitive, technique (see below). In addition, examination of their polytene preparations stained with anti-HDAC1 reveal a large, intensely staining band at the base of each chromosome arm (see Figure 2 of Pile and Wasserman (2000)) immediately adjacent to the chromocenter. In strains subject to PEV this arrangement is altered by the formation of an illicit heterochromatic/euchromatic junction that may create novel HDAC1 binding sites or perhaps the cellular response to such an illicit junction is to recruit HDAC1. Unfortunately, the distribution of HDAC1 in a strain subject to PEV was not determined.

Recently, two techniques have been developed which offer much better resolution. One relies on the ability of the DNA methyl transferase (DAM) from *E. coli* to methylate adenine in the DNA sequence GATC (van Steensel et al., 2001; van Steensel and Henikoff, 2000). This residue is not normally methylated in eukaryotes. The DAM protein is fused to the protein of interest and transformed into tissue culture cells or whole organisms and the DNA at a specific location is analyzed with restriction enzymes that recognize GATC and are either sensitive or insensitive to adenine methylation. Differences in digestion patterns between controls containing the DAM protein alone and cells transformed with the DAM-fusion imply the protein under study is directing the fusion protein to that particular region. The DAM-fusion methylates GATC over a 2500 base pair region thus providing enhanced resolution over the staining of polytene chromosomes with antibodies. However, this technique still has limitations. In some cases, in gene poor regions of chromosomes, one

may be able to conclude, with confidence, that the subject protein is associated with a particular gene. However, in other regions, the genes are very tightly packed and the results may be ambiguous regarding which of two, or perhaps three, genes the protein is associated. Obviously, its resolution is further limited by the existence of GATC sites that can be methylated. Statistically, these sites should occur every 256 base pairs however, in some sites in the genome, these sites may occur rarely. Another drawback is that, in some cases in *Drosophila*, especially with some chromatin proteins, it has been difficult or impossible to obtain germ-line transformants of the fusion proteins. For example, while GAGA, dSIR2 (van Steensel et al., 2001) and SU(VAR)3-9 (S. Ner, personal communication) DAM-fusion transformants have been successfully recovered, only one transformant of an HP1 fusion was obtained despite several thousand attempts (van Steensel, personal communication). I have injected over 10,000 *Drosophila* embryos in an attempt to generate germ-line transformants of either a carboxy or amino-terminal DAM fusion of HDAC1 without obtaining a single transformant (unpublished observations). However, if a transformant can be produced, then a valuable resource is available to quickly assess whether the protein associates with potential new targets as they come under scrutiny. Somewhat surprisingly, this technique has been little used since its was initially reported (van Steensel and Henikoff, 2000). Perhaps the difficulty in obtaining germ-line transformants is greater than it appears or the limits of its resolution have discouraged its use when a technique with superior resolution is available.

The technique most widely used to ask whether a protein is associated with a specific DNA sequence is formaldehyde cross-linked Chromatin

Immuno-Precipitation (X-ChIP). This technique has been used in eukaryotic cells, from the yeasts, *S. cerevisiae* (Hecht et al., 1996) and *S. pombe* (Noma et al., 2001), to a variety of tissue culture cells from various higher organisms (Nielsen et al., 2001). It has also been successfully applied to *Drosophila* embryos prior to about 16 hours of development. After 16 hours the ability of the formaldehyde to penetrate the embryo and cross-link the proteins and DNA drops precipitously (Cavalli et al., 1999; Orlando and Paro, 1993).

X-ChIP is based on the assumption that formaldehyde can rapidly penetrate the nucleus of a cell or small organism and cross-link the proteins and DNA with minimal disruption of the normal distribution patterns of the proteins in the nucleus. Following cross-linking with formaldehyde, the DNA is sheared by sonication into fragments of an average size of between 500 and 1000 base pairs. The protein/DNA complexes are precipitated with an antibody specific for a particular protein, the cross-links reversed and the DNA that was precipitated analyzed either by PCR or Southern Blots. There are two further assumptions upon which this technique is based: one, that all regions of the DNA are equally susceptible to shearing by sonication after fixation with formaldehyde; and two, that the epitope the antibody recognizes is available to bind the antibody and is not buried in a protein/protein or protein/DNA complex. Failure of either of these assumptions to be true will result in a false negative, either because the region under study is more susceptible to shearing by sonication and therefore is preferentially sheared and eliminated from the analysis or the antibody will not precipitate the protein because the epitope is hidden. However, when this technique does provide a positive indication that

a protein is associated with a specific sequence then this is generally accepted as strong evidence that a protein is indeed associated with the region analyzed.

The resolution of this technique is extremely good when PCR is used to analyze the immuno-precipitated DNA. Employing quantitative PCR and statistical analysis, some have reported resolution down to the level of the nucleosome, about 150 base pairs (Rundlett et al., 1998). However, in most studies, the reports generally employ primers that amplify products of between 200 and 500 base pairs. More recently Real-Time PCR has been employed to analyze the products of X-ChIP. This technique promises to bring the resolution down to under 100 base pairs and produce results that can be quantified precisely since the kinetics of the entire PCR are monitored and quantified accurately during the amplification process (Milne et al., 2002).

X-Chip analysis with an anti-HDAC1 antibody was employed in one study to ask whether HDAC1 was present at variety of promoters and coding regions in *Drosophila* SL-2 cells (Breiling et al., 2001). The report looked at two genes that were expressed, and six genes that were not, in this cell line. As expected, HDAC1 was present at the promoter and the 5' coding regions of all six genes that were not being expressed. Unexpectedly, HDAC1 was also present at the active genes *Abdominal-B* (*Abd-B*) and the locus that codes for the subunit of RNA polymerase II with a relative molecular mass of 140,000 (*RpII140*). However, its distribution appeared to be somewhat different from that observed at repressed loci. In the case of *Abd-B*, HDAC1 appears to be strictly localized to the coding region of the gene and was not found in the promoter regions. The primers employed to analyze *RpII140* overlap the proximal promoter and the 5' coding region and therefore localization strictly to

the coding region was not confirmed, nor was it ruled out. The results appear to confirm HDAC1's role as a general transcriptional repressor. However, the unexpected finding that HDAC1 also associates with active genes led the authors to suggest that perhaps HDAC1 plays a role in the regulation of active genes. HDAC1 may regulate transcription either by acting with histone acetyltransferases (HATs) to modulate the level of histone acetylation or alternatively, to regulate the activity of some of the general transcription factors (GTFs) that are known to be acetylated as well.

In this study I employed X-ChIP to ask whether HDAC1 is associated with specific regions of the *white⁺* gene in *Drosophila* and whether this association is altered when the *white⁺* gene is subject to silencing as a result of PEV. In addition, I asked whether the associations observed were altered by the presence of a mutation in HDAC1 that is a strong suppressor of PEV. The data show that when the *white⁺* locus is silenced due to PEV, HDAC1 is very strongly associated with the 1000 base pair region immediately 5' to the *white⁺* coding region. In addition, a 500 base pair region approximately 6.0 kb 5' to the transcriptional start is also very strongly associated with HDAC1. The increased association with HDAC1 was completely abolished by a mutation in HDAC1 that suppresses PEV and the levels of association return to those observed when *white⁺* was in its normal location. These results suggest that HDAC1 is acting directly at the site of the variegating gene and is an essential part of the silencing mechanism observed in PEV.

Material and Methods

Drosophila Strains

Three strains were analyzed by formaldehyde cross-linked chromatin immuno-precipitation (X-ChIP): _Oregon-R_ (OR-R), a wild-type strain; _In(1)w^{m4}_ (w^{m4}), a strain bearing an X chromosome inversion which variegates for the _w$^+$_ gene; and _In(1)w^{m4} ; HDAC^{326}/TM3 Sb Ser_ (w^{m4} ; 326), a strain bearing the X chromosome inversion and heterozygous for a mutation in _HDAC1_. All mutations are described either in the text or can be found in Lindsley and Zimm (1992).

The flies were reared at 25°C on standard yeast-sucrose-corn meal-agar medium to which a mold inhibitor, Tegosept (methyl-p-hydroxybenzoate), and antibiotics were added. Several thousand flies of the appropriate genotype were added to population cages and embryos for analysis were collected on agar plates supplemented with a paste made from live yeast. In order to eliminate any eggs retained by the females for a prolonged period after fertilization, fresh collection plates were added and the flies allowed to lay eggs for approximately three hours and this first collection was discarded. New plates were added and the flies were allowed to lay eggs for four hours. The plates were removed and held at 25°C for 12 hours and then processed for X-ChIP. Accordingly all X-ChIP experiments were conducted on chromatin from 12 - 16 hour embryos.

Chromatin Immuno-Precipitation (X-ChIP)

Sonication

The protocol I employed for X-ChIP was modified from those of Cavelli et al. (1999) and Mazo (unpublished-provided by Dr. H. Brock with the permission of Dr. Mazo). Approximately 1.0 gram of embryos were dechorionated by washing in 3% NaOCl in Embryo Wash Buffer (EWB, 0.03 % Triton X100, 0.4% NaCl) for three minutes and then extensively washed with EWB. The embryos were transferred to a 50 ml. conical tube and washed once with 0.01% Triton X100 in phosphate-buffered saline (PBS). The PBS was removed and 10 ml. of Cross-linking Solution (1.8% formaldehyde, 50 mM HEPES, 0.5 mM EGTA, 100 mM NaCl pH 8.0) and 30 ml. of heptane were added and vigorously shaken for 15 min. Embryos were pelleted by spinning at 1000 rpm on a tabletop centrifuge and the Cross-linking Solution/heptane was removed. Fifty ml. of Stop Solution (PBS, 0.125 M glycine, 0.01% Triton X100) were added and the tube was briefly shaken. The embryos were allowed to sediment without centrifugation and the Stop Solution removed. Ten ml. of Wash Solution A (10mM HEPES pH 7.6, 10 mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0, 0.25% Triton X100) were added and the embryos were washed for 10 min. on a rotator. Wash Solution A was removed and replaced by 10 ml. of Wash Solution B (10 mM HEPES pH 7.6, 200 mM NaCl, 1 mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0, 0.01% Triton X100) and the embryos were washed for an additional 10 min. on a rotator. The embryos, in Wash Solution B, were transferred to a round bottomed centrifuge tube, allowed to sediment and Wash Solution B removed. Sonication Buffer (10 mM HEPES pH 7.6, 1 mM

EDTA pH 8.0, 0.5 mM EGTA pH 8.0) was added to 5.5 ml. and then 0.5 gm. glass beads (Sigma G8893) and protease inhibitors (1.1 μl of 10 mg/ml Aprotinin, 1.1 μl of 10 mg/ml Leupeptins, 5.5 μl of 1 mg/ml Pepstatin A, 63.2 μl of 50 mM PMSF, 55 μl of 100 mM Benzamidine) were added and the tube placed on ice.

The embryos were sonicated on a Sonic 300 Dismembrator using the microtip at the maximum setting of 35%. The optimal sonication procedure was determined empirically by monitoring the average size of the genomic DNA on agarose gels after a series of 30 sec. sonication pulses. The protocols suggest the optimal average size for genomic DNA for immuno-precipitation is between 500 and 1000 base pairs. I determined that a regimen of six 30 sec. sonications, with a pause of 90 sec. between each pulse, produced genomic DNA with an average size of about 1000 base pairs. Further 30 sec. pulses did not significantly reduce the average size of the DNA. The tube was maintained on ice throughout the procedure. Samples were transferred to 1.5 ml. tubes and centrifuged at 4°C for 15 min. at maximum on a tabletop centrifuge. The supernatants were either processed immediately or flash frozen and stored at -80°C for no more than a few days before being further processed.

Samples were prepared for immuno-precipitation by mixing with an equal volume of 6.0 M Urea and dialyzing at 4°C for 4 hours in 1.0 liter of ChIP Dialysis Buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0, 10% glycerol, 1% Triton X100, 0.1% Na-desoxycholate w/v) supplemented with protease inhibitors (11.5 ml. of 50 mM PMSF and 10.0 ml. of 100 mM Benzamidine). The Dialysis Buffer was replaced with fresh Dialysis Buffer, supplemented with protease inhibitors, and the samples dialyzed overnight at

4°C. The samples were centrifuged at maximum speed on a tabletop centrifuge at 4°C and the supernatants divided into 1.0 ml aliquots and stored at -80°C.

Immuno-precipitation

The sonicated extract was thawed on ice and 100 μl per immuno-precipitation reaction was removed to a 1.5 ml tube and protease inhibitors added (2.0 μl of 50 mM PMSF, 0.1 μl of 1.0 mg/ml Aprotinin, 0.2 μl of 1 mg/ml Pepstatin A). The extract was pre-cleared by adding 10 μl of a PAS/DNA slurry and rotated for 30 min. at 4°C. The PAS/DNA slurry was made by washing 100 mg of protein-A-sepharose beads (PAS) with milli-Q dH$_2$O, removing the water and adding 600 μg of sonicated herring sperm DNA, 0.33 mg/ml of BSA and TE pH 8.0 to make a final volume of 800 μl. After pre-clearing, the extract/PAS/DNA slurry was centrifuged for 1.0 min. at 4000 rpm and the supernatant removed to a new 1.5 ml tube. Five μl of an antibody produced against a peptide identical to the 20 amino acids at the carboxy-terminal tail of *Drosophila* HDAC1 (Abcam Limited, ab1767) were added to an extract from each of the three strains to be tested. In addition control extracts from each strain, to which no antibody was added, were processed. The extracts were rotated overnight at 4°C to allow antibody binding.

The immune complexes were collected by adding 40 μl of PAS/DNA slurry to each reaction, including the no antibody control, and rotating for 2 to 3 hours at 4°C. The beads were pelleted by gentle centrifugation (1000 rpm for 1.0 min.) and the supernatants removed. The supernatant from the no antibody control tube was saved and served as the source for the Input DNA for

subsequent PCR studies. The beads were washed for 5 min. at 4 °C with each of the following buffers:

Low Salt Immune Complex Wash Buffer = 0.1% SDS, 1.0 % Triton X100, 2.0 mM EDTA pH 8.0, 20 mM Tris-HCl pH 8.1, 150 mM NaCl;

High Salt Immune Complex Wash Buffer = 0.1% SDS, 1.0 % Triton X100, 2.0 mM EDTA pH 8.0, 20 mM Tris-HCl pH 8.1, 500 mM NaCl;

LiCl Immune Complex Wash Buffer = 0.25 M LiCl, 1.0% NP40, 1.0 % Na-desoxycholate, 1.0 mM EDTA pH 8.0, 10 mM Tris-HCl pH 8.1;

TE = 10 mM Tris-HCl pH 8.0, 1.0 mM EDTA pH 8.0;

TE = 10 mM Tris-HCl pH 8.0, 1.0 mM EDTA pH 8.0.

After the last wash buffer was removed, 250 μl of freshly made Elution Buffer (1.0% SDS, 0.1 M NaHCO$_3$) was added, the mixture was vortexed briefly, and the immune complexes were eluted by rotating for 15 min. at room temperature. The mixture was centrifuged for 2 min. at 9000 rpm and the supernatant removed to a fresh tube. A second elution was performed with another 250 μl aliquot of Elution Buffer and the eluates combined.

In order to reverse the formaldehyde-induced cross-links, 20 μl of 5 M NaCl was added to each eluate and the tubes incubated at 65°C overnight. The DNA in the samples was ethanol precipitated, with the addition of 20 μg glycogen as a carrier, and resuspended in 50 μl TE pH 8.0.

Polymerase Chain Reaction Analysis (PCR)

The DNA precipitated in the above reactions was analyzed with PCR employing primers pairs for specific regions of the *D. melanogaster* genome 5' to the start of w^+ gene transcription (see Figure 1). The primer pairs for the

Proximal promoter were:

GATTCCGGGGCCTGAGATGAGGTGC/GGTACTTCAAATACCCTTGGATC

G; for the Distal Promoter were:

GTTGTCTGTCACTAGATCGGCCC/GCACCTCATCTCAGGCCCCGGAATC;

and, for the 5' Distal Region were

CGACTCTGCGTCGCTGTCTCG/GTATGCAGCA GAATTAGCAGAAG. Two

microliters of DNA from each sample was amplified according to the following

protocol: Step 1 = 94°C for 1 min., Step 2 = 92°C for 30 sec., Step 3 = 58°C for 30

sec., Step 4 = 75°C for 1 min., Step 5 = repeat Step 2 to Step 4 29 times, Step 6 =

75°C for 5 min. Under these conditions a single band of DNA, approximately

500 base pairs long, was produced by each primer pair.

The amount of DNA produced by PCR was quantified by running

aliquots of each PCR reaction on an agarose gel and staining with SYBR Green 1

according to the manufacturer's instructions (Molecular Probes). SYBR Green 1

is a fluorescent stain that is specific for double-stranded DNA and its signal is

linear to the amount of DNA present in all ranges reported. The amount of

DNA produced after 30 PCR cycles was quantified on a Storm 860 Phospho-

Imager (Amersham Pharmacia Biotech) by excitation at 450 nm and measuring

emission at 520 nm. Initial experiments had determined that, under the PCR

conditions employed here, the DNA produced at 30 cycles was within the linear

range of amplification (see Figure 2). For each fly strain and each primer pair

the amount of DNA produced by PCR in the mock treated, "No Antibody"

control, immuno-precipitation was compared to the amount produced by the

anti-HDAC1 antibody and the results expressed as "fold enhancement".

Results

As reported in Chapter 2, specific amino acid substitutions in *Drosophila* HDAC1 cause strong dominant suppression of *white⁺* gene variegation in the w^{m4} strain. However, since HDAC1 is involved in a large (and growing) number of protein complexes and binds to, and perhaps regulates, a large number of genes, it was of considerable interest to determine whether mutations in *HDAC1* cause suppression by acting directly on the variegating locus or whether suppression is indirect and the result of deregulation of one or more HDAC1-regulated genes.

I addressed this question by employing formaldehyde-cross-linked chromatin immuno-precipitation (X-ChIP). In brief, this technique relies on the ability of formaldehyde to cross-link closely associated proteins and DNA. The cross-linking distance of formaldehyde is essentially zero, since it does not contain a linker, and thus only proteins in very close proximity to the DNA will be cross-linked to it. After treatment with formaldehyde, the cross-linked proteins and DNA are sonicated to shear the DNA into fragments, ideally of an average size of between 500 and 1000 base pairs, which are then challenged with an antibody to the protein of interest. After allowing sufficient time for binding, the antibody/protein/DNA complexes are precipitated by binding the antibody with Protein A linked to agarose beads. Gentle centrifugation allows the entire complex to be precipitated. After extensive washing the cross-links are reversed by heat treatment and the precipitated DNA is analyzed. One invariably includes a mock treated sample ("No Antibody") that was exposed to the Protein A agarose beads alone to provide a baseline for non-specific precipitation of DNA by the protein A coupled to the agarose beads. The

amount of a specific DNA sequence precipitated in the mock treated sample is compared to the amount precipitated by the antibody employing PCR amplification and primers specific for the region of interest. If the protein is bound to the sequence of interest then one should observe a several fold enhancement in the amount of the specific sequence in the antibody treated sample when compared to the mock treated sample. This technique has been very successfully applied to a wide variety of systems, in particular yeast and eukaryotic cell lines, and has revolutionized our understanding of chromatin structure. However, there has been a dearth of reports on its application in multicellular organisms. One of the obvious drawbacks to using multicellular organisms as a substrate for X-ChIP is that a particular protein may be associated with a specific DNA sequence in only one tissue type and even that may occur at only a particular time during development. Accordingly, the number of cells in which the protein of interest is associated with the target sequence may be small and will not produce a strong enough signal to be significantly different from the mock treated sample. Thus, the mosaic nature of epigenetic states in a multicellular organism may render this technique unusable for the analysis of many or most proteins in intact complex organisms.

In the present case I thought I could employ X-ChIP to ask if HDAC1 is associated with the *white*$^+$ gene in w^{m4} because some of the characteristics of PEV suggest the signature variegating expression pattern is caused by a phenomenon occurring throughout the organism and not solely in the tissues in which the variegating gene is expressed. First, in *Drosophila*, clonal analysis of variegation of the *white*$^+$ gene in the eye and temperature shift studies of several genes all point to a very early determinative event in establishing the silencing

associated with PEV (see Chapter 1 for details). While this has only been studied for a few genes, the results consistently point to some event occurring about the time of cellular blastoderm formation, regardless of the loci involved, which is approximately the time heterochromatin first appears in *Drosophila* embryos and the zygotic genome becomes transcriptionally active (Lawrence, 1992). Thus if PEV is the result of "heterochromatinization" of a variegating gene, it may occur in all cells at roughly the same time, around blastoderm formation, and well before the embryos are harvested (12 to 16 hours) in my experiments. Second, microscopy studies have examined the extent of spreading of heterochromatin in the polytene chromosomes of *Drosophila*. There was a strong correlation between the extent of spreading in the polytenes and the proportion of cells in which a variegating gene is expressed (see Chapter 1 for details). This correlation, between polytene chromosome morphology in the salivary glands late 3rd instar larvae and the expression of variegating genes in the cells of the adult, suggests that, once beyond cellular blastoderm, the architecture of the chromatin surrounding the new heterochromatic/euchromatic junction may be the same, or roughly the same, in all cells regardless of tissue type or developmental stage. Third, mutations in *HDAC1* suppress several different variegating genes (see Chapter 2, Table 1). It is possible that mutations in *HDAC1* indirectly suppress each of the other genes via deregulation of a different set of genes, but a more parsimonious explanation is that mutations in *HDAC1* have their effect via a common mechanism that occurs throughout the genome and suppresses these genes regardless of the tissue in which they are expressed. This could be the early

"determinative" event in PEV that clonal analysis and temperature shift studies have identified.

I employed X-ChIP in 12 to 16 hour *Drosophila* embryos and a X-ChIP quality, commercial antibody, specific for *D. melanogaster* HDAC1 (Abcam Limited, ab1767) to ask if HDAC1 is associated with specific regions of the variegating *white*⁺ gene and further, if this association was altered in the *HDAC1* mutations that suppressed PEV. Figure 1 is a diagrammatic representation of the genomic region 5' to the *white*⁺ gene. The *white*⁺ gene promoter has been extensively characterized (Davison et al., 1985; Levis et al., 1985; Pirrotta et al., 1985).

I chose three regions to examine for the presence of HDAC1. The first region, the Proximal Promoter, is the 500 bp immediately 5' to the start of *white*⁺ gene transcription and contains the minimal promoter for the locus. The second region, the Distal Promoter, is the 500 base pairs immediately 5' to the Proximal Promoter. Finally I chose a 500 base pair region about 6.0 kilobases 5' to the transcriptional start of *white*⁺, the 5'Distal Region. I chose the Proximal and Distal Promoter regions because they should show whether HDAC1 associates with the regulatory regions of the *white*⁺ gene when it is subject to PEV. I chose the 5' Distal region because comparative analysis of the three regions might provide some insight into the molecular mechanism of PEV. While the molecular mechanism underlying PEV has not been elucidated, the most enduring theory is that silencing spreads out from the heterochromatic breakpoint by altering the structure of chromatin. If "spreading" involves a continuous structural change in the chromatin emanating out from the breakpoint, then one might expect to see new protein associations, such as with

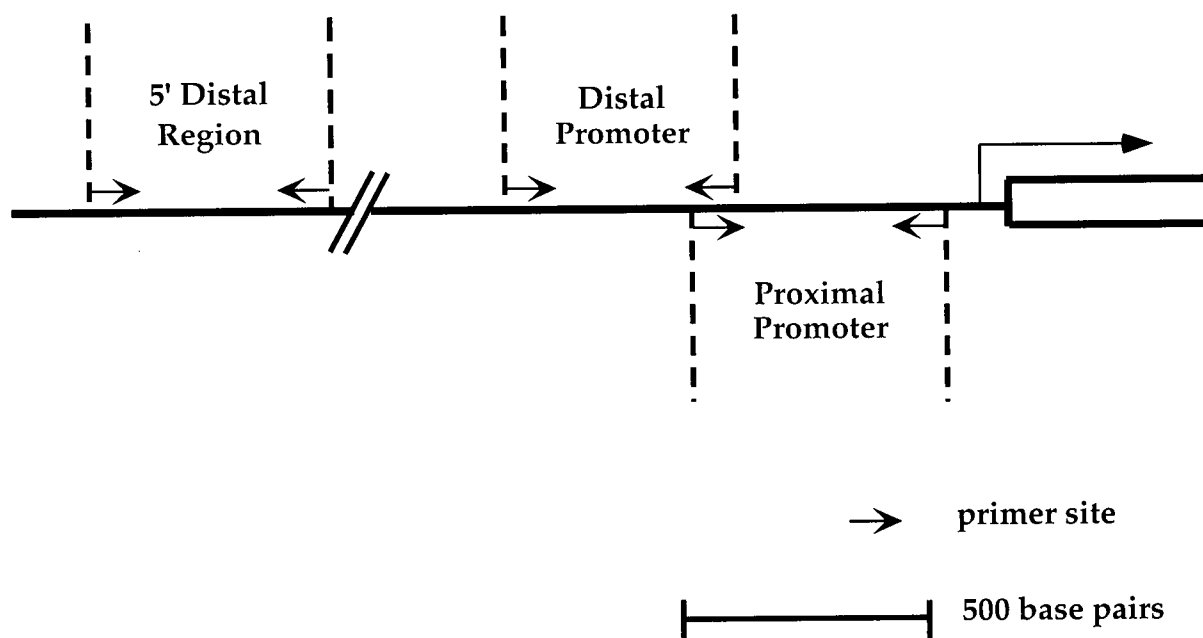**Figure 1.** Diagramatic representation of the genomic region 5' to the w+gene in D. melanogaster. The regions analyzed by X-ChIP and PCR are indicated. The filled arrow indicates the start of transcription and the boxed region shows the first exon.

HDAC1, not only at the promoter of a silenced variegating gene, but also further away from the breakpoint, in this case, further 5' to the *white*$^+$ gene. On the other hand, if HDAC1's new associations were just at specific sequences, for example at promoter sequences, then any model attempting to explain PEV would have to accommodate such selective associations. Accordingly, as a first attempt at examining the question of spreading I chose the 5' Distal Region.

For comparative purposes I examined these regions from the *white*$^+$ gene in three strains. First, in the wild-type strain *Oregon-R* (OR-R), where the *white*$^+$ gene is in its normal location near the tip of the X chromosome. Second, in the inversion strain, *In(1)w*^{m4} (*w*^{m4}). In this strain, the inversion relocates the *white*$^+$ gene to within about 25 kilobases of the newly formed heterochromatic/euchromatic junction (Tartof et al., 1984) and as a result the *white*$^+$ gene is subject to PEV. The *white*$^+$ gene is strongly silenced at this location and is only expressed in approximately 5-15% of the eye pigment cells. Finally, the regions were examined in the inversion strain which bears the *HDAC1*^{326} mutation, *In(1)w*^{m4}; *HDAC1*^{326}*/TM3SbSer* (*w*^{m4}; *326*). PEV is strongly suppressed as a result of the mutation in *HDAC1* and the *white*$^+$ gene is expressed in 80-90% of the eye pigment cells (see Chapter 2, Table1).

I employed PCR to analyze the products of X-ChIP reactions. In order to compare relative differences between the products of PCR reactions, one must ensure the products are quantified during the linear phase of the PCR amplification. To determine the linear phase of PCR under the conditions I employed, I used the primers specific for each region and quantified the product produced after a set number of PCR cycles. An example is shown in Figure 2, which shows the results of PCR amplification of the Distal Promoter
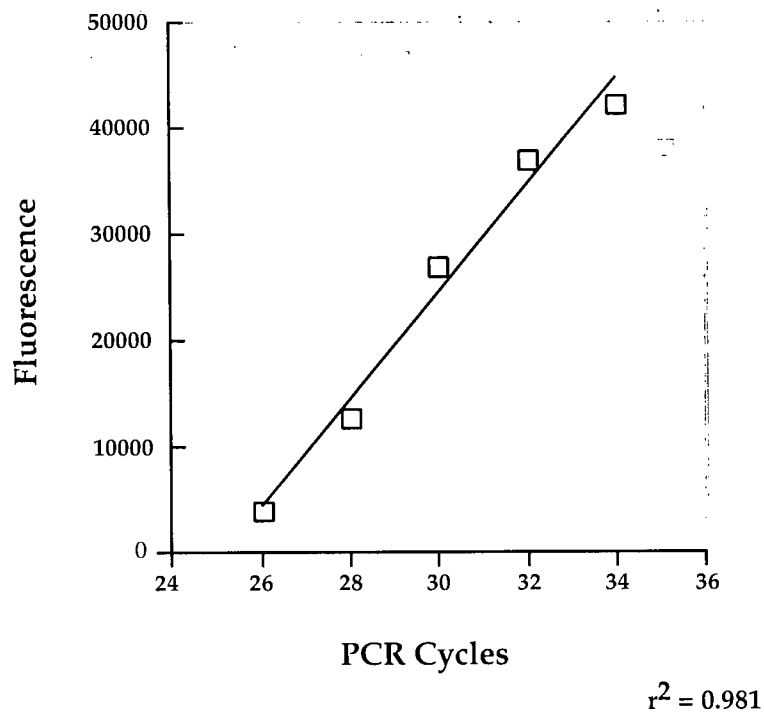
$r^2 = 0.981$

Figure 2   Graph of the relationship between fluorescence (Amount of DNA) and PCR Cycle in the Distal Promoter Region (see Figure 1). The amount of DNA was quantified on a Storm 860 Phospho-Imager (see Materials and Methods for details).

region. For all regions under the conditions I employed, amplification was linear between 26 and 32 cycles. Accordingly, in all subsequent experiments, the PCR was terminated after 30 cycles and the amount of the product visualized on an agarose gel stained by ethidium bromide and quantified using SYBR Green 1 and a phospho-imager (see Materials and Methods for details).

The DNA I analyzed by PCR came from three sources for each strain. The first was total genomic DNA that had been cross-linked, sonicated and the cross-links reversed (Input DNA). The second was the DNA precipitated from the Input DNA by the protein A agarose beads alone (No Antibody) and the third was the DNA precipitated from the Input DNA by the anti-HDAC1 antibody (anti-HDAC1). Each DNA source was analyzed by PCR using primers specific for each region. Figure 3 presents the results obtained from analysis of the Proximal Promoter region, Figure 4 the Distal Promoter region and Figure 5 the 5' Distal region. It is apparent from the agarose gels (panel A, Figures 3, 4, and 5) that in each region, relative to the No Antibody control, there is considerably more PCR product in the anti-HDAC1 lane of w^{m4} than there is in the OR-R lane. It is also apparent that, in the inversion strain bearing the HDAC1 mutation, $w^{m4};326$, the amount of the PCR product in the anti-HDAC1 lane returns to approximately the levels observed in OR-R. Thus, under conditions where the white$^+$ gene is silenced due to PEV, HDAC1 is strongly associated with the promoter and regulatory sequences in the 1.0 kilobase immediately 5' to the white$^+$ gene (panel A in Figure 3 and 4). This association is not restricted to the sequences adjacent white$^+$ and HDAC1 is also found at the 5' Distal region, some 6.0 kilobases 5' to white$^+$ (panel A in Figure 5).

A

	Input	No Antibody	anti HDAC1

OR-R

w^{m4}

$w^{m4}; 326$

B

Strain	No Antibody	Anti HDAC1	Fold Enhanced
OR-R	2115	11,542	5.5
w^{m4}	1154	22,530	19.5
$w^{m4}; 326$	1983	11,025	5.6

C

Proximal Promoter

(Bar graph: y-axis "Fold Enhancement" from 0 to 25; x-axis "Strain" with OR-R ≈ 5, w^{m4} ≈ 19.5, $w^{m4}; 326$ ≈ 5.6)
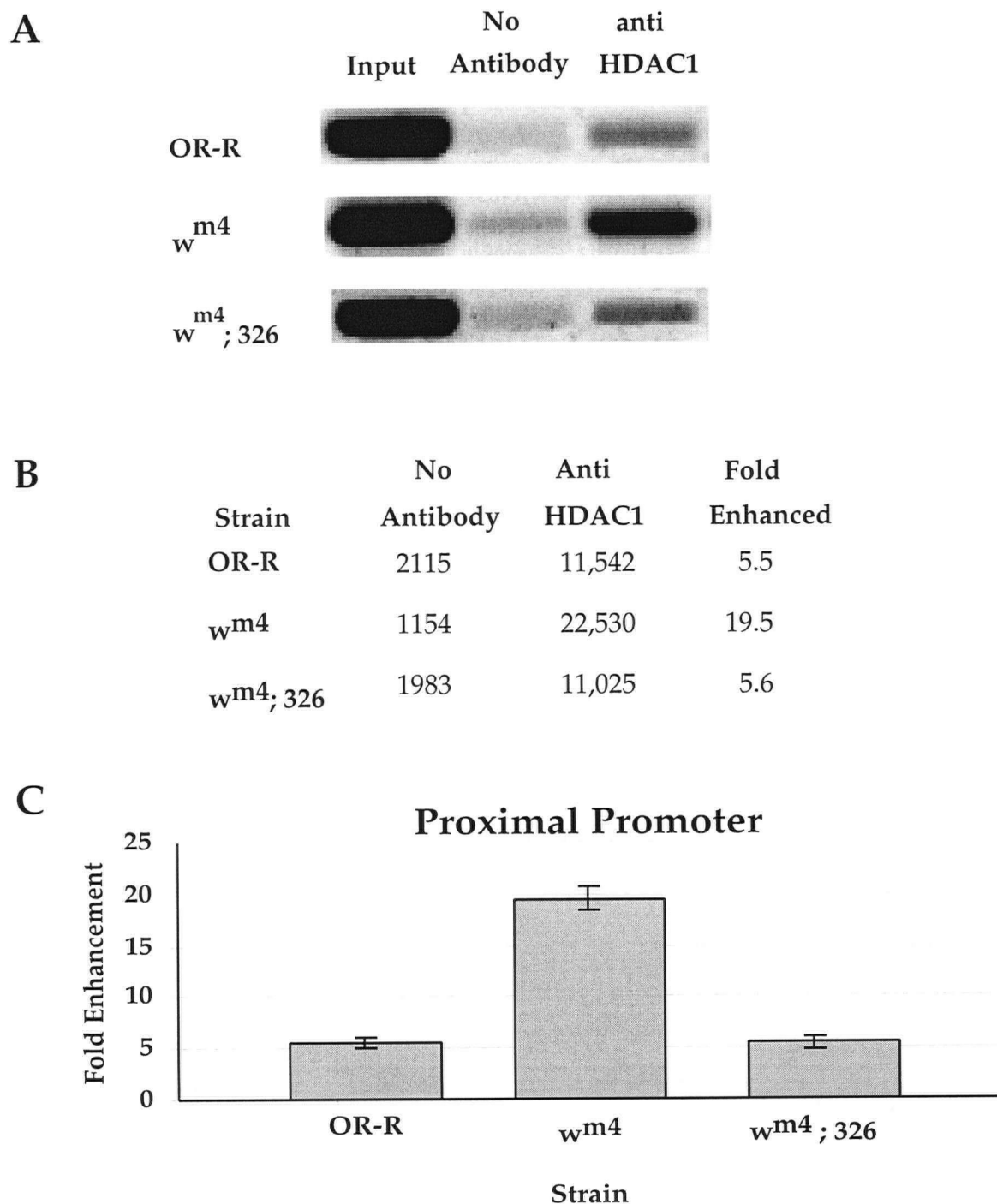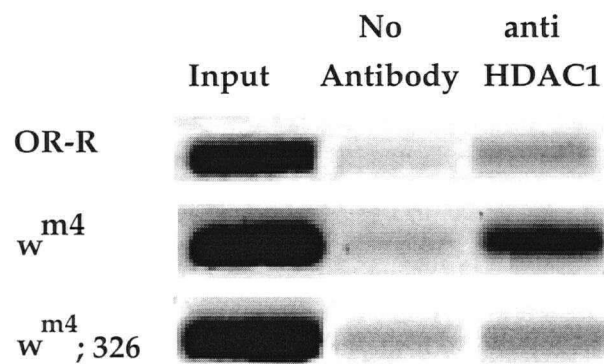
Figure 3 HDAC1 is associated with the Proximal Promoter of w^+ in all strains but the level is elevated in w^{m4}. (A) is a sample agarose gel, (B) is the amount fluorescence measured on a phospho-imager and (C) shows the level of HDAC1 enhancement in each strain relative to the "No Antibody" controls.

A

	Input	No Antibody	anti HDAC1
OR-R			
w^{m4}			
w^{m4}; 326			

B

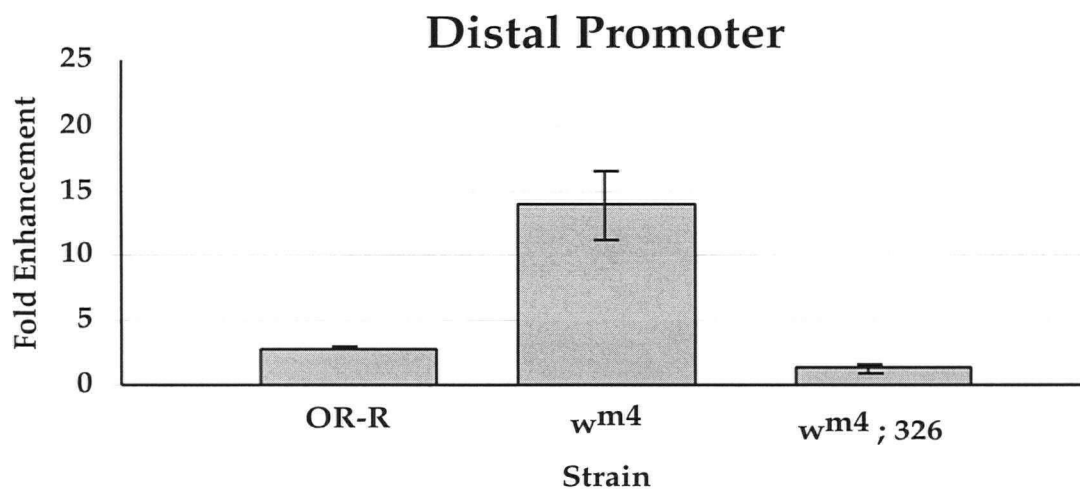| Strain	No Antibody	Anti HDAC1	Fold Enhanced
OR-R	6113	17,163	2.8
w^{m4}	2177	30,285	13.9
w^{m4}; 326	8763	11,443	1.3

C

Figure 4 HDAC1 is associated with the Distal Promoter of the w^+ gene in w^{m4} but not with the Distal Promoter of *OR-R* or w^{m4}; *326*. (A) is a sample agarose gel, (B) is the amount of fluorescence measured on a phospho-imager and (C) shows the level of HDAC1 enhancement in each strain relative to the "No Antibody" controls.

A

	Input	No Antibody	anti HDAC1

OR-R

w^{m4}

w^{m4}; 326

B

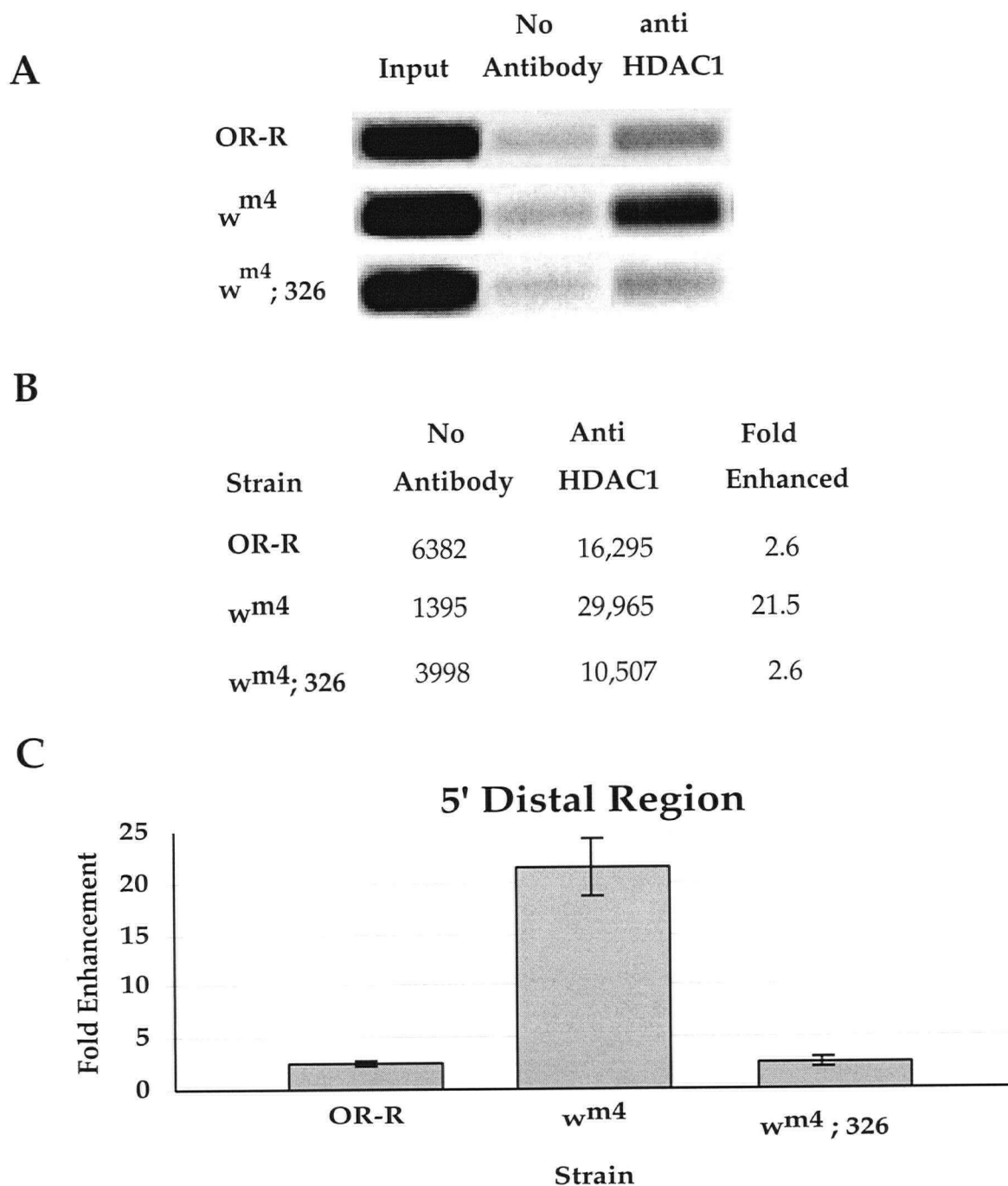| Strain	No Antibody	Anti HDAC1	Fold Enhanced
OR-R	6382	16,295	2.6
w^{m4}	1395	29,965	21.5
w^{m4}; 326	3998	10,507	2.6

C

5' Distal Region

Figure 5 HDAC1 is associated with the 5' Distal Region of the w^+ gene in w^{m4} but not with the 5 Distal Region of *OR-R* or w^{m4}; *326*. (A) is a sample agarose gel, (B) is the amount of fluorescence measured on a phospho-imager and (C) shows the level of enhancement in each strain at this region relative to the "No Antibody" controls.

In order to quantify these differences, aliquots of the PCR reactions were run on agarose gels and stained with SYBR Green 1. The amount of the products was quantified on a phospho-imager and the results are presented in tabular form (panel B, Figures 3, 4, and 5) and in graphical form (panel C, Figures 3, 4, and 5)

In *OR-R*, at the Distal Promoter region (Figure 4B and C) and the 5' Distal Region (Figure 5B and C) there was a slight enhancement in the amount of product produced from the anti-HDAC1 sample relative to the No Antibody controls, between a 2.6 and a 2.8 fold enhancement. In the Proximal Promoter region (Figure 3B and C) there was a 5.5 fold enhancement. From my data, I cannot determine whether this is due to non-specific precipitation of the DNA sequences by the antibody or represents a small but significant amount of HDAC1 bound to all three regions in *OR-R*. However, the increases are very small at the Distal Promoter and the 5' Distal Region. I have performed PCR with primers specific for other sequences in the *Drosophila* genome, unrelated to the *white*[+] gene, and all are slightly increased in the sample from the anti-HDAC1 antibody relative to the No Antibody controls (data not shown). In view of these observations I believe the slight enhancement observed at the Distal Promoter and the 5' Distal Region are likely the result of non-specific precipitation by the antibody. On the other hand, in *OR-R*, the enrichment observed at the Proximal Promoter is somewhat higher, in fact almost double that observed in the other regions. This may represent a *bone fide* site of HDAC1 binding and suggests HDAC1 may be involved in the regulation of the *white*[+] gene in its normal location on the X chromosome.

In contrast, all regions of the *white*⁺ gene in the *w*ᵐ⁴ strain show strong association with HDAC1. In *w*ᵐ⁴, the amount of PCR product for each region in the anti-HDAC1 sample was much higher than that observed in the No Antibody controls, from a 13.9 fold enrichment at the Distal Promoter (Figure 4B and C) to a 21.5 fold enrichment at the 5' Distal Region (Figure 5B and C). This represented an increase of roughly 5 fold over the levels observed in *OR-R*. The level of enhancement was not uniform throughout the regions tested, suggesting that different levels of HDAC1 may be associated with different regions. While the differences are significant, as in the case of the Distal Promoter (13.9 fold) as compared to the Proximal Promoter (19.5 fold) and the 5' Distal Region (21.5 fold), it is not clear what the implications of these difference are. Any conclusions must await the creation of a more accurate map of the distribution of HDAC1 in the *white*⁺ region of *w*ᵐ⁴ using PCR primers distributed throughout this interval. However, what is clear is that the promoter of *white*⁺, and perhaps the entire region containing the *white*⁺ gene, shows a significant increase in its association with HDAC1 when it is silenced due to PEV in the *w*ᵐ⁴ strain.

However, when the *w*ᵐ⁴ strain carries a mutation in *HDAC1* that suppresses PEV, *HDAC1*³²⁶, the increased association of HDAC1 with all regions tested is abolished. In fact, the pattern of HDAC1 association becomes identical to that seen in *OR-R*, with the Distal Promoter (Figure 4B and C) and 5' Distal Region (Figure 5B and C) showing little or no association with HDAC1 while the Proximal Promoter (Figure 3B and C) displays a slightly increased association with HDAC1.

These data provide compelling evidence that HDAC1 is specifically associated with the *white*⁺ gene in w^{m4}, but only when the gene is silenced due to PEV. In the same strain, bearing a mutation in *HDAC1*, PEV is suppressed, the *white*⁺ gene is active and HDAC1 is no longer associated with the region. This provides the first evidence that the product of a Su(var) gene is acting directly at a variegating locus and suggests HDAC1 is an essential component of the silencing mechanism at work in PEV.

It is important to note that, in the presence of $HDAC1^{326}$, pigment levels in the eye are only increased to about 85% of the amount observed in *OR-R*. This difference was not detected by the X-ChIP experiments described here and the results from *OR-R* and w^{m4}; *326* appear almost identical. The inability to detect any residual HDAC1 at the *white*⁺ promoter in the w^{m4}; *326* strain suggests that, either silencing re-establishes itself after late embryogenesis, perhaps without a requirement for HDAC1, or that the limits of sensitivity of this particular method of X-ChIP have been reached and it cannot detect relatively small differences. If the later is the case then caution must be applied when attempting to draw conclusions from small differences in apparent HDAC1 association at the various regions.

Discussion

The point mutations in *HDAC1*, described in Chapter 2, were isolated in a genetic screen for dominant mutations that suppress the variegating phenotype associated with PEV (Mottus, 1983; Sinclair et al., 1983). These mutations suppress PEV generally and are not specific modifiers of the *white⁺* gene. There is a large and growing body of evidence that HDAC1 is a member of several protein complexes and is involved in the regulation of many genes (for examples see Chapter 1 and Chapter 2). This raises the question of whether the dominant mutations in *HDAC1* characterized here are acting directly on the variegating locus or whether the suppression of PEV is indirect, the result of deregulation of one or more HDAC1-regulated genes. Several other chromatin proteins have also been identified through screens for dominant suppressors of PEV, for example HP1 (Mottus, 1983; Reuter and Wolff, 1981), SU(VAR)3-7 (Reuter et al., 1990) and SU(VAR)3-9 (Tschiersch et al., 1994). While these proteins have been shown to localize to heterochromatic regions of the genome, whether their role in suppressing PEV is direct or indirect remains speculative. In addition, recent studies with antibodies produced against the *D. melanogaster* HDAC1 protein, failed to find significant heterochromatic localization of HDAC1 (Barlow et al., 2001; Pile and Wassarman, 2000). Thus the question of whether HDAC1 or the other chromatin proteins play a direct role in the mechanism of PEV has remained open.

I decided to use the technique of X-ChIP to address this question directly. This technique has been employed with great success in single-celled organisms and in eukaryotic cells grown in tissue culture, where one can easily obtain a relatively homogeneous population of cells. It has been used less

frequently in multi-cellular organisms. In most cases, multi-cellular organisms may not be good substrates for X-ChIP since they are comprised of many different cell types, each with their own unique pattern of gene expression. This heterogeneity may obscure events occurring in a single cell type and thus render results from X-ChIP uninterpretable. The exceptions to this rule have occurred almost exclusively in studies of *Drosophila* embryos that attempted to isolate the binding sites of certain regulatory proteins, especially member of the Polycomb Group of genes, that act in early development (Orlando and Paro, 1993). However, with respect to PEV, there is some evidence that the silencing observed in this phenomenon occurs early in embryogenesis in many, and perhaps all, cells in the organism (see Chapter 1). Therefore I was of the view that PEV may be amenable to X-ChIP analysis.

I used X-ChIP and a commercially available, X-ChIP quality antibody to HDAC1, to ask whether HDAC1 is associated with specific regions of the $white^+$ gene in three strains: *Oregon-R* (*OR-R*), in which the $white^+$ gene is expressed normally; $In(1)w^{m4}$ (w^{m4}) in which the $white^+$ gene is silenced due to PEV and is only expressed in about 5-15% of the eye pigment cells; and, $In(1)w^{m4}$; $HDAC1^{326}$ (w^{m4}; 326) in which the mutation in *HDAC1* suppresses the silencing due to PEV and the $white^+$ gene is expressed in 80-90% of the eye pigment cells.

For this initial characterization, I chose to analyze three regions 5' to the start of transcription of the $white^+$ gene (Figure 1) in each strain. The first 500 base pair region, the "Proximal Promoter", is immediately 5' to the start of transcription and includes the $white^+$ gene minimal promoter (Levis et al., 1985; Pirrotta et al., 1985). The second region, the "Distal Promoter", is the region immediately 5' to the minimal promoter and contains elements involved in

regulating the *white*⁺ gene in adult tissues (Davison et al., 1985). In addition, I chose a region approximately 6.0 kilobases 5' to the *white*⁺ gene, the "5' Distal Region", which does not contain any elements known to regulate *white*⁺. The first two regions were chosen because they contain known regulatory regions of *white*⁺ and therefore might be expected to show alterations when the gene is silenced. The 5' Distal Region was chosen in order to determine whether or not any changes observed at the *white*⁺ promoter were propagated upstream of the gene.

X-ChIP analysis of the association of HDAC1 with the *white*⁺ gene when it is in its normal location at the tip of the X chromosome in the *OR-R* strain suggest low levels of HDAC1 may be associated with the Proximal Promoter region (Figure 3), but that HDAC1 is probably not associated with either the Distal Promoter region (Figure 4) or the 5' Distal Region (Figure 5). In *OR-R* the levels of HDAC1 association at the Proximal Promoter displayed a 5 fold increase over background and approximately twice as high as that observed in either the Distal Promoter and the 5' Distal Region. It would not be particularly surprising to find that HDAC1 is normally associated with the *white*⁺ gene at its promoter, where, presumably, it would act as a repressor. The only tissue in the larvae in which the *white*⁺ gene is known to be expressed, is the malpighian tubules (Levis et al., 1985; Pirrotta et al., 1985). Therefore in almost all cells of the 12-16 hour embryos, which were the substrate for X-ChIP, the *white*⁺ gene is inactive. Accordingly, I believe the 5-6 fold increases observed at the Proximal Promoter represent actual sites of HDAC1 association.

Whether the 2-3-fold enhancement observed at the Distal Promoter and the 5' Distal Region represent actual sites of HDAC1 association is more

problematic. One of the difficulties with interpreting these results stems from attempting to do X-ChIP in a whole organism, where it may be difficult, or impossible, to find a chromosomal region to use as a negative control for association with HDAC1. However, I have performed X-ChIP with a monoclonal antibody that does not have any target proteins in *Drosophila*, the T7 antigen antibody, and it non-specifically precipitates DNA from several regions of the genome at levels 2-3 fold above the No Antibody controls (unpublished observations). Therefore, I think it likely that the a 2-3 fold levels of enhancement observed in *OR-R* at the Distal Promoter and the 5' Distal Region of *OR-R* represent non-specific precipitation of DNA sequences by the anti-HDAC1 antibody and not actual sites of HDAC1 association.

In contrast, the results clearly show that in w^{m4}, where the *white*[+] gene is silenced due to PEV, HDAC1 shows a strong association with all three regions (Figure 3, 4 and 5). The levels of HDAC1 observed in w^{m4} at each region are far greater than that seen in *OR-R* and show at least a 14-fold increase in association at all regions.

However, when the dominant suppressor of PEV, $HDAC1^{326}$, is introduced into the w^{m4} strain, the increased HDAC1 association normally seen at all regions in w^{m4}, is abolished (Figures 3, 4 and 5). The levels of HDAC1 association return to that observed when the *white*[+] gene is at its normal location at the tip of the X chromosome as in the *OR-R* strain. In fact the levels of HDAC1 at each region in w^{m4}; 326 precisely mirror the levels observed in *OR-R*, including the slight enrichment observed in the Proximal Promoter region (Figure 3).

The high levels of HDAC1 associated with the three regions analyzed at the *white*$^+$ locus when it is silenced as a result of PEV in w^{m4}, which are not present when *white*$^+$ is at its normal location in *OR-R*, provides strong evidence for HDAC1's direct involvement in PEV. In addition, the observation that the association with these regions is abolished by a mutation in *HDAC1* implies that HDAC1 is an essential component of the silencing mechanism at work in PEV. This is the first evidence of a protein's direct involvement in the silencing associated with PEV.

The fact that an HDAC is present at a site does not guarantee that it is regulating the gene or phenomenon also present at the site. For example, HDAC1 has been found at active genes (Breiling et al., 2001), which was surprising, given HDAC1's suspected role as a transcriptional repressor. There could be many reasons for such an association, including something as simple as HDAC1 being stored, in an inactive form, to be quickly accessible for rapid repression of the gene. Accordingly, Robyr et al. (2002) have suggested three criteria be adopted to distinguish whether an HDAC is acting directly or indirectly on gene expression. The authors were speaking to genome wide analysis but their considerations can be easily extended to apply to X-ChIP studies of HDACs as well. The X-ChIP versions of these criteria would be: (1) X-ChIP studies to determine whether the HDAC is associated with chromatin at the suspected site; (2) since the HDAC's substrates are the histone proteins, analysis of the acetylation pattern of the histones at the site of association; and (3) transcriptional analysis of the suspected HDAC-regulated gene. This thesis has addressed two of the three criteria with respect to HDAC1's involvement in PEV. The X-ChIP studies confirm HDAC1 is associated with the euchromatic

region that variegates when, and only when, it is subject to PEV. Secondly, mutations in HDAC1, that suppress PEV, abolish the association and restore transcription to almost wild-type levels. The remaining criterion, analysis of the acetylated state of the histone proteins in the chromatin surrounding the *white*$^+$ gene, has not been a part of the study. In spite of this, I submit the evidence presented herein is compelling that HDAC1 plays an essential role in the silencing associated with PEV.

Chapter 4

Summary and General Discussion

General Discussion

Summary

The model system our laboratory has been using to investigate gene silencing is position effect variegation (PEV) in *D. melanogaster* (Grigliatti, 1991; Schotta et al., 2003; Spofford, 1976). When PEV occurs, genes normally expressed in a particular tissue are silenced in some cells of that tissue because a chromosomal rearrangement has placed the region in which the genes are located next to a breakpoint in heterochromatin. In some cells of the tissue, the genes are expressed normally, while in others, the genes are silenced, and those cells display a mutant phenotype. Accordingly, that tissue is mosaic or variegated. Importantly, the decision whether a gene will be active or inactive is made early in development and that decision is passed on to daughter cells with reasonable fidelity. Thus PEV mimics normal development in many ways. This has led our lab, and several others, to try to dissect the mechanisms underlying PEV with the hope they will shed some light on the more general silencing mechanisms that occur during normal development.

One of the conventional approaches to dissecting a phenomenon such as PEV is to isolate mutations that modify the associated phenotype. Subsequent analysis of the mutations and their effects provides some insight into that phenomenon. In the case of PEV, only a few mutations have been cloned and analyzed in any detail. However, the insights they have provided into PEV, and silencing in general, have had considerable impact. For example, *Su(var)3-9* encodes an protein that can methylate lysine 9 on histone H3. Many have suggested this creates an epigenetic mark that is passed on to daughter cells to

maintain silencing, not only in PEV, but also in other silencing systems in the cell (Czermin et al., 2001; Richards and Elgin, 2002; Schotta et al., 2002; Turner, 2002). Accordingly, the cloning and characterization of additional mutations that modify PEV holds great promise for increasing our understanding of both PEV and other silencing mechanisms employed by eukaryotic cells.

Chapter 2 presents the cloning and characterization of a gene identified in a screen for dominant mutations that suppress the silencing associated with PEV. The gene is *HDAC1*, which encodes an histone deacetylase homologous to HDAC1, from mammals, and Rpd3, from *S. cerevisiae*. Specific missense mutations in *HDAC1* suppress PEV, while hypomorphic or null alleles have no effect on the variegating phenotype. Chapter 3 provides evidence that HDAC1 is directly involved in PEV by demonstrating the protein is present on the chromatin surrounding a gene silenced as a result of PEV. However, the Su(var) mutations in *HDAC1* abolish the protein's association with the chromatin surrounding the variegating locus and restore the activity of the variegating gene.

Discussion

There are currently two widely held theories about the mechanism at the basis of PEV (Grigliatti, 1991; Schotta et al., 2003; Spofford, 1976). The most enduring is the "Spreading Hypothesis". It posits that factors normally found in heterochromatin spread out from the new chromosomal junction and create a chromatin environment that suppresses transcription. The distance the factors spread is independent in each cell and therefore in some cells the factors will spread far enough to inactivate a variegating gene, while, in other cells,

spreading will stop before reaching the gene and it will be transcribed normally.

More recently, an alternative hypothesis, the "Compartmentalization Hypothesis", has been proposed. It posits that heterochromatic regions of chromosomes are positioned in specific locations or compartments of the nucleus that exclude the factors required for transcription and therefore when a gene is in this compartment it is silenced. The mosaic phenotype of PEV occurs because the variegating gene is sometimes localized to a heterochromatic compartment, and therefore silenced, and sometime localized to its normal position, and therefore fully expressed (Csink and Henikoff, 1996).

These hypotheses are not mutually exclusive in that loci subject to PEV may be relocated to heterochromatic compartments where they are packaged as proposed by the "Spreading Hypothesis". Alternatively, it may be that a variegating locus is packaged as per the "Spreading Hypothesis" and then is relocated to an heterochromatic compartment. If either alternative is true, then a variegating gene would be fully expressed because, either it was not relocated to an heterochromatic compartment, or packaging failed to "spread" to the gene in the heterochromatic compartment or both.

Any attempt to explain the results observed here in terms of these models must also accommodate the fact that strains bearing the *HDAC1* mutations, also carry a wild-type copy of the *HDAC1* gene and therefore at least 50% of the HDAC1 in the nucleus is the wild-type protein. The data presented here cannot distinguish between these models. However, it is difficult to interpret my results based on a strict compartmentalization model. HDAC1 is not found in heterochromatin, at least in concentrations high enough to be

detected by an antibody (Barlow et al., 2001; Pile and Wassarman, 2000).
Accordingly, it would seem unlikely that the presence of HDAC1 would be a
signal to localize a normally heterochromatic region to an heterochromatic
compartment. This implies that some other factor(s) must be responsible for
localizing the heterochromatin associated with the *white*⁺ gene in w^{m4} to a
heterochromatic compartment. However, the fact that HDAC1 is found in
abundance at the *white*⁺ gene in w^{m4} implies the association with HDAC1 must
occur when the *white*⁺ gene is mis-localized. In a strict compartmentalization
model, it would be the localization to the heterochromatic compartment that
causes silencing and not the association with HDAC1. Accordingly, the
mutations in *HDAC1*, that suppress PEV, would cause misregulation of the
factor(s) responsible for localizing heterochromatin to the nuclear periphery
which would, in turn, result in the failure of the *white*⁺ gene to be localized to
the nuclear periphery. Thus the effect of the Su(var) mutations in *HDAC1*
would be indirect. While this scenario is possible, it seems unlikely. The
presence of HDAC1 at the *white*⁺ gene when it is silenced in w^{m4} and its absence
when the *white*⁺ gene is expressed in the *HDAC1* Su(var) mutations suggests a
more direct role for HDAC1.

The data presented here can be more easily explained in terms of the
"Spreading Hypothesis". The substantial body of evidence that supports this
model was reviewed in Chapter 1. In addition, there is a phenomenon in *S.
cerevisiae*, telomeric position effect variegation (TPEV) that closely resembles
PEV and also appears to involve the spreading of silencing components (see
Chapter 2). It should be noted that TPEV is also dependent on Sir2, an NAD
dependent Class III histone deacetylase, for efficient silencing (Suka et al., 2002).

Accordingly, the data presented in this thesis is consistent with the following scenario. In a chromosomal rearrangement subject to PEV, heterochromatin is assembled normally at or near the new illicit heterochromatic/euchromatic junction. This may be dependent on a complex that contains SU(VAR)3-7 that is targeted to satellite sequences by the unusual zinc fingers contained in SU(VAR)3-7. That complex contains or recruits a complex that contains HP1, SU(VAR)3-9, HDAC1 and likely several other proteins which create and stabilize the structure responsible for silencing variegating loci. The structure can spread into the adjacent euchromatin but requires the activity of each component to perpetuate its spread. HDAC1 must deacetylate K9 of histone H3, and perhaps other lysine residues, which clears the path for SU(VAR)3-9 to methylate H3 K9 that in turn creates a binding site for HP1 stabilizing the structure and silencing any loci at that location. This process would continue until the concentration of the essential components dropped below a critical level or a boundary element, in the euchromatic region of the chromosome, halted progress.

How then do mutations in *HDAC1* cause suppression of PEV? In the proposed model, HDAC1 activity is required for the silencing mechanism to spread out from the heterochromatic breakpoint. In the mutant strains, 50% of the HDAC1 protein is wild-type and should function normally. However, the remaining 50% of HDAC1 bears a mutation and is presumably defective in the ability to deacetylate histone tails. Since the mutation is a single amino acid substitution, the mutant HDAC1 may still assume its proper shape and take its place in the protein complex involved in creating the silencing structure (see Chapter 2). However, failure of the mutant HDAC1 in the protein complex to

deacetylate its targets would inhibit the action of SU(VAR)3-9 whenever it encountered an acetylated lysine 9 residue on H3 since SU(VAR)3-9's activity is blocked by acetylation (Rea et al., 2000). Accordingly a binding site would not be created for HP1, the silencing process would abort and PEV would be suppressed.

In the present case it should be noted that the *white⁺* gene is still subject to some silencing in the mutant *HDAC1* lines (in 10-20% of cells). There could be several reasons for this. The process may not be absolutely dependent on HDAC1 or the mutant HDAC1s may be hypomorphs and the residual activity allows spreading a small percentage of the time. Alternatively, halting the spread of the silencing structure may require the binding of several inactive deacetylase complexes in tandem or within a certain distance along the chromosome. Once this threshold is met silencing is not propagated beyond that point. If that occurs before the *white⁺* gene, then the gene is active, if not, silencing would spread through and perhaps beyond the *white⁺* gene rendering it transcriptionally inactive.

The results of X-Chip from the 5' Distal Region in *w^m4* (Figure 5) demonstrate that, spreading, if indeed that is the mechanism at work, spreads far beyond the *white⁺* gene, at least as far as 6.0 kilobases. Cytological evidence suggests spreading can extend as far as 80 bands on the polytene chromosomes from the breakpoint (Spofford, 1976). It is not clear what factors determine the distance that silencing spreads. It does not appear to be dependent on the presence of coding sequences or promoter regions, since the region containing the 5' Distal Region does not contain any known genes or regulatory sequences. Silencing may spread until specialized boundary type sequences are

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encountered or competition with factors creating an euchromatic environment halts its progress.

In Chapter 2, I presented a model of HDAC1 involvement in PEV based largely on speculation since, at the time of publication, the functions of other proteins involved in the silencing associated with PEV were unknown. The model proposed that, because the Su(var) mutations in HDAC1 are caused by a single amino acid change, mutant forms of HDAC1 would still fold properly and thus be able to become members of their normal complexes. However, the complexes containing mutant HDAC1 would be unable to deacetylate the target histone tails and hyperacetylation of histone tails would lead to expression of the reporter gene. Since the functions of some of the other Su(var) proteins are now known, the model of how specific mutations in HDAC1 suppress PEV requires updating.

If PEV does occur as a result of the spreading of factors from heterochromatin, then one would predict that factors, which suppress PEV, inhibit or abort the spreading process. It is now known that SU(VAR)3-9 methylation of lysine 9 on H3 (H3 K9) is inhibited if lysine 9 is acetylated and therefore HP1 will not bind (Rea et al., 2000). I have proposed that HDAC1 is required to deacetylate H3 K9 and clear the way for SU(VAR)3-9 to methylate that residue to create a binding site for HP1. I further propose that the association of SU(VAR)3-9, HP1 and other factors not only creates a repressive chromatin conformation, but also recruits additional HDAC1 which is required for propagation of the silenced conformation. Thus, the Su(var) mutations in HDAC1 suppress PEV because they occupy their place in the complex(es) but

cannot deacetylate H3 K9 and this prevents the association of both SU(VAR)3-9 and HP1.

This model makes several predictions and raises several questions that can be tested experimentally. If spreading does indeed occur then how far does the spreading extend in the w^{m4} strain? Since the *Drosophila* genome is sequenced, it is possible to generate primers for X-ChIP further 5' to the *white*[+] gene and determine how far the association with HDAC1 extends. Once the extent is determined approximately one can ask whether the association with HDAC1 ends abruptly at specific sequences, suggesting the presence of a boundary element, or if the association gradually declines, suggesting the spread is dependent on the availability of the components of the silencing mechanism. The complementary experiments can also be done. The heterochromatic/euchromatic junction in w^{m4} is known and thus one can ask, using X-ChIP, where spreading stops in an HDAC1 Su(var) mutant background.

Do certain sequences present boundaries to the spread of PEV? The existence of some type of boundary element is suggested by analysis of mutations in *Evar93D* [also known as *mod(mdg4)*]. Mutations in this gene act as strong enhancers of PEV. It is suspected the product of this gene binds to boundary elements in euchromatin creating an open chromatin conformation (Gerasimova and Corces, 2001). Thus, in mutant lines subject to PEV, heterochromatin may spread much further because boundary elements, that would normally halt the spread, are defective. Analysis of the distribution of HDAC1 or other silencing components, using X-ChIP, may provide insight into this question.

The model also proposes that HDAC1 is required to deacetylate the histone tails as one of the initial steps in the formation of silenced chromatin. Accordingly, using X-ChIP and antibodies for specific acetylated forms of the histones, one can determine the acetylation state of the histone tails. One would predict that, in the normal w^{m4} strain, the histones would be hypoacetylated in any region where HDAC1 is present and specifically that H3 K9 would not be acetylated. Conversely, in w^{m4} bearing a Su(var) mutation in *HDAC1*, one would predict the region surrounding the *white* gene would show increased acetylation, especially at H3 K9. This would also address the third test suggested to confirm that an HDAC is acting at a location where X-ChIP indicates it is localized [see Chapter 3 Discussion (Robyr et al., 2002)].

The model also makes specific predictions about what proteins should be localized to the silenced euchromatic region in w^{m4} and further predicts that a hierarchy of interactions may occur. In w^{m4}, the silenced euchromatin should be associated with SU(VAR)3-9, HP1 and perhaps SU(VAR)3-7. Antibodies exist to all three proteins and therefore X-ChIP can be employed to determine whether they are present and further to ask if their distribution precisely mirrors that of HDAC1.

Since dominant Su(var) mutations are readily available for each of the known proteins involved in the process, one can use the mutations to ask questions about the nature of the silencing complex or complexes. In the models simplest form one would predict that a mutation in HDAC1 should be epistatic to mutations in the other components. Thus, in the Su(var) HDAC1 strains, one should find that all associations of the other proteins are abolished because the activity of HDAC1 is required for one of the initial steps in

spreading. Genetic experiments suggest this may in fact be the case. A Su(var) mutation in *HDAC1* abolishes the enhancer effect of three copies of *Su(var)3-9* (Czermin et al., 2001). If indeed the components are added in a stepwise fashion then one would predict a mutation in *Su(var)3-9* would abolish the association of HP1 because SU(VAR)3-9 creates the binding site for HP1, but not the association of HDAC1. Similarly, mutations in *HP1* would not affect the distribution of HDAC1 and SU(VAR)3-9. However, I think a simple stepwise accumulation of factors, which eventually creates a silenced stretch of euchromatin, is unlikely. In *S. pombe* the spread of the SU(VAR)3-9 homologue, Clr4 and the HP1 homologue, Swi6, are mutually dependent (Noma et al., 2001). This is likely because these factors exist in complexes where it appears the action of one member of the complex reinforces and abets the function(s) of the other members. Accordingly in *D. melanogaster*, I predict that a similar situation will be found. I suspect the four proteins specifically mentioned and several others are members of one or more large complexes. The members of the complexes are dependent on each other for their localization and spread. For example, it appears that HDAC1 is a member of a complex that includes SU(VAR)3-9 and may include HP1 and SU(VAR)3-7 (Cleard et al., 1997; Schotta et al., 2002). This complex would bind in the heterochromatin at or very near to the heterochromatic/euchromatic breakpoint where HDAC1 would begin the process of silencing by deacetylating H3 K9 in an adjacent nucleosome. The actions of SU(VAR)3-9 and HP1 would then create conditions that would recruit another complex which would repeat the process until a boundary was encountered or the concentration of the complexes fell below a certain threshold.

The difference between these two scenarios is readily testable. In the stepwise model, mutations in HP1 should not affect the distribution of HDAC1, as measured by X-ChIP. On the other hand, if spreading occurs via complexes, then a mutation in any one of the members of the complex should alter the distribution of all members. Again, the distribution can be measured by X-ChIP.

The fact that HDAC1 and quite likely, SU(VAR)3-9, HP1 and perhaps other proteins, are present at the silenced euchromatin in 12 to 16 hour embryos suggests their presence may be required at the site of silencing throughout development. It has been suggested they are part of the epigenetic mark that maintains the silenced state (Hall et al., 2002). The model suggested here also implies that these proteins are involved in the initial determinative event regarding whether a reporter gene will be active or not. The question of whether these proteins function in both initiation and maintenance, or are exclusively involved in maintenance, analogous to the Polycomb Group of proteins (Breiling et al., 2001), is an interesting one that has not been addressed here or elsewhere. It may be possible to address this question employing X-ChIP and carefully staged embryos.

Flybase list over 180 loci that, when mutated, influence the variegating phenotype associated with PEV (http://flybase.bio.indiana.edu:82/). As noted in Chapter 1, many external factors, which affect basic cellular metabolism, also affect PEV. The sensitivity of the variegating phenotype to such a large number of factors suggests many may be acting indirectly. Thus one of the challenges facing investigators is determining which modifiers of PEV to clone and characterize, that is, which are likely to be modifying PEV directly and which

represent indirect effects. This thesis presents an approach to cloning essential Su(var) and E(var) loci. However, no criteria readily present themselves for determining which loci to clone and characterize first. Our lab has pursued loci that, when mutated, have a very strong effect of the variegating phenotype. However, a biochemical approach to identifying the members of complexes containing known modifiers of PEV should also be pursued.

Finally, the presence of HDAC1 and perhaps the other proteins on the silenced euchromatin throughout development suggests they may have a greater function than that suspected from the purely enzymatic activity discovered to date. These proteins are highly conserved from yeast to mammals with large tracts of amino acids almost absolutely conserved (De Rubertis et al., 1996; Eissenberg and Elgin, 2000; Mottus et al., 2000). It may be they are important structural components of chromatin and the highly conserved regions represent domains involved in creating and maintaining specific chromatin conformations. Such a role for HP1 is suggested by the studies that demonstrated altered accessibility to nucleases at the variegating locus in HP1 mutations (Cryderman et al., 1998). An adequate test of this hypothesis may have to wait until all the components of the complexes have been identified and the creation of an *in vitro* chromatin assembly system that faithfully recreates chromatin as it is found *in vivo*.

References

Alland, L., Muhle, R., Hou, H., Jr., Potes, J., Chin, L., Schreiber-Agus, N., and
DePinho, R. A. (1997). Role for N-CoR and histone deacetylase in Sin3-
mediated transcriptional repression. Nature *387*, 49-55.

Allfrey, V., Faulkner, R., and Mirsky, A. (1964). Acetylation and methylation of
histones and their possible role in the regulation of RNA sythesis. Proc
Natl Acad Sci U S A *51*, 786-794.

Annunziato, A. T., Frado, L. L., Seale, R. L., and Woodcock, C. L. (1988).
Treatment with sodium butyrate inhibits the complete condensation of
interphase chromatin. Chromosoma *96*, 132-138.

Bachman, K. E., Rountree, M. R., and Baylin, S. B. (2001). Dnmt3a and Dnmt3b
are transcriptional repressors that exhibit unique localization properties
to heterochromatin. J Biol Chem *276*, 32282-32287.

Baker, B. S., Gorman, M., and Marin, I. (1994). Dosage compensation in
Drosophila. Annu Rev Genet *28*, 491-521.

Baker, W. K. (1967). A clonal system of differential gene activity in Drosophila.
Dev Biol *16*, 1-17.

Baker, W. K. (1968). Position-effect variegation. Adv Genet *14*, 133-169.

Bannister, A. J., Zegerman, P., Partridge, J. F., Miska, E. A., Thomas, J. O.,
Allshire, R. C., and Kouzarides, T. (2001). Selective recognition of
methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature
410, 120-124.

Barlow, A. L., van_Drunen, C. M., Johnson, C. A., Tweedie, S., Bird, A., and
Turner, B. M. (2001). dSIR2 and dHDAC6: two novel, inhibitor-resistant

deacetylases in Drosophila melanogaster. Experimental Cell Research *265*, 90-103.

Bashaw, G. J., and Baker, B. S. (1996). Dosage compensation and chromatin structure in Drosophila. Curr Opin Genet Dev *6*, 496-501.

Belmont, A. S., and Bruce, K. (1994). Visualization of G1 chromosomes: a folded, twisted, supercoiled chromonema model of interphase chromatid structure. J Cell Biol *127*, 287-302.

Belyaeva, E. S., Demakova, O. V., Umbetova, G. H., and Zhimulev, I. F. (1993). Cytogenetic and molecular aspects of position-effect variegation in Drosophila melanogaster. V. Heterochromatin-associated protein HP1 appears in euchromatic chromosomal regions that are inactivated as a result of position-effect variegation. Chromosoma *102*, 583-590.

Boffa, L. C., Gruss, R. J., and Allfrey, V. G. (1981). Manifold effects of sodium butyrate on nuclear function. Selective and reversible inhibition of phosphorylation of histones H1 and H2A and impaired methylation of lysine and arginine residues in nuclear protein fractions. J Biol Chem *256*, 9612-9621.

Boggs, B. A., Cheung, P., Heard, E., Spector, D. L., Chinault, A. C., and Allis, C. D. (2002). Differentially methylated forms of histone H3 show unique association patterns with inactive human X chromosomes. Nat Genet *30*, 73-76.

Brehm, A., Miska, E. A., McCance, D. J., Reid, J. L., Bannister, A. J., and Kouzarides, T. (1998). Retinoblastoma protein recruits histone deacetylase to repress transcription [see comments]. Nature *391*, 597-601.

Breiling, A., Turner, B. M., Bianchi, M. E., and Orlando, V. (2001). General transcription factors bind promoters repressed by Polycomb group proteins. Nature *412*, 651-655.

Briggs, S. D., Xiao, T., Sun, Z. W., Caldwell, J. A., Shabanowitz, J., Hunt, D. F., Allis, C. D., and Strahl, B. D. (2002). Gene silencing: trans-histone regulatory pathway in chromatin. Nature *418*, 498.

Brosseau, G. (1970). V-type position effects for e^+ and ro^+ in *Drosophila*. Dros Inf Serv *45*, 100.

Brown, K. E., Baxter, J., Graf, D., Merkenschlager, M., and Fisher, A. G. (1999). Dynamic repositioning of genes in the nucleus of lymphocytes preparing for cell division. Mol Cell *3*, 207-217.

Brown, K. E., Guest, S. S., Smale, S. T., Hahm, K., Merkenschlager, M., and Fisher, A. G. (1997). Association of transcriptionally silent genes with Ikaros complexes at centromeric heterochromatin. Cell *91*, 845-854.

Brownell, J. E., Zhou, J., Ranalli, T., Kobayashi, R., Edmondson, D. G., Roth, S. Y., and Allis, C. D. (1996). Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. Cell *84*, 843-851.

Candido, E., Reeves, R., and Davie, J. R. (1978). Sodium butyrate inhibits histone deacetylation in cultured cells. Cell *14*, 105.

Carmen, A. A., Rundlett, S. E., and Grunstein, M. (1996). HDA1 and HDA3 are components of a yeast histone deacetylase (HDA) complex. J Biol Chem *271*, 15837-15844.

Carruthers, L. M., and Hansen, J. C. (2000). The core histone N termini function independently of linker histones during chromatin condensation. J Biol Chem *275*, 37285-37290.

Catcheside, D. (1947). The P-locus position effect in *Oenothera*. J Genet *48*.

Cattanach, B. M. (1974). Position effect variegation in the mouse. Genet Res *23*, 291-306.

Cavalli, G., Orlando, V., and Paro, R. (1999). Mapping DNA target sites of chromatin-associated proteins by formaldehyde cross-linking in *Drososphila* embryos. In Chromosome Structural Analysis: A Practical Approach, W. Bickmore, ed. (Oxford, Oxford University Press), pp. 20-37.

Christman, J. K., Weich, N., Schoenbrun, B., Schneiderman, N., and Acs, G. (1980). Hypomethylation of DNA during differentiation of Friend erythroleukemia cells. J Cell Biol *86*, 366-370.

Cleard, F., Delattre, M., and Spierer, P. (1997). SU(VAR)3-7, a Drosophila heterochromatin-associated protein and companion of HP1 in the genomic silencing of position-effect variegation. Embo J *16*, 5280-5288.

Cleard, F., Matsarskaia, M., and Spierer, P. (1995). The modifier of position-effect variegation Suvar(3)7 of Drosophila: there are two alternative transcripts and seven scattered zinc fingers, each preceded by a tryptophan box. Nucleic Acids Res *23*, 796-802.

Cleard, F., and Spierer, P. (2001). Position-effect variegation in Drosophila: the modifier Su(var)3-7 is a modular DNA-binding protein. EMBO Rep *2*, 1095-1100.

Clutterbuck, A. J., and Spathas, D. H. (1984). Genetic and environmental modification of gene expression in the brlA12 variegated position effect mutant of Aspergillus nidulans. Genet Res 43, 123-138.

Cohen, J. (1962). Position effect variegation at several closely-linked loci in *Drosophila melanogaster*. Genetics 47, 647-659.

Cryderman, D. E., Cuaycong, M. H., Elgin, S. C., and Wallrath, L. L. (1998). Characterization of sequences associated with position-effect variegation at pericentric sites in Drosophila heterochromatin. Chromosoma *107*, 277-285.

Csink, A. K., and Henikoff, S. (1996). Genetic modification of heterochromatic association and nuclear organization in Drosophila. Nature *381*, 529-531.

Czermin, B., Schotta, G., Hulsmann, B. B., Brehm, A., Becker, P. B., Reuter, G., and Imhof, A. (2001). Physical and functional association of SU(VAR)3-9 and HDAC1 in Drosophila. EMBO Rep 2, 915-919.

Davison, D., Chapman, C. H., Wedeen, C., and Bingham, P. M. (1985). Genetic and physical studies of a portion of the white locus participating in transcriptional regulation and in synapsis-dependent interactions in Drosophila adult tissues. Genetics *110*, 479-494.

De Rubertis, F., Kadosh, D., Henchoz, S., Pauli, D., Reuter, G., Struhl, K., and Spierer, P. (1996). The histone deacetylase RPD3 counteracts genomic silencing in Drosophila and yeast. Nature *384*, 589-591.

Delattre, M., Spierer, A., Tonka, C. H., and Spierer, P. (2000). The genomic silencing of position-effect variegation in Drosophila melanogaster: interaction between the heterochromatin-associated proteins Su(var)3-7 and HP1. J Cell Sci *113 Pt 23*, 4253-4261.

Dimitri, P., and Pisano, C. (1989). Position effect variegation in Drosophila melanogaster: relationship between suppression effect and the amount of Y chromosome. Genetics *122*, 793-800.

Eberl, D. F., Duyf, B. J., and Hilliker, A. J. (1993). The role of heterochromatin in the expression of a heterochromatic gene, the rolled locus of Drosophila melanogaster. Genetics *134*, 277-292.

Eissenberg, J. C., and Elgin, S. C. (2000). The HP1 protein family: getting a grip on chromatin. Curr Opin Genet Dev *10*, 204-210.

Ekwall, K., Olsson, T., Turner, B. M., Cranston, G., and Allshire, R. C. (1997). Transient inhibition of histone deacetylation alters the structural and functional imprint at fission yeast centromeres. Cell *91*, 1021-1032.

Emiliani, S., Fischle, W., Van Lint, C., Al-Abed, Y., and Verdin, E. (1998). Characterization of a human RPD3 ortholog, HDAC3. Proc Natl Acad Sci U S A *95*, 2795-2800.

Felsenfeld, G. (1996). Chromatin unfolds. Cell *86*, 13-19.

Ferreira, J., Paolella, G., Ramos, C., and Lamond, A. I. (1997). Spatial organization of large-scale chromatin domains in the nucleus: a magnified view of single chromosome territories. J Cell Biol *139*, 1597-1610.

Friedman, T. B., Owens, K. N., Burnett, J. B., Saura, A. O., and Wallrath, L. L. (1991). The faint band/interband region 28C2 to 28C4-5(-) of the Drosophila melanogaster salivary gland polytene chromosomes is rich in transcripts. Mol Gen Genet *226*, 81-87.

Gerasimova, T. I., and Corces, V. G. (2001). Chromatin insulators and

> boundaries: effects on transcription and nuclear organization. Annu Rev
> Genet *35*, 193-208.

Gibson, T. J., and Spring, J. (1998). Genetic redundancy in vertebrates:

> polyploidy and persistence of genes encoding multidomain proteins.
> Trends Genet *14*, 46-49; discussion 49-50.

Gowen, J., and Gay, E. (1934). Chromosome constitution and behavior in ever-

> sporting and mottling in *Drosophila melanogaster*. Genetics *19*, 189-208.

Grant, P. A., Duggan, L., Cote, J., Roberts, S. M., Brownell, J. E., Candau, R.,

> Ohba, R., Owen-Hughes, T., Allis, C. D., Winston, F., *et al.* (1997). Yeast
> Gcn5 functions in two multisubunit complexes to acetylate nucleosomal
> histones: characterization of an Ada complex and the SAGA (Spt/Ada)
> complex. Genes Dev *11*, 1640-1650.

Grewal, S. I., Bonaduce, M. J., and Klar, A. J. (1998). Histone deacetylase

> homologs regulate epigenetic inheritance of transcriptional silencing and
> chromosome segregation in fission yeast. Genetics *150*, 563-576.

Grewal, S. I., and Elgin, S. C. (2002). Heterochromatin: new possibilities for the

> inheritance of structure. Curr Opin Genet Dev *12*, 178-187.

Grigliatti, T. (1991). Position-effect variegation--an assay for nonhistone

> chromosomal proteins and chromatin assembly and modifying factors.
> Methods Cell Biol *35*, 587-627.

Grunstein, M. (1997). Histone acetylation in chromatin structure and

> transcription. Nature *389*, 349-352.

Grunstein, M. (1998). Yeast heterochromatin: regulation of its assembly and

> inheritance by histones. Cell *93*, 325-328.

Hall, I. M., Shankaranarayana, G. D., Noma, K., Ayoub, N., Cohen, A., and
Grewal, S. I. (2002). Establishment and maintenance of a heterochromatin
domain. Science *297*, 2232-2237.

Hall, L. M., Mason, P. J., and Spierer, P. (1983). Transcripts, genes and bands in
315,000 base-pairs of Drosophila DNA. J Mol Biol *169*, 83-96.

Hansen, J. C. (2002). Conformational dynamics of the chromatin fiber in
solution: determinants, mechanisms, and functions. Annu Rev Biophys
Biomol Struct *31*, 361-392.

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

Hassig, C. A., Tong, J. K., Fleischer, T. C., Owa, T., Grable, P. G., Ayer, D. E.,
and Schreiber, S. L. (1998). A role for histone deacetylase activity in
HDAC1-mediated transcriptional repression. Proc Natl Acad Sci U S A
95, 3519-3524.

Heard, E., Rougeulle, C., Arnaud, D., Avner, P., Allis, C. D., and Spector, D. L.
(2001). Methylation of histone H3 at Lys-9 is an early mark on the X
chromosome during X inactivation. Cell *107*, 727-738.

Hearn, M. G., Hedrick, A., Grigliatti, T. A., and Wakimoto, B. T. (1991). The
effect of modifiers of position-effect variegation on the variegation of
heterochromatic genes of Drosophila melanogaster. Genetics *128*, 785-
797.

Hecht, A., Strahl-Bolsinger, S., and Grunstein, M. (1996). Spreading of
transcriptional repressor SIR3 from telomeric heterochromatin. Nature
383, 92-96.

Heinzel, T., Lavinsky, R. M., Mullen, T. M., Soderstrom, M., Laherty, C. D., Torchia, J., Yang, W. M., Brard, G., Ngo, S. D., Davie, J. R., *et al.* (1997). A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression [see comments]. Nature *387*, 43-48.

Henikoff, S. (1992). Position effect and related phenomena. Curr Opin Genet Dev *2*, 907-912.

Henikoff, S. (1994). A reconsideration of the mechanism of position effect. Genetics *138*, 1-5.

Henikoff, S. (2000). Heterochromatin function in complex genomes. Biochim Biophys Acta *1470*, O1-8.

Hess, O. (1970). Independence between modification of genetic position effect and formation of lampbrush loops by the Y chromosome of *Drosophila hydei*. Molec Gen Genet *107*, 224-242.

Hessler, A. (1958). V-type position effects at the light locus in *Drosophila melanogaster*. Genetics *43*, 395-403.

Hinton, T. (1949). The modification of teh expression of a position effect. Am Nat *83*, 69-94.

Hinton, T., and Goodsmith, W. (1950). An analysis of phenotypic reversions at the brown locus in *Drosophila*. J Exp Zool *114*, 103-114.

Horn, P. J., and Peterson, C. L. (2002). Molecular biology. Chromatin higher order folding--wrapping up transcription. Science *297*, 1824-1827.

Horsley, D., Hutchings, A., Butcher, G. W., and Singh, P. B. (1996). M32, a murine homologue of Drosophila heterochromatin protein 1 (HP1), localises to euchromatin within interphase nuclei and is largely excluded from constitutive heterochromatin. Cytogenet Cell Genet *73*, 308-311.

Hwang, K. K., Eissenberg, J. C., and Worman, H. J. (2001). Transcriptional repression of euchromatic genes by Drosophila heterochromatin protein 1 and histone modifiers. Proc Natl Acad Sci U S A *98*, 11423-11427.

Jacobs, S. A., Taverna, S. D., Zhang, Y., Briggs, S. D., Li, J., Eissenberg, J. C., Allis, C. D., and Khorasanizadeh, S. (2001). Specificity of the HP1 chromo domain for the methylated N-terminus of histone H3. Embo J *20*, 5232-5241.

James, T. C., Eissenberg, J. C., Craig, C., Dietrich, V., Hobson, A., and Elgin, S. C. (1989). Distribution patterns of HP1, a heterochromatin-associated nonhistone chromosomal protein of Drosophila. Eur J Cell Biol *50*, 170-180.

James, T. C., and Elgin, S. C. (1986). Identification of a nonhistone chromosomal protein associated with heterochromatin in Drosophila melanogaster and its gene. Mol Cell Biol *6*, 3862-3872.

Janning, W. (1970). [Timing of heterochromatization in white-variegated Drosophila melanogaster by x-ray induced mitotic recombination in the eye anlage]. Mol Gen Genet *107*, 128-149.

Jaquet, Y., Delattre, M., Spierer, A., and Spierer, P. (2002). Functional dissection of the Drosophila modifier of variegation Su(var)3-7. Development *129*, 3975-3982.

Jenuwein, T., Laible, G., Dorn, R., and Reuter, G. (1998). SET domain proteins modulate chromatin domains in eu- and heterochromatin. Cell Mol Life Sci *54*, 80-93.

Johnson, C. A., Barlow, A. L., and Turner, B. M. (1998). Molecular cloning of Drosophila melanogaster cDNAs that encode a novel histone deacetylase dHDAC3. Gene *221*, 127-134.

Jones, P. A., and Takai, D. (2001). The role of DNA methylation in mammalian epigenetics. Science *293*, 1068-1070.

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

Kadosh, D., and Struhl, K. (1997). Repression by Ume6 involves recruitment of a complex containing Sin3 corepressor and Rpd3 histone deacetylase to target promoters. Cell *89*, 365-371.

Kadosh, D., and Struhl, K. (1998). Histone deacetylase activity of Rpd3 is important for transcriptional repression in vivo. Genes Dev *12*, 797-805.

Karpen, G. H., and Spradling, A. C. (1992). Analysis of subtelomeric heterochromatin in the Drosophila minichromosome Dp1187 by single P element insertional mutagenesis. Genetics *132*, 737-753.

Khesin, R. B., and Leibovitch, B. A. (1978). Influence of deficiency of the histone gene-containing 38B-40 region on X-chromosome template activity and the white gene position effect variegation in Drosophila melanogaster. Mol Gen Genet *162*, 323-328.

Khochbin, S., and Wolffe, A. P. (1997). The origin and utility of histone deacetylases. FEBS Lett *419*, 157-160.

Kuo, M. H., and Allis, C. D. (1998). Roles of histone acetyltransferases and deacetylases in gene regulation. Bioessays *20*, 615-626.

Lachner, M., O'Carroll, D., Rea, S., Mechtler, K., and Jenuwein, T. (2001). Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature *410*, 116-120.

Laherty, C. D., Yang, W. M., Sun, J. M., Davie, J. R., Seto, E., and Eisenman, R. N. (1997). Histone deacetylases associated with the mSin3 corepressor mediate mad transcriptional repression. Cell *89*, 349-356.

Lawrence, P. (1992). The Making of a Fly (Oxford, Blackwell Science Ltd.).

Lefevre, G. (1976). A photographic representation and interretation of the polytene chromosomes of *Drosophila melanogaster* salivary glands. In The Genetics and Biology of *Drosophila*, M. a. E. N. Ashburner, ed. (London, Academic Press), pp. 32-64.

Levis, R., Hazelrigg, T., and Rubin, G. M. (1985). Separable cis-acting control elements for expression of the white gene of Drosophila. Embo J *4*, 3489-3499.

Li, Y., Kirschmann, D. A., and Wallrath, L. L. (2002). Does heterochromatin protein 1 always follow code? Proc Natl Acad Sci U S A *99 Suppl 4*, 16462-16469.

Lindsley, D., and Zimm, G. (1992). The Genome of *Drosophila melanogaster* (San Diego, Academic Press).

Lloyd, V. K., Sinclair, D. A., and Grigliatti, T. A. (1997). Competition between different variegating rearrangements for limited heterochromatic factors in Drosophila melanogaster. Genetics *145*, 945-959.

Locke, J., Kotarski, M. A., and Tartof, K. D. (1988). Dosage-dependent modifiers of position effect variegation in Drosophila and a mass action model that explains their effect. Genetics *120*, 181-198.

Lowell, C. A., and Soriano, P. (1996). Knockouts of Src-family kinases: stiff

bones, wimpy T cells, and bad memories. Genes Dev *10*, 1845-1857.

Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F., and Richmond, T. J.

(1997). Crystal structure of the nucleosome core particle at 2.8 A

resolution. Nature *389*, 251-260.

Luo, R. X., Postigo, A. A., and Dean, D. C. (1998). Rb interacts with histone

deacetylase to repress transcription. Cell *92*, 463-473.

Madhani, H. D., and Fink, G. R. (1998). The riddle of MAP kinase signaling

specificity. Trends Genet *14*, 151-155.

Madhani, H. D., Styles, C. A., and Fink, G. R. (1997). MAP kinases with distinct

inhibitory functions impart signaling specificity during yeast

differentiation. Cell *91*, 673-684.

Magnaghi-Jaulin, L., Groisman, R., Naguibneva, I., Robin, P., Lorain, S., Le

Villain, J. P., Troalen, F., Trouche, D., and Harel-Bellan, A. (1998).

Retinoblastoma protein represses transcription by recruiting a histone

deacetylase [see comments]. Nature *391*, 601-605.

Mannervik, M., and Levine, M. (1999). The rpd3 histone deacetylase is required

for segmentation of the drosophila embryo [In Process Citation]. Proc

Natl Acad Sci U S A *96*, 6797-6801.

Martienssen, R., and Henikoff, S. (1999). The House & Garden guide to

chromatin remodelling. Nat Genet *22*, 6-7.

Messmer, S., Franke, A., and Paro, R. (1992). Analysis of the functional role of

the Polycomb chromo domain in Drosophila melanogaster. Genes Dev *6*,

1241-1254.

·Michailidis, J., Murray, N., and Graves, J. (1988). A correlation between developmental time and variegated position-effect in *Drosophila melanogaster*. Genet Res *52*, 119-123.

Milne, T. A., Briggs, S. D., Brock, H. W., Martin, M. E., Gibbs, D., Allis, C. D., and Hess, J. L. (2002). MLL targets SET domain methyltransferase activity to Hox gene promoters. Mol Cell *10*, 1107-1117.

Minc, E., Allory, Y., Worman, H. J., Courvalin, J. C., and Buendia, B. (1999). Localization and phosphorylation of HP1 proteins during the cell cycle in mammalian cells. Chromosoma *108*, 220-234.

Mizzen, C. A., Yang, X. J., Kokubo, T., Brownell, J. E., Bannister, A. J., Owen-Hughes, T., Workman, J., Wang, L., Berger, S. L., Kouzarides, T., *et al.* (1996). The TAF(II)250 subunit of TFIID has histone acetyltransferase activity. Cell *87*, 1261-1270.

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

Mottus, R. (1979) The effect of n-butyrate and n-proprionate on postion effect variegation in *Drosophila melanogaster*, BSc, University of British Columbia, Vancouver.

Mottus, R. (1983) Position effect variegation in *Drosophila melanogaster*: chemical modification and mutational analysis, MSc, University of British Columbia, Vancouver.

Mottus, R., Reeves, R., and Grigliatti, T. A. (1980). Butyrate suppression of position-effect variegation in Drosophila melanogaster. Mol Gen Genet *178*, 465-469.

Mottus, R., Sobel, R. E., and Grigliatti, T. A. (2000). Mutational analysis of a histone deacetylase in Drosophila melanogaster: missense mutations suppress gene silencing associated with position effect variegation. Genetics *154*, 657-668.

Mottus, R. C., Whitehead, I. P., O'Grady, M., Sobel, R. E., Burr, R. H., Spiegelman, G. B., and Grigliatti, T. A. (1997). Unique gene organization: alternative splicing in Drosophila produces two structurally unrelated proteins. Gene *198*, 229-236.

Nagy, L., Kao, H. Y., Chakravarti, D., Lin, R. J., Hassig, C. A., Ayer, D. E., Schreiber, S. L., and Evans, R. M. (1997). Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. Cell *89*, 373-380.

Nakayama, J., Rice, J. C., Strahl, B. D., Allis, C. D., and Grewal, S. I. (2001). Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. Science *292*, 110-113.

Narlikar, G. J., Fan, H. Y., and Kingston, R. E. (2002). Cooperation between complexes that regulate chromatin structure and transcription. Cell *108*, 475-487.

Ner, S. S., Harrington, M. J., and Grigliatti, T. A. (2002). A Role for the Drosophila SU(VAR)3-9 Protein in Chromatin Organization at the Histone Gene Cluster and in Suppression of Position-Effect Variegation. Genetics *162*, 1763-1774.

Nielsen, S. J., Schneider, R., Bauer, U. M., Bannister, A. J., Morrison, A., O'Carroll, D., Firestein, R., Cleary, M., Jenuwein, T., Herrera, R. E., and

Kouzarides, T. (2001). Rb targets histone H3 methylation and HP1 to promoters. Nature *412*, 561-565.

Noma, K., Allis, C. D., and Grewal, S. I. (2001). Transitions in distinct histone H3 methylation patterns at the heterochromatin domain boundaries. Science *293*, 1150-1155.

Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996). The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell *87*, 953-959.

Okano, M., Bell, D. W., Haber, D. A., and Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell *99*, 247-257.

Orlando, V., and Paro, R. (1993). Mapping Polycomb-repressed domains in the bithorax complex using in vivo formaldehyde cross-linked chromatin. Cell *75*, 1187-1198.

Paro, R., and Hogness, D. S. (1991). The Polycomb protein shares a homologous domain with a heterochromatin-associated protein of Drosophila. Proc Natl Acad Sci U S A *88*, 263-267.

Peters, A. H., Mermoud, J. E., O'Carroll, D., Pagani, M., Schweizer, D., Brockdorff, N., and Jenuwein, T. (2002). Histone H3 lysine 9 methylation is an epigenetic imprint of facultative heterochromatin. Nat Genet *30*, 77-80.

Peters, A. H., O'Carroll, D., Scherthan, H., Mechtler, K., Sauer, S., Schofer, C., Weipoltshammer, K., Pagani, M., Lachner, M., Kohlmaier, A., *et al.* (2001). Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. Cell *107*, 323-337.

Pile, L. A., and Wassarman, D. A. (2000). Chromosomal localization links the SIN3-RPD3 complex to the regulation of chromatin condensation, histone acetylation and gene expression. Embo J 19, 6131-6140.

Pirrotta, V., Steller, H., and Bozzetti, M. P. (1985). Multiple upstream regulatory elements control the expression of the Drosophila white gene. Embo J 4, 3501-3508.

Platero, J. S., Ahmad, K., and Henikoff, S. (1999). A distal heterochromatic block displays centromeric activity when detached from a natural centromere. Mol Cell 4, 995-1004.

Rabl, C. (1885). Uber Zelltheilung. Morph Jb 10, 214-330.

Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B. D., Sun, Z. W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C. P., Allis, C. D., and Jenuwein, T. (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature 406, 593-599.

Reeves, R., and Cserjesi, P. (1979). Sodium butyrate induces new gene expression in Friend erythroleukemic cells. J Biol Chem 254, 4283-4290.

Reid, J. L., Iyer, V. R., Brown, P. O., and Struhl, K. (2000). Coordinate regulation of yeast ribosomal protein genes is associated with targeted recruitment of Esa1 histone acetylase. Mol Cell 6, 1297-1307.

Reuter, G., Gausz, J., Gyurkovics, H., Friede, B., Bang, R., Spierer, A., Hall, L. M., and Spierer, P. (1987). Modifiers of position-effect variegation in the region from 86C to 88B of the Drosophila melanogaster third chromosome. Mol Gen Genet 210, 429-436.

Reuter, G., Giarre, M., Farah, J., Gausz, J., Spierer, A., and Spierer, P. (1990). Dependence of position-effect variegation in Drosophila on dose of a gene encoding an unusual zinc-finger protein. Nature *344*, 219-223.

Reuter, G., and Spierer, P. (1992). Position effect variegation and chromatin proteins. Bioessays *14*, 605-612.

Reuter, G., and Wolff, I. (1981). Isolation of dominant suppressor mutations for position-effect variegation in Drosophila melanogaster. Mol Gen Genet *182*, 516-519.

Richards, E. J., and Elgin, S. C. (2002). Epigenetic codes for heterochromatin formation and silencing: rounding up the usual suspects. Cell *108*, 489-500.

Robyr, D., Suka, Y., Xenarios, I., Kurdistani, S. K., Wang, A., Suka, N., and Grunstein, M. (2002). Microarray deacetylation maps determine genome-wide functions for yeast histone deacetylases. Cell *109*, 437-446.

Rundlett, S. E., Carmen, A. A., Kobayashi, R., Bavykin, S., Turner, B. M., and Grunstein, M. (1996). HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription. Proc Natl Acad Sci U S A *93*, 14503-14508.

Rundlett, S. E., Carmen, A. A., Suka, N., Turner, B. M., and Grunstein, M. (1998). Transcriptional repression by UME6 involves deacetylation of lysine 5 of histone H4 by RPD3. Nature *392*, 831-835.

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

Sabbattini, P., Lundgren, M., Georgiou, A., Chow, C., Warnes, G., and Dillon, N. (2001). Binding of Ikaros to the lambda5 promoter silences transcription through a mechanism that does not require heterochromatin formation. Embo J 20, 2812-2822.

Sadoni, N., Langer, S., Fauth, C., Bernardi, G., Cremer, T., Turner, B. M., and Zink, D. (1999). Nuclear organization of mammalian genomes. Polar chromosome territories build up functionally distinct higher order compartments. J Cell Biol 146, 1211-1226.

Sambrook, J., Fritsch, E., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, NY, Cold Spring Harbor Press).

Sass, G. L., and Henikoff, S. (1999). Pairing-dependent mislocalization of a Drosophila brown gene reporter to a heterochromatic environment. Genetics 152, 595-604.

Schotta, G., Ebert, A., Dorn, R., and Reuter, G. (2003). Position-effect variegation and the genetic dissection of chromatin regulation in Drosophila. Semin Cell Dev Biol 14, 67-75.

Schotta, G., Ebert, A., Krauss, V., Fischer, A., Hoffmann, J., Rea, S., Jenuwein, T., Dorn, R., and Reuter, G. (2002). Central role of Drosophila SU(VAR)3-9 in histone H3-K9 methylation and heterochromatic gene silencing. Embo J 21, 1121-1131.

Schultz, J. (1936). Variegation in *Drosophila* and the inert chromosome regions. Proc Natl Acad Sci U S A 22, 27-33.

Schultz, J. (1956). The relation of the heterochromatic chromosomes regions to the nucleic acids of the cell. Cold Spring Harb Symp Quant Biol *21*, 307-328.

Sinclair, D., Mottus, R., and Grigliatti, T. (1983). Genes which suppress position-effect variegation in *Drosophila melanogaster* are clustered. Mol Gen Genet *191*, 326-333.

Sinclair, D. A., Clegg, N. J., Antonchuk, J., Milne, T. A., Stankunas, K., Ruse, C., Grigliatti, T. A., Kassis, J. A., and Brock, H. W. (1998). Enhancer of Polycomb is a suppressor of position-effect variegation in Drosophila melanogaster. Genetics *148*, 211-220.

Smothers, J. F., and Henikoff, S. (2000). The HP1 chromo shadow domain binds a consensus peptide pentamer. Curr Biol *10*, 27-30.

Spierer, P., Spierer, A., Bender, W., and Hogness, D. S. (1983). Molecular mapping of genetic and chromomeric units in Drosophila melanogaster. J Mol Biol *168*, 35-50.

Spofford, J. (1976). Position-effect Variegation in *Drosophila*. In The Genetics and Biology of *Drosophila*, M. a. E. N. Ashburner, ed. (New York, Academic Press), pp. 955-1018.

Spotswood, H. T., and Turner, B. M. (2002). An increasingly complex code. J Clin Invest *110*, 577-582.

Spradling, A. C., and Karpen, G. H. (1990). Sixty years of mystery. Genetics *126*, 779-784.

Stern, C., and Kodani, M. (1955). Studies on the position effect at the cubitus interruptus locus of *Drosophila melanogaster*. Genetics *40*, 343-373.

Strahl, B. D., and Allis, C. D. (2000). The language of covalent histone modifications. Nature 403, 41-45.

Suka, N., Luo, K., and Grunstein, M. (2002). Sir2p and Sas2p opposingly regulate acetylation of yeast histone H4 lysine16 and spreading of heterochromatin. Nat Genet 32, 378-383.

Suka, N., Suka, Y., Carmen, A. A., Wu, J., and Grunstein, M. (2001). Highly specific antibodies determine histone acetylation site usage in yeast heterochromatin and euchromatin. Mol Cell 8, 473-479.

Sun, F. L., Cuaycong, M. H., and Elgin, S. C. (2001). Long-range nucleosome ordering is associated with gene silencing in Drosophila melanogaster pericentric heterochromatin. Mol Cell Biol 21, 2867-2879.

Talbert, P. B., and Henikoff, S. (2000). A reexamination of spreading of position-effect variegation in the white-roughest region of Drosophila melanogaster. Genetics 154, 259-272.

Tartof, K. D., Hobbs, C., and Jones, M. (1984). A structural basis for variegating position effects. Cell 37, 869-878.

Taunton, J., Hassig, C. A., and Schreiber, S. L. (1996). A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p [see comments]. Science 272, 408-411.

Tschiersch, B., Hofmann, A., Krauss, V., Dorn, R., Korge, G., and Reuter, G. (1994). The protein encoded by the Drosophila position-effect variegation suppressor gene Su(var)3-9 combines domains of antagonistic regulators of homeotic gene complexes. Embo J 13, 3822-3831.

Tumbar, T., Sudlow, G., and Belmont, A. S. (1999). Large-scale chromatin unfolding and remodeling induced by VP16 acidic activation domain. J Cell Biol *145*, 1341-1354.

Turner, B. M. (1998). Histone acetylation as an epigenetic determinant of long-term transcriptional competence. Cell Mol Life Sci *54*, 21-31.

Turner, B. M. (2002). Cellular memory and the histone code. Cell *111*, 285-291.

van Steensel, B., Delrow, J., and Henikoff, S. (2001). Chromatin profiling using targeted DNA adenine methyltransferase. Nat Genet *27*, 304-308.

van Steensel, B., and Henikoff, S. (2000). Identification of in vivo DNA targets of chromatin proteins using tethered dam methyltransferase. Nat Biotechnol *18*, 424-428.

Vidal, M., and Gaber, R. F. (1991). RPD3 encodes a second factor required to achieve maximum positive and negative transcriptional states in Saccharomyces cerevisiae. Mol Cell Biol *11*, 6317-6327.

Vogelauer, M., Wu, J., Suka, N., and Grunstein, M. (2000). Global histone acetylation and deacetylation in yeast. Nature *408*, 495-498.

Volpe, T. A., Kidner, C., Hall, I. M., Teng, G., Grewal, S. I., and Martienssen, R. A. (2002). Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science *297*, 1833-1837.

Wang, L., Mizzen, C., Ying, C., Candau, R., Barlev, N., Brownell, J., Allis, C. D., and Berger, S. L. (1997). Histone acetyltransferase activity is conserved between yeast and human GCN5 and is required for complementation of growth and transcriptional activation. Mol Cell Biol *17*, 519-527.

Weiler, K. S., and Wakimoto, B. T. (1995). Heterochromatin and gene expression in Drosophila. Annu Rev Genet *29*, 577-605.

Workman, J., and Kingston, R. (1998). Alterations of nucleosome structure as a mechanism of transcriptional regulation. Annu Rev Biochem *67*, 545-579.

Wu, J., Carmen, A. A., Kobayashi, R., Suka, N., and Grunstein, M. (2001). HDA2 and HDA3 are related proteins that interact with and are essential for the activity of the yeast histone deacetylase HDA1. Proceedings of the National Academy of Sciences of the United States of America *98*, 4391-4396.

Wustmann, G., Szidonya, J., Taubert, H., and Reuter, G. (1989). The genetics of position-effect variegation modifying loci in Drosophila melanogaster. Mol Gen Genet *217*, 520-527.

Xu, G. L., Bestor, T. H., Bourc'his, D., Hsieh, C. L., Tommerup, N., Bugge, M., Hulten, M., Qu, X., Russo, J. J., and Viegas-Pequignot, E. (1999). Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature *402*, 187-191.

Xue, Y., Wong, J., Moreno, G. T., Young, M. K., Cote, J., and Wang, W. (1998). NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. Mol Cell *2*, 851-861.

Yang, W. M., Inouye, C., Zeng, Y., Bearss, D., and Seto, E. (1996a). Transcriptional repression by YY1 is mediated by interaction with a mammalian homolog of the yeast global regulator RPD3. Proc Natl Acad Sci U S A *93*, 12845-12850.

Yang, X. J., Ogryzko, V. V., Nishikawa, J., Howard, B. H., and Nakatani, Y. (1996b). A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. Nature *382*, 319-324.

Zhang, Y., LeRoy, G., Seelig, H. P., Lane, W. S., and Reinberg, D. (1998). The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities. Cell *95*, 279-289.

Zuckerkandl, E. (1974). A possible role of "inert" heterochromatin in cell differentiation. Action of and competition for "locking" molecules. Biochimie *56*, 937-954.

Appendix I

List of Abbreviations

bw^{vDe2}	brown variegated of Demerec 2
DUb80	*D. melanogaster* ubiquitin fusion protein 80
e	*ebony*
E(var)s	enhancer of position effect variegation
GCN5	histone acetyltransferases from *S. cerevisiae*
H3 K9	lysine nine of histone H3
H3 mK9	methylated lysine nine of histone H3
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDAC1	histone deacetylase one
HOX	vertebrate homeotic genes
In(1)sc^{s1}	inversion one scute^{s1}
In(1)w^{m4}	inversion one white mottled four
In(3LR)TM3 Δ2-3 Sb	inversion three left right bearing the transposase source Δ2-3 and *Stubble*
ISW1	chromatin remodeling protein
MAD	matrix associated deacetylase bodies
Mi-2	ATP dependent nucleosome remodeling factor
N-CoR	nuclear receptor corepressor

NuRD	nucleosome remodeling complex
P-1.8	a fly strain bearing a P element inserted 1.8 kb 5' to the start of HDAC1
p300/CBP	transcriptional coactivator
PCAF	histone acetyltransferase
PcG	Polycomb Group of proteins
PCR	polymerase chain reaction
PEV	position effect variegation
P-UTR	a fly strain bearing a P element inserted into the 5' untranslated region of *HDAC1*
RB	retinoblastoma protein
RPD3	reduced potassium dependency three
ry^{506}	an allele of the *rosy* gene from *D. melanogaster*
ry^{RK}	an allele of the *rosy* gene from *D. melanogaster*
Sb	the *Stubble* gene from *D. melanogaster*
Sb^{v}	a variegating allele of *Stubble*
Ser	the *Serrate* gene from *D. melanogaster*
SMRT	silencing mediator for retinoic acid and thyroid hormone receptors
SNF2	sucrose non-fermenting two from *S. cerevisiae*
Su(var)s	suppressor of position effect variegation
SWI2	an ATP dependent helicase
$TAF_{II}230/250$	transcription associated factor II
Tb	the *Tubby* gene from *D. melanogaster*

TM3	a multiply inverted third chromosome which suppresses recombination in *D. melanogaster*
TM6	a multiply inverted third chromosome which suppresses recombination in *D. melanogaster*
tsp	temperature sensitive period
YY1	yin/yang transcriptional corepressor and activator