Mutational Analysis of a Histone Deacetylase in *Drosophila melanogaster*: Missense Mutations Suppress Gene Silencing Associated With Position Effect Variegation

Randy Mottus, Richard E. Sobel[1] and Thomas A. Grigliatti

Department of Zoology, University of British Columbia, Vancouver, British Columbia V6T 1Z4, Canada

Manuscript received June 26, 1999
Accepted for publication October 5, 1999

ABSTRACT

For many years it has been noted that there is a correlation between acetylation of histones and an increase in transcriptional activity. One prediction, based on this correlation, is that hypomorphic or null mutations in histone deacetylase genes should lead to increased levels of histone acetylation and result in increased levels of transcription. It was therefore surprising when it was reported, in both yeast and fruit flies, that mutations that reduced or eliminated a histone deacetylase resulted in transcriptional silencing of genes subject to telomeric and heterochromatic position effect variegation (PEV). Here we report the first mutational analysis of a histone deacetylase in a multicellular eukaryote by examining six new mutations in *HDAC1* of *Drosophila melanogaster*. We observed a suite of phenotypes accompanying the mutations consistent with the notion that HDAC1 acts as a global transcriptional regulator. However, in contrast to recent findings, here we report that specific missense mutations in the structural gene of *HDAC1* suppress the silencing of genes subject to PEV. We propose that the missense mutations reported here are acting as antimorphic mutations that "poison" the deacetylase complex and propose a model that accounts for the various phenotypes associated with lesions in the deacetylase locus.

THE basic unit of chromatin is the nucleosome, which consists of ~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 that 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 that are targeted to the genes they regulate by members of the complex with 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 HATs, histone deacetylases (HDACs) also exist as members of large multiprotein complexes. However, an unexpected finding was that some HDAC complexes, 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 corepressor 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), which 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 that 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 predic-

Corresponding author: Thomas A. Grigliatti, Department of Zoology, University of British Columbia, 6270 University Blvd., Vancouver, British Columbia V6T 1Z4, Canada. E-mail: grigliat@zoology.ubc.ca

[1] *Present address:* Prostate Center, Jack Bell Research Center, Vancouver General Hospital, 2660 Oak St., Vancouver, British Columbia V6H 3Z6, Canada.

tion 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 derepression of the targets. This also appeared to be the case. Mutational analysis in *Saccharomyces 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 derepression of silenced genes was confounded when it was reported, in both *S. cerevisiae* and Drosophila, that mutations that 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 *Drosophila melanogaster*. This is the first instance of a mutational analysis of an HDAC in a multicellular eukaryotic organism. In contrast to previous findings, we report that specific missense mutations in the structural gene of *HDAC1* suppress silencing and increase the expression of a w^+ gene subject to PEV. We propose that these missense mutations are acting as antimorphic 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 missense, 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, multidomain family of proteins that forms part of a large multiprotein 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.

MATERIALS 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 (for reduced potassium dependency 3) 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 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, because 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 use that nomenclature in this article.

The *HDAC1* mutations that suppress PEV (hereafter called the *Su*(*var*) *HDAC1*s) 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*) *HDAC1*s. One insert line, l(3)04556 (hereafter called P-UTR), was almost completely lethal under normal culture conditions with all members 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*. Because 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. F_1 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 and batches of ~100 eggs were counted out on construction paper and placed in shell vials. A minimum of five shell vials were set up for each mutant strain. The construction paper was removed after 3 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 was 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 F_1 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 14 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 be 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^+ Δ*2-3*] males. The *TM3* ry^{RK} *Sb e P*[ry^+ Δ*2-3*] chromosome carries a *P*-element transposase source (Δ*2-3*) that 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^+ Δ*2-3*] males were collected and crossed to +/+; ry^{506}/ry^{506} females and ry^- F_2 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 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 manufacturer's instructions (Life Technologies). Poly(A) RNA was subsequently isolated employing the Oligotex mRNA mini kit produced by QIAGEN (Chatsworth, CA) following the manufacturer's 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, Buckinghamshire, UK), and probed with DNA labeled with [^{32}P]dATP using Boehringer Mannheim's (Laval, Quebec) 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 were determined by reprobing 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 *Su*ppressors of position effect *var*iegation [*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 (Reuter and Wolff 1981; Sinclair *et al.* 1983; Locke *et al.* 1988), 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 because its product is required for deposition of pigment, the eyes of flies in the w^{m4} strain generally have ~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*) *HDAC1*s or individually as $HDAC1^{303}$, $HDAC1^{313}$, $HDAC1^{326}$, and $HDAC1^{328}$]. All are strong dominant sup-

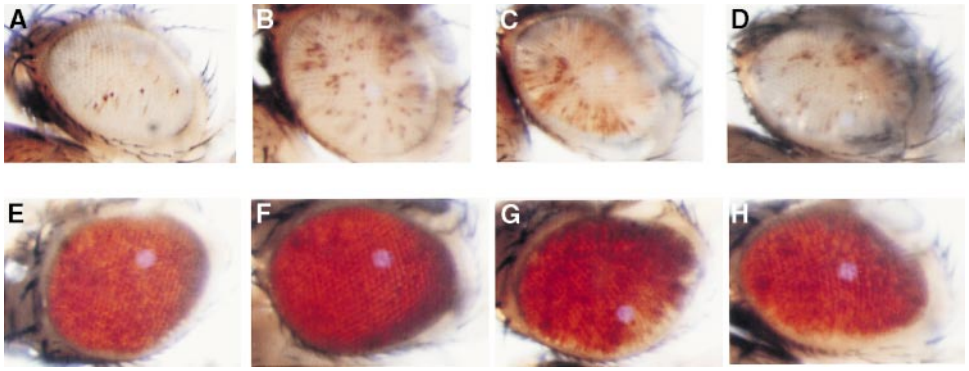

Figure 1.—Examples of eyes from male flies bearing the In(1)w^{m4} chromosome and third chromosomes of the following constitutions: (A) + /+; (B) *P-UTR*/+; (C) *HDAC1*^{def8}/+; (D) *HDAC1*^{def24}/+; (E) *HDAC1*^{303}/+; (F) *HDAC1*^{313}/+; (G) *HDAC1*^{326}/+; and (H) *HDAC1*^{328}/+.

pressors of PEV and, in addition to the dominant phenotype, all four alleles are recessive lethal. In *w*^{m4} strains bearing the *Su*(*var*) *HDAC1*s, pigments in the eyes of both males and females are increased from 5 to 15% to 60 to 90% of the pigment levels observed in the wild-type strain OR-R (Figure 1 and Table 1).

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*) *HDAC1*s, *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*) *HDAC1*s suppress the gene silencing associated with PEV and are not mutations in factors that specifically modify the *w*$^{+}$ gene.

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.

Mapping the *Su*(*var*) *HDAC1*s: We mapped the recessive lethality associated with the *Su*(*var*) *HDAC1*s to 64B17-64C13-15 employing deficiencies and confirmed the *Su*(*var*) 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 that causes strong dominant enhancement of PEV but is homozygous viable and fertile. However, complementation analysis with the *Su*(*var*) *HDAC1*s 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*) *HDAC1*s represented two different genes.

We then crossed the *Su*(*var*) *HDAC1*s 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*) *HDAC1*s 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*) *HDAC1*s, it has no dominant effect on variegation of *w*$^{+}$ in the In(1)w^{m4} strain (Figure 1 and Table 4). Because 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*) *HDAC1*s. Alternatively, it was possible that the *Su*(*var*) *HDAC1*s, 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.

First, 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 recov-

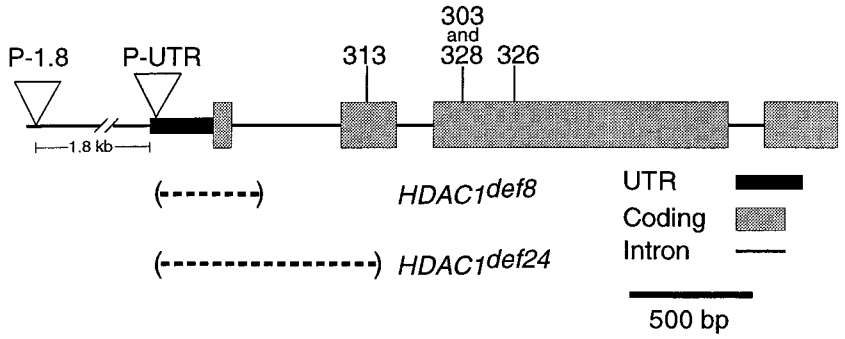

Figure 2.—Genomic organization of the *HDAC1* gene. The approximate location of each of the *Su*(*var*) *HDAC1* group mutations is shown, the insertion points of the *P* elements in the P-1.8 and P-UTR strains are indicated by the triangles, and dashed lines indicate the extent of the deficiencies in the deletion strains.

ered 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*) *HDAC1*s. 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. Because 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*) *HDAC1*s also have a lethal lesion in the *HDAC1* gene.

Second, to determine whether the *Su*(*var*) *HDAC1*s, 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 display 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 $<1.6 \times 10^{-2}$ map units apart, a distance representing ~4–7 kb of DNA in a typical region of the Drosophila genome (Lefevre 1976). Because transcript analysis has shown that there are no other transcripts within ~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.

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 $HDAC1^{328}$/*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.

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 lesions 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. To further examine these phenotypes we generated $HDAC1^{303}$/*P-UTR* flies. In this cross, under carefully maintained culture conditions, adult males eclosed at ~40% of expected and females at ~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; allila that were larger than normal; and a reduction in the number of sex combs on the legs of the males 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 and is consistent with its proposed role as a global transcriptional regulator. It also suggests that the *Su*(*var*) *HDAC1*s retain at least some of their functions, because 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*) *HDAC1*s.

Because P-UTR and the *Su*(*var*) *HDAC1*s 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. To further characterize the requirements for *HDAC1*, we determined the developmental time at which *HDAC1* is required for survival in *D. melanogaster*. Because P-UTR is a very strong hypomorph

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.

(Mannervik and Levine 1999) and we were unable to determine whether or not the *Su*(*var*) *HDAC1*s 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 the 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 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 favor the second of the above three possibilities. We also conducted lethal phase analyses of the *Su*(*var*) *HDAC1*s. $HDAC1^{313}$ 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 ~50% of larvae bearing $HDAC1^{303}$ and $HDAC1^{328}$ died during the larval period while ~50% survived into pupation. This is consistent with the sequencing data (see below) that demonstrated that 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*) *HDAC1*s appeared to have a dominant semilethal 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 that are 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 ~50% the level of their genotypically identical female siblings in the same cross. Males in crosses involving the other *Su*(*var*) *HDAC1*s also survived at significantly lower levels than females. Because 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 hypertranscription of the male X chromosome. The male X chromosome adopts a special conformation that 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*) *HDAC1*s 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 that was originally employed in the screen for *Su*(*var*) mutations. The results of this analysis are presented in Figure 2 (accession no. 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

TABLE 3

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

	HDAC1^{313} R30C	*HDAC1*^{303} *HDAC1*^{328} C98Y	*HDAC1*^{326} P204S
D. melanogaster	GHPMKPHRIRM	FNVGEDCPVFDGL	SFHKYGEYFPGTG
HDAC1 mutant strain	-------C---	------Y------	---------S---
Homo sapiens HDAC1	-------R---	------C------	---------P---
S. cerevisiae RPD3	-------R---	----D-C------	-------F-P---

A dashed line indicates identity.

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 *S. cerevisiae* 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 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 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* that 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 the members of the *Su*(*var*) *HDAC1*s 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). Because 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 because it is lethal when homozygous. One possible explanation is that, although this mutation is a hypomorph that 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*) *HDAC1*s and therefore represented potential improper excisions. DNA sequence analysis has shown we generated two deficiencies that begin at the insertion point of P-UTR and remove amino-terminal coding regions of *HDAC1*; *HDAC1*^{def8} deletes ~440 bp and *HDAC1*^{def24} deletes ~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: Because 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 hypertranscription 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 mater-

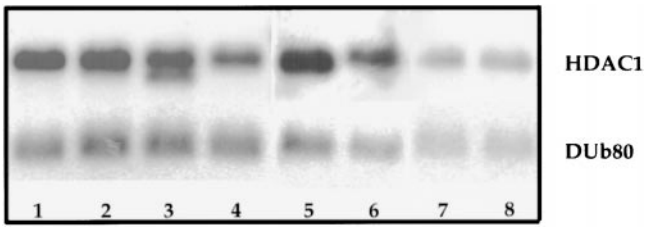

Figure 4.—Northern analysis of poly(A) RNA isolated from adults bearing *HDAC1* mutations. Lanes 1 and 6 are from wild-type female controls and lane 7 is from wild-type male controls. Lanes 2–4 are from females of the constitution *HDAC1*^{def8}/*TM3 Sb Ser*, *HDAC1*^{def24}/*TM3 Sb Ser*, and *P-UTR*/*TM3 Sb Ser*, respectively. Lanes 5 and 8 are from *HDAC1*^{326} females and males, respectively. The approximate amount of poly(A) RNA loaded in each lane was determined by reprobing the blots with a probe specific for the message for the ribosomal protein DUb80. Loading in lanes 1–4 is approximately equivalent and shows 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 poly(A) RNA as lane 6, and when taken into account, the levels of HDAC1 poly(A) RNA in *HDAC1*^{326} females and control females (lane 6) are approximately equivalent, as are the amounts in *HDAC1*^{326} males (lane 8) and control males (lane 7).

nal contribution of *HDAC1* in the P-UTR strain was approximately fivefold less than wild type. In a strain heterozygous for the deficiency, *HDAC1*^{def8}, the message produced from the deleted chromosome is reduced in amount and is evident as a widening of the 2.2-kb band produced from the nondeleted 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. Because the smaller transcripts in both deficiency strains are very unlikely to produce functional proteins, we believe these mutations represent null alleles.

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 a histone deacetylase gene, which reduce or eliminate histone deacetylase activity, ought to lead to increased levels of histone acetylation that, in turn, would lead to derepression 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 effects 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 that have the following characteristics: (1) they are members of a closely related gene family; (2) they encode multidomain 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 *et al.* 1997; Madhani and Fink 1998). Examples of this class of gene are certainly not 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 that 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, multiprotein 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. Because 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 com-

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*) *HDAC1*s
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 semilethal; in rare survivors, PEV strongly suppressed
Su(*var*) *HDAC1*s point mutations	Strong supressors	Lethal	Lethal
$HDAC1^{def8}$ deletion	No effect	Lethal	Lethal
$HDAC1^{def24}$ deletion	No effect	Lethal	Lethal

[a] For a complete description of mutations, see text.

pletely 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 multiprotein 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 have 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 analyses 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.

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 mutant 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 multiprotein 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*) *HDAC1*s 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. Because 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 effect on PEV because in heterozygotes, wild-type *HDAC1*, produced from the nondeleted 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*) *HDAC1*s to P-1.8, the strain bearing the *P*-element insertion 1.8 kb 5′ to the *HDAC1* gene, flies

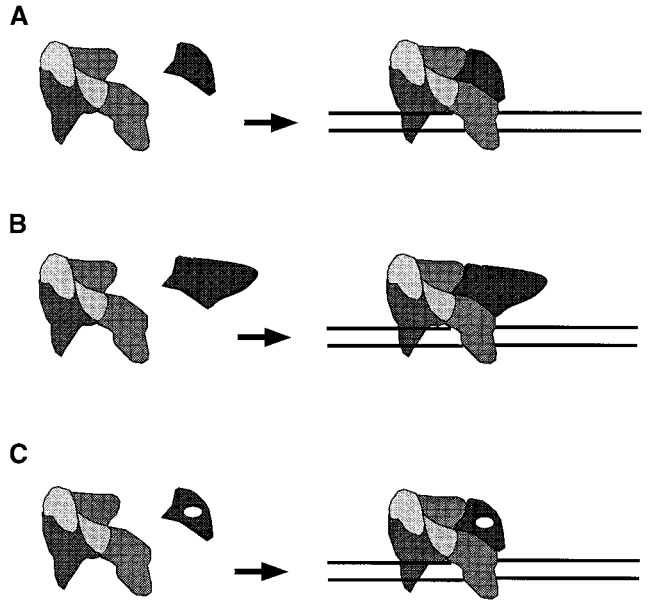

Figure 5.—A model proposed to explain the phenotypes observed in the various kinds of *HDAC1* mutations. (A) Normally, HDAC1 participates in a number of different multiprotein complexes that usually act to repress gene activity. The specific set of genes regulated by each of the complexes is determined by the DNA binding corepressors associated with the complex. In strains that are heterozygous for a complete null allele, such as a deficiency heterozygote, the nondeleted homologue produces normal HDAC1, although probably in reduced amounts, which takes its usual place in the complexes and functions normally. Thus, the structure of the complex in wild-type strains or deficiency heterozygote strains is the same. (B) In cases where no HDAC1 is available, and only when no HDAC1 is available, as in the homozygous P-1.8 strain that does not produce any HDAC1 in the eye imaginal disk or in haploid yeast strains that bear deficiencies for the *RPD3* gene, another HDAC family member takes the place of the HDAC1 homologue in the complexes. The complexes are still targeted to the genes they regulate because the DNA binding corepressors continue to participate in the complexes. However, because the incorrect HDAC is present in the complexes, aberrant deacetylation patterns are produced at regulated genes and this results in abnormal transcription, including repression of genes subject to PEV and TPEV. (C) In the *Su*(*var*) *HDAC1* strains, the single amino acid substitution does not substantially alter the conformation of HDAC1 and, accordingly, it can still bind to the complexes. However, the activity of the mutant HDAC1 is compromised and therefore it acts as a dominant negative and "poisons" the complex. Targeting occurs normally but the complex is unable to function, resulting in hyperacetylation at regulated genes. At loci subject to PEV this results in substantial increases in gene expression.

bearing both mutations were viable and fertile and showed a weak-to-moderate suppression of PEV. Because 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*) *HDAC1*s are producing a product. Third, in the complementation and recombination studies, heterozygotes bearing both the P-UTR chromosome and the *Su*(*var*) *HDAC1*s 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. Because 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*) *HDAC1*s are producing a product that 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*) *HDAC1*s displayed a dominant maternal effect reduction in the viability of males, regardless of their phenotype, a reduction that was not observed in crosses with the deficiency strains, implies that the *Su*(*var*) *HDAC*s are producing a protein product because this observed maternal effect 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 derepression 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). Because 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 derepression 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, because 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 that 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, another 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 investigated underappreciated. 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 many possible roles a protein may have because of its membership in one or more multiprotein machines.

This work was supported by the National Sciences and Engineering Research Council of Canada grant 3005-99 to T.A.G.

LITERATURE CITED

Alland, L., R. Muhle, H. Hou, Jr., J. Potes, L. Chin *et al.*, 1997 Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression. Nature **387:** 49–55.

Allfrey, V. G., R. Faulkner and A. E. Mirsky, 1964 Acetylation and methlylation of histones and their possible role in the regulation of RNA synthesis. Proc. Natl. Acad. Sci. USA **51:** 786–794.

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

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

Brehm, A., E. A. Miska, D. J. McCance, J. L. Reid, A. J. Bannister *et al.*, 1998 Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature **391:** 597–601.

Brownell, J. E., J. Zhou, T. Ranalli, R. Kobayashi, D. G. Edmondson *et al.*, 1996 Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. Cell **84:** 843–851.

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

De Rubertis, F., D. Kadosh, S. Henchoz, D. Pauli, G. Reuter *et al.*, 1996 The histone deacetylase RPD3 counteracts genomic silencing in Drosophila and yeast. Nature **384:** 589–591.

Felsenfeld, G., 1996 Chromatin unfolds. Cell **86:** 13–19.

Gibson, T. J., and J. Spring, 1998 Genetic redundancy in vertebrates: polyploidy and persistence of genes encoding multidomain proteins. Trends Genet. **14:** 46–49.

Grant, P. A., L. Duggan, J. Cote, S. M. Roberts, J. E. Brownell *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., M. J. Bonaduce and A. J. Klar, 1998 Histone deacetylase homologs regulate epigenetic inheritance of transcriptional silencing and chromosome segregation in fission yeast. Genetics **150:** 563–576.

Grigliatti, T., 1991 Position-effect variegation: an assay for nonhistone chromosomal proteins and chromatin assembly and modifying factors, pp. 587–627 in *Functional Organization of the Nucleus*, edited by B. Hamkalo and S. Elgin. Academic Press, San Diego.

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.

Hassig, C. A., J. K. Tong, T. C. Fleischer, T. Owa, P. G. Grable *et al.*, 1998 A role for histone deacetylase activity in HDAC1-mediated transcriptional repression. Proc. Natl. Acad. Sci. USA **95:** 3519–3524.

Heinzel, T., R. M. Lavinsky, T. M. Mullen, M. Soderstrom, C. D. Laherty *et al.*, 1997 A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. Nature **387:** 43–48.

Henikoff, S., 1992 Position effect and related phenomena. Curr. Opin. Genet. Dev. **2:** 907–912.

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

Kadosh, D., and K. Struhl, 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 K. Struhl, 1998 Targeted recruitment of the Sin3-Rpd3 histone deacetylase complex generates a highly localized domain of repressed chromatin in vivo. Mol. Cell. Biol. **18:** 5121–5127.

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

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

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

Laherty, C. D., W. M. Yang, J. M. Sun, J. R. Davie, E. Seto *et al.*, 1997 Histone deacetylases associated with the mSin3 corepressor mediate mad transcriptional repression. Cell **89:** 349–356.

Lefevre, G., 1976 A photographic representation and interpretation of the polytene chromosomes of *Drosophila melanogaster* salivary glands, pp. 32–64 in *The Genetics and Biology of Drosophila*, edited by M. Ashburner and E. Novitski. Academic Press, London.

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

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

Locke, J., M. A. Kotarski and K. D. Tartof, 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 P. Soriano, 1996 Knockouts of Src-family kinases: stiff bones, wimpy T cells, and bad memories. Genes Dev. **10:** 1845–1857.

Luo, R. X., A. A. Postigo and D. C. Dean, 1998 Rb interacts with histone deacetylase to repress transcription. Cell **92:** 463–473.

Madhani, H. D., and G. R. Fink, 1998 The riddle of MAP kinase signaling specificity. Trends Genet. **14:** 151–155.

Madhani, H. D., C. A. Styles and G. R. Fink, 1997 MAP kinases with distinct inhibitory functions impart signaling specificity during yeast differentiation. Cell **91:** 673–684.

Magnaghi-Jaulin, L., R. Groisman, I. Naguibneva, P. Robin, S. Lorain *et al.*, 1998 Retinoblastoma protein represses transcription by recruiting a histone deacetylase. Nature **391:** 601–605.

Mannervik, M., and M. Levine, 1999 The rpd3 histone deacetylase is required for segmentation of the Drosophila embryo. Proc. Natl. Acad. Sci. USA **96:** 6797–6801.

Mizzen, C. A., X. J. Yang, T. Kokubo, J. E. Brownell, A. J. Bannister *et al.*, 1996 The TAF(II)250 subunit of TFIID has histone acetyltransferase activity. Cell **87:** 1261–1270.

Mottus, R. C., I. P. Whitehead, M. O'Grady, R. E. Sobel, R. H. L. Burr *et al.*, 1997 Unique gene organization: alternative splicing in *Drosophila* produces two structurally unrelated proteins. Gene **198:** 229–236.

Nagy, L., H. Y. Kao, D. Chakravarti, R. J. Lin, C. A. Hassig *et al.*, 1997 Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. Cell **89:** 373–380.

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

Reuter, G., and P. Spierer, 1992 Position effect variegation and chromatin proteins. Bioessays **14:** 605–612.

Reuter, G., and I. Wolff, 1981 Isolation of dominant suppressor

mutations for position-effect variegation in Drosophila melanogaster. Mol. Gen. Genet. **182:** 516–519.

Rundlett, S. E., A. A. Carmen, R. Kobayashi, S. Bavykin, B. M. Turner *et al.*, 1996 HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription. Proc. Natl. Acad. Sci. USA **93:** 14503–14508.

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

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

Taunton, J., C. A. Hassig and S. L. Schreiber, 1996 A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science **272:** 408–411.

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

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

Wang, L., C. Mizzen, C. Ying, R. Candau, N. Barlev *et al.*, 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.

Workman, J. L., and R. E. Kingston, 1998 Alteration of nucleosome structure as a mechanism of transcriptional regulation. Annu. Rev. Biochem. **67:** 545–579.

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

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

Communicating editor: S. Henikoff