Essential role of *Drosophila Hdac1* in homeotic gene silencing

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Deacetylation of the N-terminal tails of core histones plays a crucial role in gene silencing. *Rpd3* and *Hda1* represent two major types of genes encoding trichostatin A-sensitive histone deacetylases. Although they have been widely found, their cellular and developmental roles remain to be elucidated in metazoa. We show that *Drosophila Hdac1*, an *Rpd3*-type gene, interacts cooperatively with Polycomb group repressors in silencing the homeotic genes that are essential for axial patterning of body segments. The biochemical copurification and cytological colocalization of HDAC1 and Polycomb group repressors strongly suggest that HDAC1 is a component of the silencing complex for chromatin modification on specific regulatory regions of homeotic genes.

Pc-G | histone deacetylase | *Ubx* | PRE

The identities of body segments along the anteroposterior axis are specified by a subgroup of homeobox-containing genes, including those originally identified in Antennapedia and bithorax complexes in *Drosophila* and their orthologs in other animal species (1). The spatial and temporal patterns of expression of these genes are strictly controlled during development. Alterations in their levels or domains of expression result in partial or complete homeotic transformations of certain body parts, e.g., the transformations of haltere to wing or antenna to leg (2, 3).

Two groups of antagonizing trans-acting genes are known to exert dosage-sensitive enhancing or suppressing effects on homeotic phenotypes caused by inappropriate homeotic gene expression (4, 5). The chromosomal proteins encoded by several trithorax group (trx-G) and Polycomb group (Pc-G) genes directly regulate transcription of homeotic genes, either positively (trx-G) or negatively (Pc-G) (6, 7). Some of these activities appear to be exerted at the level of nucleosomal organization, thereby affecting the accessibility of cis-regulatory sequences to the transcriptional machinery. For example, the trx-G proteins BRAHMA, MOIRA, OSA, and SNR1 are components of an ATP-dependent, chromatin-remodeling complex of the SWI/SNF family (8, 9). SWI/SNF complexes have been shown to increase the fluidity of the chromatin, thus facilitating the binding of transcriptional factors (10). Such activities of the SWI/SNF complexes can be blocked by a repressor complex, PRC1, which contains at least three Pc-G proteins, POLYCOMB (PC), POLYHOMEOTIC (PH), and POSTERIOR SEX COMBS (PSC) (11).

In addition to remodeling nucleosomes, trx-G and Pc-G proteins are also involved in the maintenance of homeotic gene expression (6, 7), a crucial step in cellular memory of the determined states. Although little is known about the mechanisms of cellular memory at the molecular level, the acetylation and deacetylation of the N-terminal lysine residues of nucleosomal core histones have been implicated. The stable maintenance of an artificially activated transgene controlled by a linked Fab-7 response element, which is critical for regulation of the homeotic gene *Abdominal-B* (*Abd-B*) by Pc-G and trx-G proteins, is correlated with hyperacetylation of histone H4 (12, 13). In addition, the *Drosophila* MI-2 homolog, which is a component of nucleosomal-remodeling histone deacetylase complexes (14), was identified by its direct interaction with the HUNCHBACK (HB) repressor protein (15). HB proteins are essential for establishing the silenced state of homeotic genes in early embryos, and *Mi-2* mutations enhance the homeotic phenotypes caused by *hb* and Pc-G mutations at various developmental stages (15, 16). Furthermore, the *Drosophila* homolog of YY1 (which recruits HDAC2 in mammals for transcription repression) is encoded by the Pc-G gene *pleiohomeotic* (17, 18). Although these observations suggest the involvement of histone deacetylation in homeotic gene silencing, evidence supporting a direct role of HDAC is still lacking.

Two HDAC families, which are distinguishable by their sensitivity to trichostatin A (TSA) or dependence on NAD cofactor, have been found in many eukaryotes (19, 20). The TSA-sensitive family has sequence similarity to prokaryotic enzymes involved in acetoin utilization and acetyl-polyamine catabolism (21, 22). Based on sizes and sequence similarities, the TSA-sensitive family can be divided further into RPD3 and HDA1 types, which may form different protein complexes with overlapping functions on histone substrates (19, 23). In support of their roles in gene silencing, the association of RPD3-type HDACs with various repressors or corepressors has been demonstrated (24). Despite the expanding knowledge on HDACs, the physiological functions of individual enzymes are not fully understood and their roles in metazoan development remain largely unexplored (25).

In this study, we examined the roles of four *Drosophila* HDACs in homeotic gene silencing. We found that HDAC1 (an RPD3-type HDAC) shows specific genetic interactions with Pc-G mutations to enhance ectopic expression of homeotic genes, biochemical associations with Pc-G proteins, and cytological colocalization with Pc-G proteins on salivary gland polytene chromosomes. From these results, we conclude that HDAC1 plays an essential role in homeotic gene silencing.

Materials and Methods

***Drosophila* Strains and Genetic Interactions.** We follow the nomenclature of Mottus *et al.* (26) for *Hdac1* and its alleles throughout this report. This numeric system has been adopted for three other fly HDACs, and we agree that it is a more comprehensible system with which to designate the members of a multigene family. Mutant stocks were obtained from the following sources: esc^{10} and the bxd-14 transgenic line (W. Bender, Harvard University); Psc^{1} and $Hdac1^{P\text{-}UTR}$ (or $rpd3^{04556}$; Bloomington Stock Center); Pc^{4} (J. Kennison, National Institutes of Health); $E(z)^{63}$ (R. Jones, Southern Methodist University); *Hdac1* alleles (R. Mottus, University of British Columbia); *Df(3L)10H* (A. Nose, University of Tokyo); and Psc^{e24} (T. Wu, Harvard University). About 10 pairs of flies per vial were used for crosses. The crosses were maintained at 25°C and transferred every 2 days. To quantitate the *Pc* mutant phenotype, we counted the sex comb teeth on the second and third legs of male progeny under ×200 magnification.

Abbreviations: trx-G, trithorax group; Pc-G, Polycomb group; TSA, trichostatin A.

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Antibody Preparation. C-terminal regions of the PC protein (codons 205–390) or HDAC1 protein (codons 435–521) were cloned into pET15b (Novagen) and expressed in bacteria. After SDS/PAGE, gel slices containing recombinant PC and HDAC1 proteins were prepared as antigen for injection into rabbits. Antibodies were affinity-purified by using native recombinant proteins coupled to affi-gel 10 (Bio-Rad). Antibodies were eluted with 0.1 M glycine (pH 2.5) and dialyzed against a buffer containing 20 mM Tris·HCl (pH 8), 150 mM NaCl, and 10% glycerol. For immunoblotting, both antibodies were diluted 1:500, followed by enhanced chemiluminescence detection (Amersham Pharmacia).

Immunostaining. Whole-mount embryos were incubated with culture supernatant containing UBX (FP.3.38; 1:2 dilution) or ABD-B (1A2E9; 1:5 dilution) mAb. For disk staining, imaginal discs from wandering third-instar larvae were fixed and stained with SCR (6H4B; 1:1,000 dilution of ascites) or UBX antibody. Labeling was detected by the ABC detection method (Vector Laboratories) and diaminobenzidine staining. Individual ventral nerve cords or imaginal discs then were dissected and mounted. For polytene chromosome staining, salivary glands were dissected, fixed, and squashed (27). For double immunofluorescence staining, PSC mAbs IF4 (0.1 mg/ml purified IgG) and 6E8 (3:10 dilution of culture supernatant) or affinity-purified rabbit HDAC1 antibody (1:50) was used. Negative controls for the specificity of the HDAC1 antibody were as follows: antibody was used to stain polytene chromosomes from *Hdac1*$^{P\text{-}UTR}$ homozygous larvae, or antibody was preincubated with purified recombinant HDAC1 before staining of polytene chromosomes from wild-type larvae (data not shown). The secondary antibodies were conjugated with Cy5 and rhodamine Red-X for anti-mouse and anti-rabbit antibodies, respectively. Images were obtained from a Zeiss LSM310 confocal microscope.

Purification of PC Complexes. A stable S2 cell line that contains a metallothionein promoter-driven *Pc* with a FLAG epitope tag at the end of the coding sequences (details will be provided elsewhere) was established. Nuclear extracts were prepared as described (28). Nuclear proteins extracted with 10% $(NH_4)_2SO_4$ were precipitated by 40% $(NH_4)_2SO_4$. The pellet was dissolved in buffer A (25 mM Hepes, pH 7.5/150 mM NaCl/0.1 mM DTT/1 mM PMSF/5 μg/ml each of aprotinin, leupeptin, and pepstatin A) containing 0.1% Tween 20. This fraction was passed over a FLAG antibody column (M2; Kodak) three times, the column was washed extensively, and bound proteins were eluted with buffer A that contained 375 μg/ml FLAG peptide. DTT and glycerol were added to 1 mM and 10% concentrations, respectively. In a typical experiment, ≈15–20 ml of nuclear extracts was prepared from 6–10 liters of cells ($2–4 \times 10^6$/ml). This procedure achieved a purification of ≈500-fold.

HDAC Assays. *In vitro* acetylation of core histones (Boehringer Mannheim) was carried out with [^{3}H]acetyl CoA (4.9 Ci/mmol; Amersham Pharmacia) and truncated p300/CBP proteins (codons 1195–1673) purified from bacteria (29). Acetylated histones were recovered by TCA precipitation and extensive dialysis against water. The specific activity was ≈25,000 dpm/μg. HDAC assays were carried out with 1 μg of core histones in 50-μl reactions as described (30).

Immunoprecipitation. Nuclear extracts were prepared from 0- to 18-h Oregon R embryos. Extracts (50 μl) were diluted 10-fold with buffer A (50 mM Hepes, pH 7.6/100 mM KCl/1 mM $MgCl_2$/1 mM EDTA/0.25% Tween 20/1 mM PMSF/50 μg/ml aprotinin/50 μg/ml leupeptin/10 μg/ml pepstatin A). The samples were centrifuged for 15 min in a microcentrifuge to remove insoluble material. The extracts were preincubated with Protein A-Sepharose beads and then incubated with either purified HDAC1 antibody or preimmune serum at 4°C for 3 h. Protein A-Sepharose beads were prewashed with buffer A, added to the extract, and incubated at 4°C for 2.5 h. Beads were washed six times with buffer A, four times with buffer B (50 mM Tris·HCl, pH 7.6/150 mM NaCl/0.25% Tween 20/1% Nonidet P-40), and analyzed by immunoblotting.

Table 1. Genetic interactions between Pc-G and *Hdac1* mutations

Maternal mutation	Pc-G/Bal	Pc-G/*Hdac1*
Df(3L)10H	1.79* (*Pc*^{4}; 104)	4.25 (*Pc*^{4}; 116)
Hdac1$^{P\text{-}UTR}$	2.70 (*Pc*^{4}; 152)	3.94 (*Pc*^{4}; 184)
	0.19 (*Psc*^{1}; 152)	0.48 (*Psc*^{1}; 112)
	0 (*esc*^{10}; 132)	0 (*esc*^{10}; 184)
	0 (*E(z)*^{63}; 104)	0 (*E(z)*^{63}; 152)
Pc^4	1.03 (*Hdac1*$^{P\text{-}UTR}$; 84)	2.78 (*Hdac1*$^{P\text{-}UTR}$; 160)
	1.36 (*Df(3L)10H*; 296)	2.93 (*Df(3L)10H*; 392)
Hdac1^{303}	2.45 (*Pc*^{4}; 100)	4.74 (*Pc*^{4}; 110)
Hdac1^{313}	2.42 (*Pc*^{4}; 83)	5.30 (*Pc*^{4}; 128)
Hdac1^{326}	1.71 (*Pc*^{4}; 89)	0.40 (*Pc*^{4}; 253)
Hdac1^{def8}	1.94 (*Pc*^{4}; 101)	2.58 (*Pc*^{4}; 149)

*The numbers shown here are ectopic sex comb teeth per leg. They are derived by dividing the total numbers of sex comb teeth in second and third legs of male progeny by the total numbers of legs examined. The paternal genotypes and numbers of legs examined are shown in parentheses. The degree of leg transformations appears to be significantly higher when the maternal genome carries *Hdac1* mutation, suggesting a maternal effect of *Hdac1*. The weak effect of *Hdac1*^{def8} might be related to perdurance of maternal products (data not shown). *Hdac1* heterozygotes do not have ectopic sex comb teeth.

Results

Genetic Interactions Between *Hdac1* and Pc-G Mutations. In *Drosophila*, five potential genes encoding TSA-sensitive HDACs have been identified (31). Of these five, *Hdac1* and *Hdac3* are Rpd3 types, *Hdac2* and *Hdac4* are Hda1 types, and *CG10899* appears to diverge significantly from both types. Because the expressions of homeotic genes are oppositely controlled by Pc-G and trx-G proteins in a dosage-sensitive manner, it is possible to assess the roles of these HDACs in homeotic gene regulation by examining the genetic interactions between Pc-G or trx-G mutations and HDAC mutations. In a preliminary study, we examined deficiencies that delete four of five potential HDAC genes (a deficiency for *Hdac3* is not available currently) for genetic interactions with *Pc*. We found that only *Df(3R)10H*, which deletes *Hdac1*, showed a significant genetic interaction with a *Pc* mutation, resulting in a more than 2-fold increase in ectopic sex comb teeth on the second and third legs of male adults (Table 1). In addition, this deficiency substantially reduced the frequency of mesothoracic transformation typically found in trx-G mutants (data not shown). These results are consistent with a negative role for *Hdac1* in homeotic gene regulation.

Df(3R)10H deletes not only *Hdac1*, but several other genes as well (32). To show that deletion of the *Hdac1* gene is responsible for the genetic interactions with *Pc*, we examined genetic interactions between Pc-G mutants and *Hdac1*$^{P\text{-}UTR}$, a semilethal mutant with a P element inserted at +47 of *Hdac1* (33). As shown in Table 1, *Hdac1*$^{P\text{-}UTR}$ also shows dosage-sensitive genetic interactions with *Pc* and *Psc* mutations, indicating that *Hdac1* is important in regulating the function of homeotic genes. In contrast to the results with *Pc* and *Psc*, no genetic interactions were observed between *Hdac1*$^{P\text{-}UTR}$ and *extra sex combs* (*esc*) or *Enhancer of zeste* [*E(z)*] mutations. The differences in the genetic interactions with the Pc-G mutations might reflect the presence of two physically distinct complexes formed by these proteins,

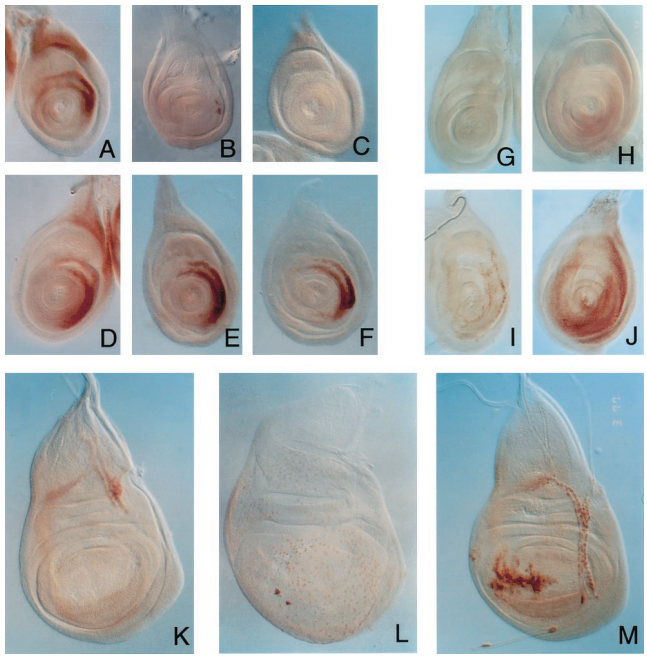

Fig. 1. Synergistic effects of *Pc* and *Hdac1* mutations on ectopic expressions of SCR and UBX proteins in imaginal discs. First (*A*, *D*, *G*, and *I*), second (*B*, *E*, *H*, and *J*), and third (*C* and *F*) leg discs and wing discs (*K–M*) from wild type (*K*), Pc^4 heterozygotes (*A–C*, *G*, *H*, and *L*), $Pc^4/Hdac1^{303}$ double heterozygotes (*D– F*, and *M*), or $Pc^4/Hdac1^{326}$ double heterozygotes (*I* and *J*) were stained with either SCR (*A–F*) or UBX (*G–M*) antibody. Similar to $Pc^4/Hdac1^{303}$, ectopic expressions of SCR and UBX also were observed for trans-heterozygotes carrying each of the three *Hdac1* alleles ($Hdac1^{P\text{-}UTR}$, $Hdac1^{313}$, and $Hdac1^{def8}$). Although there was no ectopic SCR expression, $Pc^4/Hdac1^{326}$ double heterozygotes showed more extensive UBX misexpression (*I* and *J*) than other alleles. *Hdac1* mutations alone do not show ectopic expressions of UBX or SCR in discs.

because the PC and PSC proteins copurify in one Pc-G complex and the ESC and E(Z) proteins copurify in a different Pc-G complex (34). It is interesting to note that *Df(3R)10H* and $Hdac1^{P\text{-}UTR}$ by themselves did not cause leg transformation. Thus, the homeotic effects of *Hdac1* appear to manifest themselves only in combination with *Pc-G* mutations as noted for several other *Pc-G*, including *Enhancer of Polycomb*, *Suppressor 2 of zeste*, and *Mi-2* (15, 35).

Further support for the role of *Hdac1* in homeotic gene regulation was obtained by analyzing several newly characterized *Hdac1* mutations (26). As observed for $Hdac1^{P\text{-}UTR}$, two missense mutations ($Hdac1^{303}$ and $Hdac1^{313}$) and one small deletion ($Hdac1^{def8}$) enhanced the Pc mutant phenotype. Surprisingly, one missense mutation, $Hdac1^{326}$, suppressed the Pc phenotype significantly. As shown below, this unexpected suppression probably resulted from a stronger effect on ectopic expression of posterior homeotic genes, causing repression of more anterior ones.

Misexpression of Homeotic Genes During Development. To demonstrate that the effect of *Hdac1* mutations is exerted at the level of expression of homeotic genes, we compared the expressions of SEX COMBS REDUCED (SCR) and ULTRABITHORAX (UBX) proteins in wild-type and *Pc* mutant imaginal discs. SCR proteins normally are expressed at high levels in the first leg discs, but are not expressed in the second and third leg discs. In Pc^4 mutant heterozygotes, however, SCR proteins also could be detected at low levels in second and third leg discs (Fig. 1 *B* and *C*). Consistent with the increase in ectopic sex comb teeth, dramatic increases in the levels of SCR proteins were observed in the second and third leg discs from Pc^4 mutant heterozygotes that were also heterozygous for any of the *Hdac1* alleles (Fig. 1 *E* and *F*) except $Hdac1^{326}$ (data not shown). In addition, UBX proteins were marginally detectable only in the peripodial membranes of imaginal wing discs of wild-type or Pc^4 mutant heterozygous larvae (Fig. 1 *K* and *L*). In larvae heterozygous for both Pc^4 and an *Hdac1* mutation, high levels of UBX proteins were observed in the medial sections of the wing discs proper (Fig. 1*M*). In contrast to the lack of ectopic SCR expression in Pc^4 heterozygotes carrying the $Hdac1^{326}$ allele, a much stronger effect on ectopic UBX expression was observed; UBX protein levels in both first and second leg discs were increased substantially (Fig. 1, compare *G* and *H* with *I* and *J*). It is highly likely that the expanded UBX expression reduces SCR expression, resulting in suppressed Pc phenotype (i.e., reduced numbers of ectopic sex comb teeth) in $Pc^4/Hdac1^{326}$ trans-heterozygotes. These results strongly suggest that *Hdac1* acts cooperatively with *Pc* to repress homeotic genes during larval and pupal development.

Experiments also were performed to explore the role of *Hdac1* in regulating the embryonic expressions of two homeotic genes, *Abd-B* and *Ubx*. *ABD-B* proteins normally are expressed in a graded fashion in the posterior part of ventral nerve cord, starting from parasegment 10 (PS10) (ref. 36; also see Fig. 2*A*). Although this pattern was not altered in homozygous $Hdac1^{303}$ mutants (Fig. 2*B*), significant levels of ABD-B proteins were observed in more anterior parasegments of homozygous Psc^{e24} mutants (Fig. 2*C*). Much higher levels of ectopic ABD-B proteins were found in Psc^{e24} $Hdac1^{303}$ double mutants (Fig. 2, compare *C* and *D*), indicating a synergistic effect of *Hdac1* and *Psc* on *Abd-B* repression. Consistent (but less striking) effects also were observed on UBX protein levels. The anterior boundary of the UBX expression domain is PS5, with the exception of a small cluster of cells in the middle of PS4 that also express UBX proteins (Fig. 2*E*). Although homozygous Psc^{e24} mutants only showed sporadic low levels of UBX expression in more anterior parasegments, Psc^{e24} $Hdac1^{303}$ double mutants showed significantly higher levels of ectopic UBX expression in more cells (Fig. 2, compare *G* and *H*). In PS5, more cells with higher levels of UBX proteins were observed in the double mutants than were observed in either of the single mutants (Fig. 2 *F–H*). In contrast, UBX expression was reduced substantially in the abdominal parasegments of the double mutants compared with that in the single mutants, presumably reflecting *Ubx* repression by more extensive ectopic expression of ABD-B and possibly ABD-A (37). These data indicate that *Hdac1* is essential for homeotic gene silencing in embryos.

Cofractionation of HDAC1 and Pc-G Proteins. The genetic interactions between *Hdac1* and Pc-G mutations suggested that they might be physically associated. We tested this idea by examining whether HDAC1 and PC proteins could be copurified from cultured *Drosophila* cells. A permanent S2 cell line was established that expresses a PC protein with a FLAG-epitope tag at the C-terminal end under the control of a metallothionein promoter. Nuclear extracts prepared from induced cells were passed over a FLAG antibody column. After the addition of FLAG peptide, tagged PC and its associated proteins were eluted (Fig. 3*A*). Using ^{3}H-labeled, acetylated core histones as substrates to assay these fractions, we found that HDAC activity eluted with the same profile as PC (Fig. 3*B*). In addition, this activity was sensitive to the HDAC-specific inhibitor TSA (38).

To determine the identity of the HDAC associated with PC, we immunoblotted with an affinity-purified antibody against the C-terminal part of HDAC1. As shown in Fig. 3*C*, HDAC1 was detected in the eluted fraction. In addition, we found that substantial amounts of PSC and PH also were copurified (Fig. 3*C*), consistent with previous findings that they are components of large PC protein complexes (11, 39). Much lower amounts of

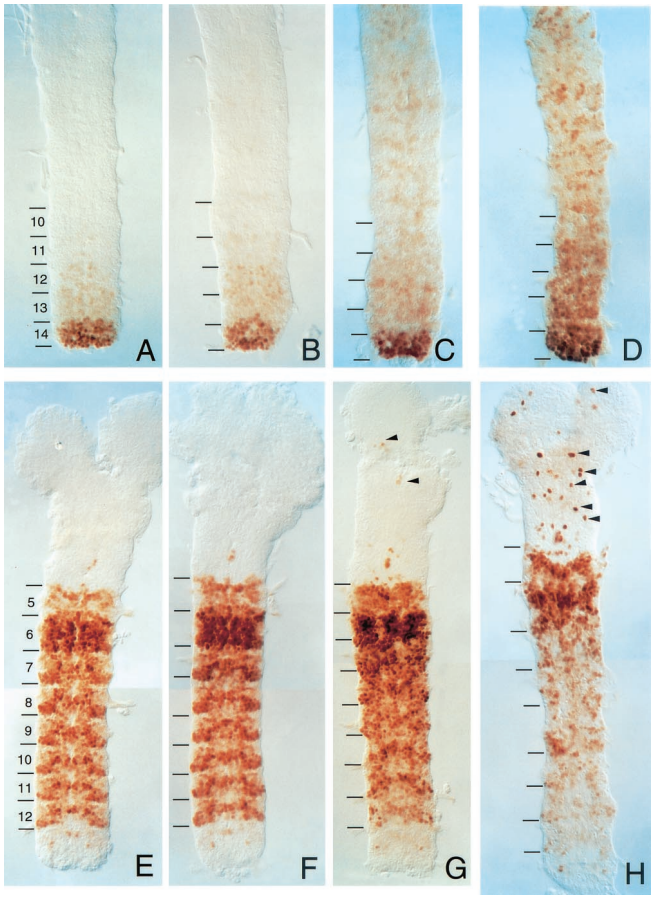

Fig. 2. Hdac1 mutations enhance the homeotic effects of *Psc* mutations in embryos. Wild-type embryos (*A* and *E*) or embryos homozygous for *Hdac1*^{303} (*B* and *F*), *Psc*^{e24} (*C* and *G*), or *Psc*^{e24} *Hdac1*^{303} (*D* and *H*) were stained with either ABD-B (*A–D*) or UBX antibody (*E–H*). Ventral nerve cords of these embryos were dissected out and displayed with the anterior to the top. In wild-type embryos, ABD-B expression normally is restricted to PS10–14, with a gradual increase toward the posterior end (*A*), whereas strong and highly modulated UBX expression is restricted to PS5–12, with weak expression in small clusters of cells in PS4 and PS13 (*E*). These patterns were not affected in *Hdac1*^{303} (*B* and *F*) or *Df(3R)10H* homozygotes (data not shown). ABD-B expression extended to more anterior PS at low levels in *Psc*^{e24} homozygotes (*C*) and at much higher levels in *Psc*^{e24} *Hdac1*^{303} double homozygotes (*D*). Similarly, ectopic UBX expression was somewhat sporadic and at lower levels in *Psc*^{e24} homozygous embryos (arrowheads in *G*), but was more extensive and much stronger in *Psc*^{e24} *Hdac1*^{303} double homozygotes (arrowheads in *H*). In addition, UBX expression became stronger in PS5, but reduced in abdominal parasegments in both *Psc*^{e24} homozygotes (*G*) and *Psc*^{e24} *Hdac1*^{303} double homozygotes (*H*). The effects appeared to be much stronger in double mutants than in single mutants. The designation of genotypes was based on the frequency of the novel patterns produced from heterozygous parents, i.e., 20 to ≈25% and ≈3.3% (11 of 336 embryos) for single and double mutants, respectively. Although *Hdac1* mutants did not show aberrant homeotic gene expressions, their ventral nerve cords were slightly distorted in the abdominal region.

another Pc-G protein, SEX COMBS ON MID-LEG (SCM), were detected in our preparations. A similar result also was seen previously (11). Thus, our results indicate that HDAC1 is associated with the PC protein complexes in cultured cells.

To examine whether HDAC1 is associated with PC complexes in embryos, we immunoprecipitated HDAC1 proteins from embryonic nuclear extracts. As shown in Fig. 3*D*, PC was detected when we used an HDAC1 antibody for the immunoprecipitation, but not when we used a preimmune serum. These results further support the idea that the associations between PC and HDAC1 proteins are physiologically relevant.

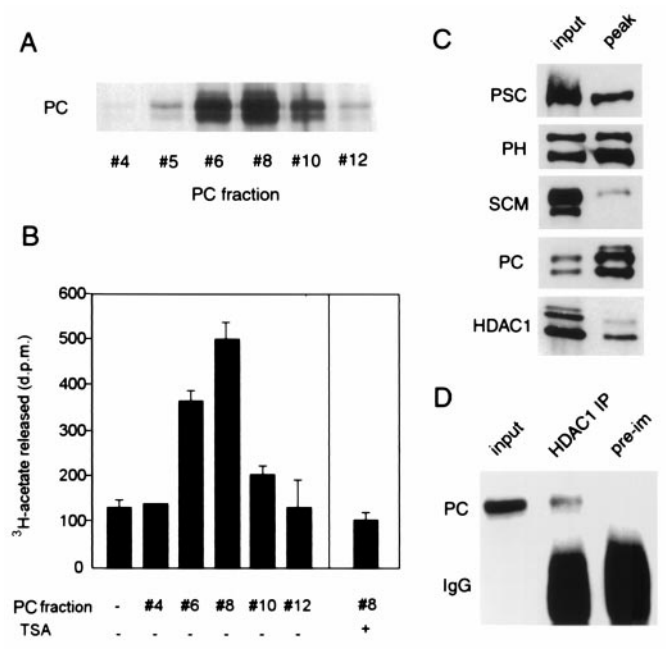

Fig. 3. Association of HDAC1 with PC. (*A*) Elution profile of PC. The silver-stained polyacrylamide gel corresponding to the portion with PC protein is shown. Column fraction is indicated. (*B*) Activity profile of HDAC in eluted fractions. Aliquots of indicated fractions were assayed for HDAC activity with ^{3}H-labeled acetylated core histones in the absence or presence of TSA (100 ng/ml). Values shown are the averages of duplicate experiments. (*C*) Column input (1 μl) and peak fraction (5 μl) were analyzed by immunoblotting with various antibodies. Note that tagged PC was detected by an anti-FLAG antibody. (*D*) Coimmunoprecipitation of HDAC1 and PC from embryonic nuclear extracts. Embryonic nuclear extracts (lane 1) and immunoprecipitates of the extracts with affinity-purified HDAC1 antibody (lane 2) or preimmune serum (lane 3) were analyzed by immunoblotting with an anti-PC antibody. The positions for PC and IgG are indicated.

Colocalization of HDAC1 and Pc-G Proteins on Polytene Chromosomes. Given the genetic and biochemical interactions between *Hdac1* and *Pc*, it might be anticipated that a fraction of HDAC1 will colocalize with Pc-G complexes on polytene chromosomes. In previous studies, ≈100 common binding sites were identified for several Pc-G proteins (7). At least 70% of these sites (identified by staining with PSC mAbs) also stained with the HDAC1 antibody (Table 2), including the Antennapedia complex at 84AB and the bithorax complex at 89E. These results suggest that HDAC1 proteins act together with a substantial fraction of the Pc-G complex. However, the relative intensities of the signals for PSC and HDAC1 at these sites do not always correlate, suggesting a regulatory, rather than a constitutive function. Furthermore, HDAC1 is much more widely distributed along the chromosomes than is PSC (Fig. 4), consistent with its role in global gene regulation and/or chromatin structure (41–44).

The colocalizations of HDAC1 and PSC were examined further on polytene chromosomes from a transgenic line that carries a *Ubx* upstream cis-regulatory region (i.e., bxd-14) inserted at 62A. This insert contains a functional PRE (45, 46) and creates a new Pc-G-binding site (refs. 47 and 48; also see Fig. 4 *A* and *D*). Staining with both PSC and HDAC1 antibodies revealed that a new PSC site coincides with a new HDAC1-binding site (Fig. 4, compare *C* and *F*). This new binding site is beside an HDAC1 site present in the wild-type chromosome, creating a broader signal of HDAC1 at this site (Fig. 3, compare *B* and *E*). These results strongly suggest that HDAC1 and Pc-G proteins are recruited to this ectopic PRE.

Table 2. Distributions and relative intensities of HDAC1 at PSC sites on polytene chromosomes

2D	++	**26E**	−	59F	−	84D	+
4C	−	**28A**	−	**61A**	++	**84E**	++
5A	+	**32EF**	+	**61C**	+	84F	+
8A	+	**33F**	−	**61F**	++	86C	−
8B	+	**35AB**	++	**63E**	+	88A	−
8D	++	**35DE**	+	65D	−	**89C**	+
8F/9A	+	**37A**	+	69C	++	**89E**	+
11A	++	**38F**	+	**69D**	−	**90E**	−
11B	++	*42A*	+	**70A**	++	**93E**	−
14B	−	*44A*	+	70D	+	**94EF**	+
21AB	+	**48A**	−	**76C**	−	**96B/C**	++
22A	++	**49EF**	++	**77E**	+	**98D**	++
22B	+	**51C**	+	**82E**	+++	**99A**	++
24A	+	**56C**	++	**83C**	++	**100A**	−
25E	−	**57B**	−	**84AB**	+	**100B**	+

The major PSC sites detected by a combination of monoclonal antibodies 1F4 and 6E8 are listed. Note that there are some differences between our list and the sites previously published (40). The bold, plain, and italicized letters indicate major, minor, and sites undetected by the earlier work, respectively. The relative staining intensity of HDAC1 at these sites is indicated by the number of ''+.'' The absence of HDAC1 staining is indicated by ''−.''

Discussion

In this report, we provide evidence that HDAC1, which is an RPD3-type HDAC, is directly involved in homeotic gene silencing. Our results indicate that *Hdac1* mutations act like Pc-G mutations in that they enhance the effects of Pc-G mutations and suppress the effects of trx-G mutations. Deletions of three other HDAC genes failed to show such interactions. Thus, among these HDACs, HDAC1 may play a unique role in homeotic gene silencing. However, we cannot exclude the possibility that inclusion of adjacent genes in these HDAC deletions might obscure their effects on homeotic genes or that they might be involved in certain regulatory aspects that are not amenable to our tests.

We have also shown that the effects of *Hdac1* mutations are exerted directly on homeotic genes rather than their targets, because altered expression patterns of SCR, UBX, and ABD-B proteins were observed at embryonic or larval stages. It is possible that *Hdac1* mutations reduce the levels of Pc-G proteins, thus leading to enhanced misexpression of homeotic genes. We believe that this is unlikely, because the overall intensity of PSC staining is not altered significantly on polytene chromosomes of *Hdac1* mutants (unpublished observation). However, we note that *Hdac1* mutations alone do not show significant homeotic effects. Neither do *Mi-2*, *E(Pc)*, or *Su(z)2* mutations (15, 35). This might reflect a functional difference from other Pc-G genes. It is also possible that the effect of this subgroup of genes on homeotic genes is obscured by pleiotropic effects on other target genes.

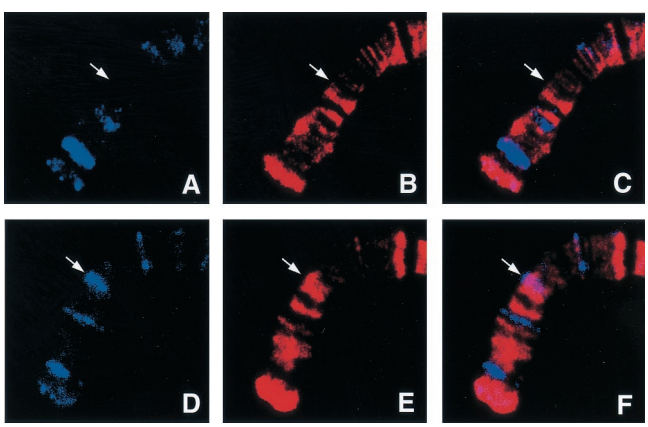

Fig. 4. Colocalization of PSC and HDAC1 at a bxd PRE. Polytene chromosomes from wild type (*A–C*) or a transgenic line carrying a bxd-14 PRE at 62A (*D–F*) were labeled simultaneously with mouse monoclonal PSC antibodies (*A* and *D*) and affinity-purified rabbit HDAC1 antibody (*B* and *E*). The merged images are also shown (*C* and *F*). The insertion site is indicated by the arrow. Only the tip of chromosome arm 3L, which includes 62A, is shown. The specificity of HDAC1 labeling was confirmed by negative labeling of polytene chromosomes from $Hdac1^{P\text{-}UTR}$ homozygous larvae or from wild-type larvae with blocked antibody.

That the silencing effect by HDAC1 is mediated through physical association with Pc-G protein complexes is strongly supported by the following observations. First, functional HDAC1 was copurified with PC complexes from *Drosophila* S-2 cells by immunoaffinity chromatography. Second, HDAC1 and PC were coimmunoprecipitated from embryonic nuclear extracts. Third, double immunofluorescence staining showed that PSC and HDAC1 coexist on sites corresponding to Antennapedia and bithorax gene complexes and on an ectopic site corresponding to a well characterized *Ubx* PRE. Therefore, HDAC1 appears to be recruited to homeotic genes with PC complexes. In contrast to our findings, HDAC activity has not been detected in some Pc-G complexes previously isolated from *Drosophila*, *Xenopus*, and human cells (11, 49, 50). However, considerable amounts of Pc-G proteins remain in other chromatographic fractions and many Pc-G genes are redundant in vertebrates (11, 51), suggesting that different Pc-G complexes may exist. This is consistent with the apparent diversity of *Drosophila* Pc-G complexes discussed below.

Our results do not imply that there is a direct physical interaction between HDAC1 and any Pc-G proteins that have been characterized to date. It is possible that an adapter-like molecule might be involved. In several organisms, direct interactions or cytological colocalization between HDAC1 and SIN3A proteins has been demonstrated (24, 44). However, there is currently no evidence that SIN3A is required for homeotic gene repression. The observations that HDAC1 is not detected at about 30% of PSC sites and that HDAC1 intensity does not always correlate with that of remaining PSC sites suggest that HDAC1 is not constantly associated with the PC complexes. This is consistent with a catalytic rather than a structural function. Different levels of HDAC1 staining might reflect varying degrees of repression at these sites. Because more acetyl groups need to be removed when a gene becomes repressed from an active state, it is also possible that higher levels of HDAC1 are required for the initiation of a repressed state than for the maintenance of a repressed state. Thus, the significance of the relative levels of HDAC1 should be interpreted with caution.

It is also important to note that chromatin immunoprecipitation experiments have revealed substantial variations in the relative ratios of PC, PSC, and PH proteins on different response sequences (52). Contrary to the generalized role for repression, certain combinations of these proteins have been found at active genes (52). In addition, both genetic and biochemical studies indicate that many components of Pc-G complexes remain to be identified (4, 11). The apparent diversity of Pc-G complexes indicates that further analyses of their components and their structure–function relationships are essential for a better understanding of their molecular and cellular functions.

The recent finding that a purified Pc-G complex, PRC1, can block the activity of SWI/SNF chromatin-remodeling complexes suggests that a potential function of Pc-G complexes is to restrict fluidity of nucleosomes on homeotic genes (11). Additional functions appear to be necessary to account for other properties of the Pc-G complexes. For example, the stable transmissions of active and inactive states of a PRE-driven transgene have been found to correlate with the presence and

absence of histone H4 hyperacetylation, respectively. This suggests that the state of histone acetylation is affected by Pc-G and trx-G complexes (13). Our results indicate that the deacetylation of histones can be partially, if not entirely, fulfilled by HDAC1 associated with PC complexes. We propose that this activity is essential for stable maintenance of the silenced state of homeotic genes.

During early embryogenesis, transiently expressed HB repressor proteins are believed to be responsible for initial establishment of the silenced domain for homeotic genes (16). A recent study has shown that MI-2, a HB-interacting protein, coimmunoprecipitates with HDAC1 (15, 53), implicating HDAC1 in the initiation step as well. It is possible that HDAC1 plays crucial roles in both initiation and maintenance of silencing by establishing epigenetic marks on the chromatin of homeotic genes, thus allowing silenced states to be perpetuated throughout development.

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