CtBP Levels Control Intergenic Transcripts, PHO/YY1 DNA Binding, and PcG Recruitment to DNA

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ABSTRACT

Carboxy-terminal binding protein (CtBP) is a well-known corepressor of several DNA binding transcription factors in *Drosophila* as well as in mammals. CtBP is implicated in Polycomb Group (PcG) complex-mediated transcriptional repression because it can bind to some PcG proteins, and mutation of the *ctbp* gene in flies results in lost PcG protein recruitment to Polycomb Response Elements (PREs) and lost PcG repression. However, the mechanism of reduced PcG DNA binding in CtBP mutant backgrounds is unknown. We show here that in a *Drosophila* CtBP mutant background, intergenic transcripts are induced across several PRE sequences and this corresponds to reduced DNA binding by PcG proteins Pleiohomeotic (PHO) and Polycomb (Pc), and reduced trimethylation of histone H3 on lysine 27, a hallmark of PcG repression. Restoration of CtBP levels by expression of a CtBP transgene results in repression of intergenic transcripts, restored PcG binding, and elevated trimethylation of H3 on lysine 27. Our results support a model in which CtBP regulates expression of intergenic transcripts that controls DNA binding by PcG proteins and subsequent histone modifications and transcriptional activity. J. Cell. Biochem. 110: 62–69, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: POLYCOMB; TRANSCRIPTION; NON-CODING RNA; REPRESSION

Hox gene expression in the *Drosophila* bithorax (BX-C) complex (*Ubx*, *abd-A*, and *abd-B* genes) is governed by both spatially and temporally regulated expression of the segmentation genes. After decay of the segmentation gene products, *hox* gene expression patterns are maintained by Polycomb Group (PcG) and Trithorax Group (TxG) proteins [Maeda and Karch, 2006]. PcG proteins comprise at least two complexes, PRC1 and PRC2, which are involved in the maintenance of the silent state, whereas TxG proteins maintain active gene expression [Simon and Tamkun, 2002]. PcG complexes bind to specific DNA regions termed Polycomb Response Elements (PREs) to mediate their effects on transcriptional repression. These elements are sometimes referred to as memory elements or maintenance elements (ME) because often they can switch between repressed and active modes depending upon the function of either PcG or TxG proteins, respectively [Rank et al., 2002]. The mechanism of specific DNA binding by PcG complexes was long considered an enigma because the individual PcG proteins do not possess DNA binding site specificity. This conundrum was partially solved with the molecular cloning of the cDNA encoding *Drosophila* PcG protein, Pleiohomeotic (PHO),

which contains four zinc fingers and binds to specific DNA sequences within numerous PREs [Mihaly et al., 1998]. This suggested the role of specific DNA binding proteins in recruitment of PcG complexes to DNA. Association of PHO with E(z), Polyhomeotic (Ph), and Polycomb (Pc) [Mohd-Sarip et al., 2002; Wang et al., 2004], components of the PRC2 and PRC1 complexes, suggested a role for PHO in Polycomb recruitment to DNA [Schuettengruber et al., 2007; Schwartz and Pirrotta, 2007]. However, the molecular mechanism of PHO recruitment of PcG proteins to DNA is still not clear [Poux et al., 2001; Savla et al., 2008].

Yeast two-hybrid studies have shown direct interaction of *Xenopus* Pc with the corepressor protein, C-terminal binding protein (CtBP) [Sewalt et al., 1999]. *Drosophila* repressors such as Snail, Knirps, and Krüppel exert short-range transcriptional repressive activity by recruiting CtBP to DNA [Nibu and Levine, 2001; Nibu et al., 2003], but the association of CtBP with some PcG proteins suggests a role in PcG-mediated gene repression. We previously showed by transgenic studies that human YY1, the vertebrate counterpart of *Drosophila* PHO, is able to mediate transcriptional silencing in a PcG-dependent fashion and can phenotypically

correct PHO mutant flies [Atchison et al., 2003]. We also found that, like PHO, YY1 is able to recruit PcG complexes to PRE sequences [Srinivasan and Atchison, 2004; Wilkinson et al., 2006]. Interestingly, we also showed that YY1 interacts with CtBP and is involved in PcG repression [Atchison et al., 2003]. Subsequently, we found that in a heterozygous CtBP mutant background (homozygous *ctbp* mutation is lethal), there is loss of YY1 DNA binding to PRE sequences, lost PcG recruitment, and decreased histone modification marks pertaining to Polycomb regulation [Srinivasan and Atchison, 2004]. This suggested a unique but still unknown role of CtBP in controlling PcG-mediated gene regulation.

Recently, it was found that gene regulation in the BX-C is accompanied by non-coding transcription through *cis*-regulatory sequences, and expression of these transcripts changes dynamically throughout development [Lempradl and Ringrose, 2008]. The first non-coding transcripts in the fly BX-C complex were described more than two decades ago [Lewis, 1985; Celniker and Lewis, 1987]. The pattern of non-coding transcription was similar to that of *hox* genes and was collinear with the regulatory domains of the *hox* gene clusters. The segmentation genes regulate these early transient transcripts as segmentation gene mutations alter the expression pattern of early non-coding RNAs (ncRNAs) [Sanchez-Herrero and Akam, 1989]. These transcripts may antagonize PcG function because there is a tight correlation between the reversal of PcG-mediated silencing and non-coding transcription through regulatory regions of the BX-C [Bae et al., 2002; Bender and Fitzgerald, 2002; Hogga and Karch, 2002; Rank et al., 2002; Schmitt et al., 2005]. Recruitment of Ash1 (a TxG protein) by transcripts located in the *Ubx* region mediates transcriptional activation [Sanchez-Elsner et al., 2006]. It has also been proposed that transcriptional elongation of *bxd* ncRNAs by TAC1 (a TxG protein) represses *Ubx* transcription [Petruk et al., 2006].

In this study, we demonstrate that short intergenic transcripts across PRE$_D$, *sex combs reduced* (*scr*) and *engrailed* (*en*) PRE regions are induced in *ctbp*$^{+/-}$ flies compared to wild-type flies. This is accompanied by reduced occupancy by PcG proteins at these PRE sequences, and reduced histone H3 trimethylation of lysine 27. We further show that these phenomena are reversed when transgenic CtBP expression is induced in the mutant background, suggesting that optimum levels of CtBP are essential for repression of intergenic transcripts and for PcG-mediated gene regulation.

MATERIALS AND METHODS

DROSOPHILA LINES, TRANSGENIC CtBP FLY LINE AND CROSSES

The *BGUZ/CtBP;TM3SB* and *ctbp*^{03463} fly lines were described previously [Srinivasan and Atchison, 2004; Wilkinson et al., 2006]. For generation of hspFLAG-dCtBP transgenic flies, CtBP full-length cDNA was cloned into *hsp70*-driven *pry*-derived vectors [Atchison et al., 2003] and coinjected with a transposase expressing plasmid (phsπ) into dechorionated *ry*^{506} embryos (Genetic Services, Inc.). Eclosed flies were backcrossed to *ry*^{506} and progeny were screened for transgene incorporation by appearance of *ry*$^+$ eyes. For CtBP rescue experiments, *hspFLAG-dCtBP* flies were crossed to *ctbp*^{03463} flies and embryos were heat-shocked twice for 30 min at 37°C with an interval of 2 h.

ANALYSIS OF PRE-SEQUENCES FOR PUTATIVE PHO/YY1 BINDING SITES

Based on analysis and identification of various PRE regions in *Drosophila* by Ringrose et al. [2003], we chose PRE$_D$, *scr*, and *en* PRE regions for evaluating YY1/PHO binding sites. The details of the PRE sequences used for analysis are as follows. PRE$_D$: the sequence starts at 12,589,500 bp and ends at 12,590,299 bp. It is 23,000 bp upstream of the *bxd* gene that is known to be a Polycomb-regulated gene. The *en* PRE: the sequence starts at 6,592,700 bp and ends at 6,593,199 bp. *Engrailed* is the nearest gene regulated by this PRE element. The *Scr* PRE: the sequence spans 2,711,922–2,712,492 and is upstream of the protein coding region. Sequence analyses were done on version 3.1 of the *Drosophila melanogaster* genome. We used the MathInspector database (available with the Genomatix Software package), which predicts transcription factor binding sites (http://www.genomatix.de/), using a Core Similarity of 0.9–0.95 with the Matrix Similarity "Optimized." The "core sequence" of a matrix is defined as the highest conserved positions of the matrix. The Matrix Similarity was determined by the highest conserved nucleotide of each position in the matrix of the binding sequence. Since YY1/PHO binding sites are quite degenerate, we chose to use "Optimized" Matrix Similarity, to minimize false positives. It should be noted that the binding sites analyzed for the PRE$_D$ sequence corresponded to the sites validated by Fritsch et al. [1999].

RNA EXTRACTION AND RT–PCR ANALYSIS

Embryos were dechorionated with 50% bleach (Chlorox^{TM}) and then washed twice with PBS containing 0.01% Triton X-100. RNA was extracted from embryos using TRIZOL (Invitrogen) according to the manufacturer's protocol with minor modifications. Briefly, the washed embryo pellet was resuspended and homogenized in 200–300 μl of TRIZOL reagent using plastic pestles. The volume of TRIZOL was made up to 1 ml, samples were incubated at room temperature (RT) for 5 min, 200 μl of chloroform was added, and vigorously vortexed for 10 min. The remaining steps were performed according to the manufacturer's protocol. RNA pellets were dissolved in DEPC-treated water and 5 μg of total RNA was taken for first-strand cDNA synthesis (Superscript II Kit; Invitrogen) using both random hexamers and oligo(dT) primers according to the manufacturer's protocol. Conventional PCR was performed with Taq polymerase (ABI Biosystems) using primers (Table I) encompassing the PRE regions. Real-time PCR was performed with a Light Cycler System (Roche Molecular Biochemicals) at least twice in duplicate using SYBR Green (Sigma, St. Louis, MO). Normalization was performed with respect to β-actin and relative amounts were further calculated based on C$_T$ values of wild-type controls.

CHROMATIN IMMUNOPRECIPITATION

Drosophila embryos from various egg-lays were dechorionated with 50% bleach, collected in PBS containing 0.04% Triton X-100, and subsequently fixed with 2% formaldehyde in 50 mM HEPES (pH 7.6), 1 mM EDTA, 0.5 mM EGTA, and 100 mM NaCl along with 3 volumes of *n*-heptane by vigorous rocking at RT for 15 min. Embryos were neutralized with PBS containing 0.125 M glycine and 0.01% Triton X-100 for 5 min at RT. Fixed embryos were washed with Wash Solution A (10 mM HEPES, pH 8.0, 10 mM EDTA, 0.5 mM EGTA, and

TABLE I. Primers used for RT-PCR analyses to detect intergenic transcripts across PRE-D, scr and en PRE sequences.

Primer name	Primer sequence
PRE_D sense 1F	CAGCCGTGCGGTATGGAGAG
PRE_D sense 1R	CTTGCAAAAGCGGCTATGAAAAG
PRE_D sense 2F	ATAAAACCCAGTGCGAAATGC
PRE_D sense 2R	TGCGCGTAGTCTTATCTGTATCTCG
PRE_D sense 3F	TAAAGCGAGAGCGATCCGAGC
PRE_D sense 3R	AAACGGCCATTACGAACGACAG
PRE_D sense 6F	TTCGGGCTTGTATTCGTGTTTTG
PRE_D sense 6R	TTACGGCCCTTTTATAGATGTTGC
PRE_D sense 5F	TACGCACGTCAGACTTGGAATAGC
PRE_D sense 5R	CAAGCCCGAAAAAGAAGAAGAAGC
PRE_D sense 4F	AAAACGGCCATTACGAACGACAG
PRE_D sense 4R	GACGTGCGTAAGAGCGAGATACAG
PRE_D antisense 1F	CGGCCCTTTTATAGATGTTGCAAC
PRE_D antisense 1R	CCGCCGCTTCTTCTTCTTTTTC
PRE_D antisense 2F	ATAAAACCCAGTGCGAAATGC
PRE_D antisense 2R	TGCGCGTAGTCTTATCTGTATCTCG
PRE_D antisense 3F	GCAAACATGGGCAAACACAACC
PRE_D antisense 3R	CAGCTCCGTCGCCATAACTGTC
PRE_D antisense 4F	CGTAATGGCCGTTTTAAGTGCG
PRE_D antisense 4R	TAAGCAAACATGGGCAAACACAAC
PRE_D antisense 5F	GCCCAGTGAAAATTTGGCAGC
PRE_D antisense 5R	ACAGCCGTGCGGTATGGAGAG
Scr 1F	AATCGGTCGAATTATTTAGCAAC
Scr 1R	ACTTCATCGGCAGTCTTGGAG
Scr 2F	GTAATTTTTATTTTTTGTTGC
Scr 2R	GCCCCTGCTTTCTACCATCTCC
Scr 3F	GAGAGGCCTTTGATTGTGTGG
Scr 3R	TCGACCGATTATGGAAAACTG
Scr 4F	ATGCAGCTGGGAAATCGTTGG
Scr 4R	GGCCTCTCCTGGTTTATCTTTG
Scr 5F	AATGGCTGATTTGGGTTCTCTG
Scr 5R	ATAGGCCACCGGGTAACATTTG
Engrailed 1F	ATCGTGTATTTAGCGTATTTTTG
Engrailed 1R	CAACTTTATCGACACCACCTTTAG
Engrailed 4F	CAGCATGCGCATAATAAAGTC
Engrailed 4R	TTTCGCCGGCTCACTCACTC
Engrailed 5F	GTTCGCATGGGGCAGGTGAC
Engrailed 5R	TAGCTGGCGAAGTGTGTGCG
Engrailed 6F	CTCGCTCGCTCGCACACAC
Engrailed 6R	TGACTACTTCGGAATCGCAGC

TABLE II. Primers used for ChIP analyses across PRE-D, scr and en regions.

Primer name	Primer sequence
$ChIP\_PRE_D$ F	TAAAGCGAGAGCGATCCGAGC
$ChIP\_PRE_D$ R	CAAGCCCGAAAAAGAAGAAGAAGC
ChIP_scr F	TCGACCGATTATGGAAAACTG
ChIP_scr R	ATGCAGCTGGGAAATCGTTGG
ChIP_eng F	ATCGTGTATTTAGCGTATTTTTG
ChIP_eng R	TTTCGCCGGCTCACTCACTC

volume of 200 μl and Proteinase K to a final concentration of 0.5 mg/ml and incubation at 45°C for 2 h. Subsequently, 8 μl of 5 M NaCl was added and cross-linking was reversed at 65°C overnight.

DNA was extracted using the QIAquick Purification Kit (Qiagen). Real-time PCR was done using the SYBR Green system with primers encompassing different PRE regions to test for promoter occupancy. The primers used for chromatin immunoprecipitation (ChIP)-PCR are listed in Table II.

RESULTS

INDUCTION OF INTERGENIC TRANSCRIPTS IN CtBP$^{+/-}$ FLIES

Previously, using ChIP assays of chromatin isolated from fly embryos, and by immunofluorescence studies of salivary polytene chromosomes, we showed that in a *Drosophila* heterozygous CtBP mutant background, binding of PcG proteins to numerous PRE sequences is reduced compared to wild-type samples [Srinivasan and Atchison, 2004]. The mechanism of lost PcG DNA binding in *ctbp*$^{+/-}$ samples was perplexing because the wild-type *ctbp* allele should still provide roughly half the amount of CtBP protein. Several studies have shown that ncRNAs or intergenic transcripts are responsible for switching memory elements from a repressive mode to the active mode by interfering with PcG-mediated gene silencing [Hogga and Karch, 2002; Rank et al., 2002]. Therefore, we hypothesized that CtBP may serve to repress transcription arising from promoters that flank PRE sequences, and a 50% loss of CtBP may be sufficient to derepress these promoters allowing transcription to occur across PRE sequences thus interfering with PcG recruitment to PRE sequences.

Based on computational analysis followed by experimental validation, many PRE sequences were identified by Ringrose et al. [2003]. Previous work from our lab showed that CtBP levels regulated YY1 DNA binding and PcG recruitment to different PREs in the fly genome [Srinivasan and Atchison, 2004]. Based on these data, we chose the PRE$_D$, *scr*, and *en* PRE sequences for our analyses. These sequences all contain PHO binding sites (see Figs. 1 and 2) and all exhibit reduced PcG DNA binding in a *ctbp*$^{+/-}$ background [Srinivasan and Atchison, 2004]. To search for potential transcripts across PREs that might be upregulated due to reduction in CtBP levels, we isolated RNA from fly embryos containing wild-type levels of CtBP (*ry*^{506}) and from embryos containing heterozygous mutant levels of CtBP (*ctbp*$^{+/-}$ and *BGUZ/CtBP;TM3SB*). Using several primer pairs across the PRE$_D$ sequence, we observed increased transcripts with most primer pairs in the *ctbp*$^{+/-}$ samples compared to wild type (Fig. 1). For instance, sense primer pair 2 (2S) showed an increase in transcript levels in *ctbp*$^{+/-}$ and *BGUZ/*
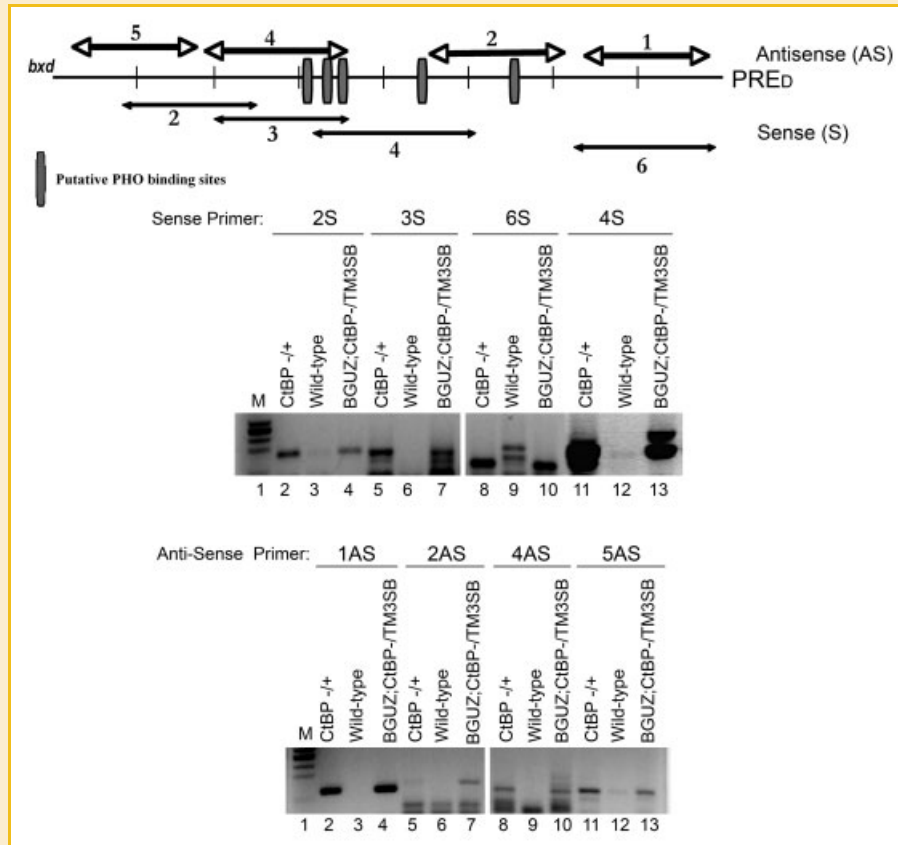
0.25% Triton X-100) for 10 min at RT and then with Wash Solution B (10 mM HEPES, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, and 0.01% Triton X-100) for 10 min at RT. Embryos were resuspended in buffer containing 10 mM HEPES (pH 8.0), 1 mM EDTA, and 0.5 mM EGTA along with protease inhibitors (Sigma). Glass beads of 106-μm (G-4649; Sigma) were added and sonication was carried out at 6 W output power for 8 pulses of 20 s each (Ultrasonic Processor, Sonics & Materials, CT).

Sonicated chromatin (50–200 μg) was diluted in 16.7 mM Tris–HCl, pH 8.1, 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS, 1.1% Triton X-100 along with protease inhibitors (Sigma) and precleared for 1–2 h with protein A-ssDNA slurry (200 μg salmon sperm DNA/ protein A agarose/0.5 mg/ml BSA) and 1% of the total chromatin was taken as the input sample. Immunoprecipitation was carried out at 4°C overnight with antibodies (anti-Polycomb, sc-25762; Santa Cruz Biotechnology; acetyl-K9 07-352, trimethyl K27 07-449, Upstate Biotechnology; or PHO antiserum, kindly provided by Dr. Judy Kassis). The DNA/protein/antibody complex was isolated using protein A agarose beads. Immune complexes were washed successively with low salt, high salt, and LiCl wash buffers followed by twice with TE. Two hundred microliters of elution buffer (0.1 M NaHCO$_3$ and 1.0% SDS) was added to the beads along with RNaseA (50 μg/ml) and incubated at 37°C for 30 min followed by the addition of 4 μl of 0.5 M EDTA, 8 μl of 1 M Tris–HCl, pH 6.5, to a

Fig. 1. CtBP controls intergenic transcripts at the PRE$_D$ sequence. The upper panel shows the PRE$_D$ region with predicted PHO binding sites (vertical rectangles). Double headed arrows show the location of primers used to detect RNA transcripts by RT–PCR along the PRE$_D$ sequence. The middle and lower panels show the results of RT–PCR to detect RNA transcripts induced in CtBP mutant backgrounds compared to wild-type flies. RNA was isolated 14 h post-egg-lay. Genotypes of samples are shown above the lanes as well as primer pairs. Sizes of amplified products for sense primers 2S, 3S, 4S, and 6S are 166, 160, 175, and 121 bp, respectively. Antisense amplified products 1AS, 2AS, 4AS, and 5AS are 137, 156, 138, and 139 bp, respectively.

CtBP;TM3SB lines compared to CtBP wild-type embryos (Fig. 1, middle panel, lanes 2–4). Likewise, primer pairs 3S, 4S, and 6S yielded transcripts in CtBP mutant backgrounds that were almost absent in wild-type embryos (Fig. 1, middle panel, lanes 5–13). In a similar manner, antisense primers 1AS, 2AS, 4AS, and 5AS detected transcripts in CtBP mutant backgrounds that were almost absent in CtBP wild-type embryos (Fig. 1, lower panel, lanes 2–13). Thus, in a CtBP mutant background, transcripts are induced across the PRE$_D$ sequence.

These results were not unique to the PRE$_D$ sequence. Similar results were obtained with two primer sets (3 and 5) at the *scr* PRE sequence (Fig. 2A, lanes 1–6), as well as the *en* PRE sequence (Fig. 2B, primer set 5). Time-course studies showed that transcripts across the PRE$_D$ sequence were elevated in CtBP mutant backgrounds at 3, 10, and 14 h post-egg-lay but were near wild-type levels at 6 h (Fig. 1A, supplemental data). Time-course studies at the *scr* PRE showed highest transcript inductions at 3 and 10 h post-egg-lay compared to wild type (Fig. 1B, supplemental data) and at the *en* PRE at 14 h (Fig. 1C, supplemental data). Interestingly, all transcripts were detected with cDNA synthesized by random priming but not with oligo(dT) priming suggesting that these

transcripts do not contain polyA tails (data not shown). In summary, we have detected intergenic transcripts across several PRE sequences that appear to be regulated by the levels of CtBP within the embryo.

RESTORATION OF CtBP LEVELS REVERSE TRANSCRIPT INDUCTION

To determine whether reduced levels of CtBP in the CtBP mutant backgrounds were indeed responsible for induction of transcripts, we crossed our *ctbp*$^{+/-}$ flies with a transgenic line containing a heat-shock inducible *ctbp* transgene (*hsp*FLAG-dCTBP). Embryos were collected and either left untreated, or heat-shocked to induce CtBP expression. Strikingly, upon induction of CtBP, expression of the intergenic transcripts across the PRE$_D$ sequence was reduced (Fig. 3, top panel, compare lanes 2 and 3, 6 and 7, 10 and 11, 14 and 15; bottom panel, lanes 2 and 3, 6 and 7, 10 and 11). As expected, very little change was observed in embryos where *ctbp*$^{+/-}$ flies were crossed with wild-type flies followed by heat-shock (Fig. 3, top panel, compare lanes 4 and 5, 8 and 9, 12 and 13, 16 and 17; bottom panel, lanes 4 and 5, 8 and 9, 12 and 13). Therefore, our data indicate that CtBP levels play a crucial role in regulation of expression of transcripts across PRE sequences.
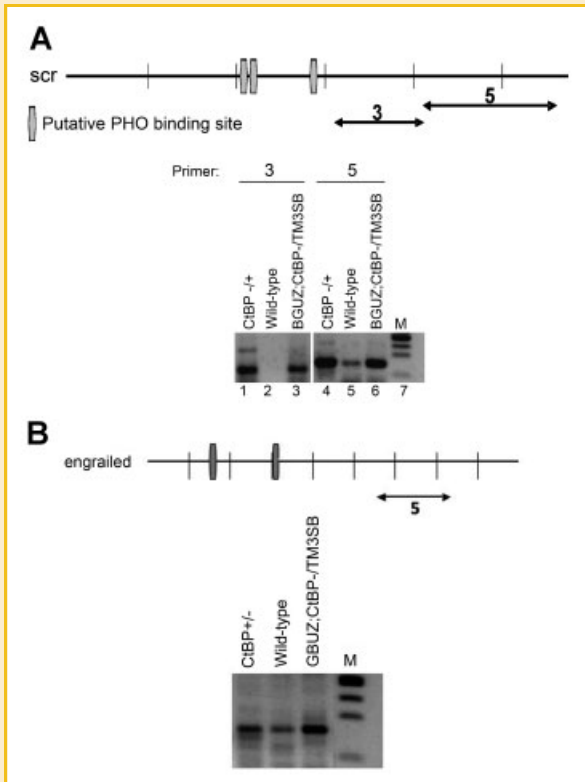
Fig. 2. CtBP controls intergenic transcripts at the *scr* (A) and *engrailed* (B) PRE sequences. Maps of the *scr* and *engrailed* PRE regions are shown in the top panels with putative PHO binding sites represented as vertical rectangles. Primer pairs are represented by double-headed arrows. Lower panels show RT–PCR results with RNA isolated from 14 h embryos and the genotype of each sample shown above the lanes. Amplified products for *scr* primer pairs 3 and 5 are 122 and 150 bp, respectively, and the *en* amplified product is 152 bp.

Fig. 3. Expression of transgenic CtBP restores repression of intergenic transcripts. *ctbp*$^{+/-}$ mutant flies were crossed to either FLAG–dCtBP transgenic flies or wild–type flies. Embryos were harvested at 3 h post–egg–lay, either left untreated or heat–shocked to induced CtBP expression, and RNA was isolated at 15 h and subjected to RT–PCR with the primers shown above the lanes. Genotypes and treatments are shown above each lane.

INTERGENIC TRANSCRIPTS CORRELATE WITH LEVELS OF PHO AND Pc DNA BINDING AND WITH HISTONE MODIFICATIONS

Previously we showed that YY1 is the functional mammalian homolog of *Drosophila* PHO and that YY1 can correct phenotypic defects in *pho* mutants flies [Atchison et al., 2003]. We also found that CtBP is required for YY1 or PHO DNA binding at PRE sequences and for subsequent recruitment of PcG proteins to DNA [Srinivasan and Atchison, 2004]. Our results above indicate that lowered levels of CtBP expression cause increased expression of intergenic transcripts across PRE regions. Therefore, we wanted to investigate whether induction of these transcripts resulted in the loss of PHO DNA binding at PRE sequences and subsequent loss of recruitment of PcG complexes. We performed ChIP experiments with wild-type and *ctbp*$^{+/-}$ embryos using various antibodies and DNA was quantitated by qPCR using primers that amplify the PRE$_D$, *scr*, and *en* PRE regions (Fig. 4). As anticipated, we found that in a CtBP mutant background, there was a reduction in occupancy by PHO at the PRE$_D$, *scr*, and *en* sequences compared to the wild type (Fig. 4A–C). This correlated with concomitant reduction in Polycomb (Pc) DNA binding and reduction in trimethylation of histone H3 on lysine 27 which is characteristic of PcG function (Fig. 4A–C). Interestingly, there was a corresponding increase in histone H3
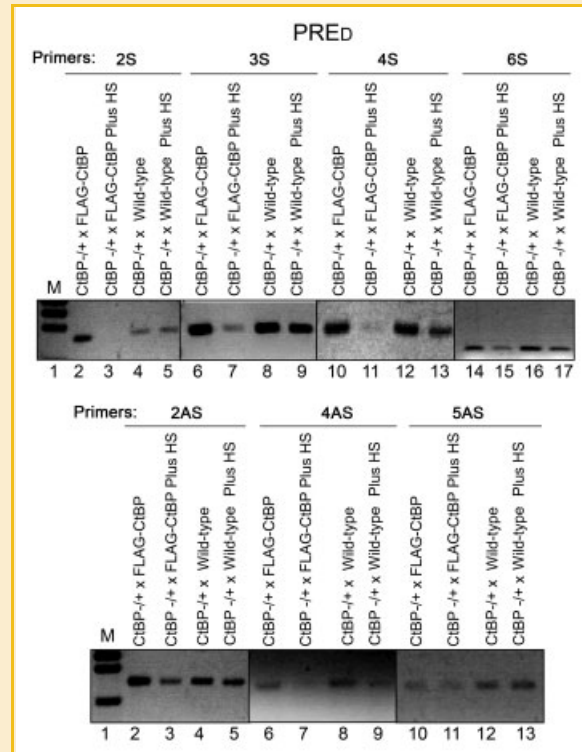
acetylation on lysine 9 at the PRE$_D$ sequence that correlates with active chromatin (Fig. 4A).

CtBP MEDIATES PHO AND Pc BINDING TO PREs

We showed above that reduced levels of CtBP resulted in increased expression of intergenic transcripts across PRE sequences as well as loss of PHO binding. In addition, we showed that expression of intergenic transcripts could be reversed by overexpression of CtBP. Based on these results, we questioned whether increased CtBP levels in a *ctbp*$^{+/-}$ background would revert PHO and Pc binding to wild-type levels. To test this, *ctbp*$^{+/-}$ mutant flies were crossed with our transgenic *hsp*FLAG-dCTBP line. Embryos were collected at 3 h, heat-shocked multiple times to induce CtBP expression, and chromatin was prepared 10 h post-egg-lay for ChIP assays with various antibodies. PCR was performed with primers spanning the PRE$_D$, *scr*, and *en* PRE sequences. Strikingly, after heat-shock we found significant increases in DNA binding by PHO and Pc to each PRE sequence, and a corresponding increase in levels of H3 trimethylation on lysine 27 compared to the non-heat-shock control samples (Fig. 5A–C). These results indicate that upon CtBP induction there is recovery of PcG complexes on PRE sequences that parallels repression of intergenic transcripts. Our results provide a direct correlation between levels of CtBP protein, upregulation of intergenic transcripts, and promoter occupancy by PRC complexes.
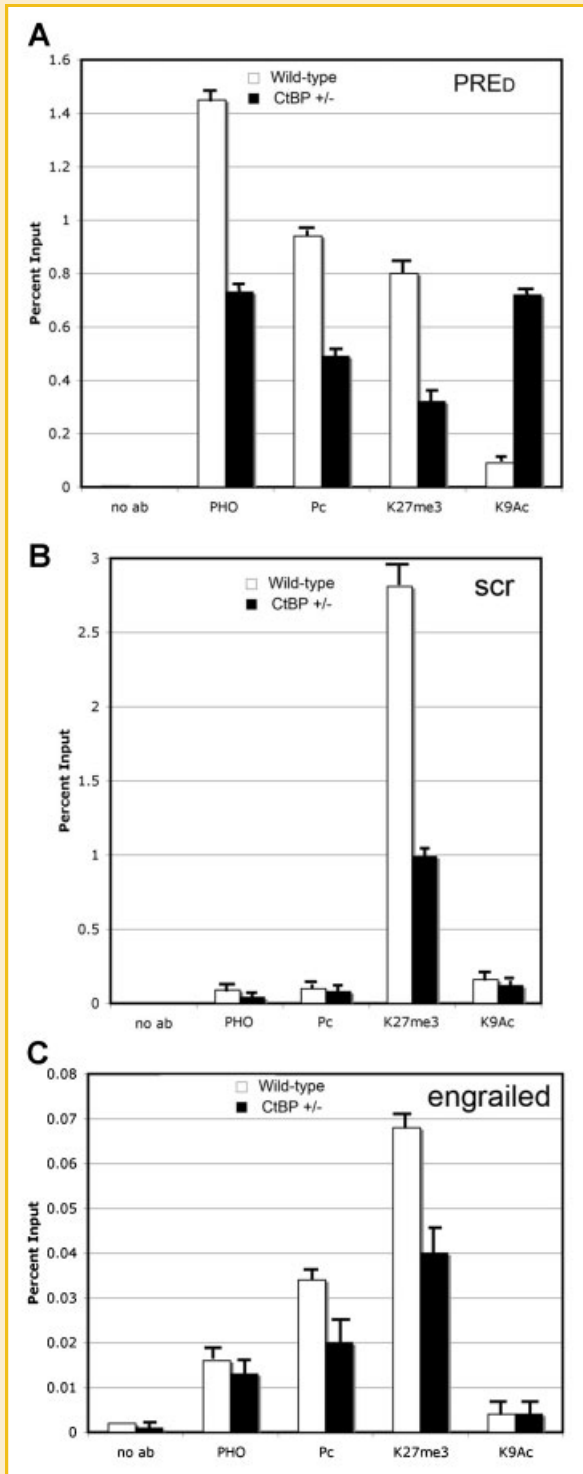
Fig. 4. CtBP levels control PHO and Pc DNA binding and histone modifications. Wild-type and *ctbp*^{+/−} embryos were harvested at 14 h and processed for ChIP assay with antibodies against PHO, Pc, H3 trimethyl-lysine 27, or H3 acetyl-lysine 9 followed by PCR with primers to the PRE$_D$ (A), *scr* (B), or *engrailed* (C) PRE sequences.
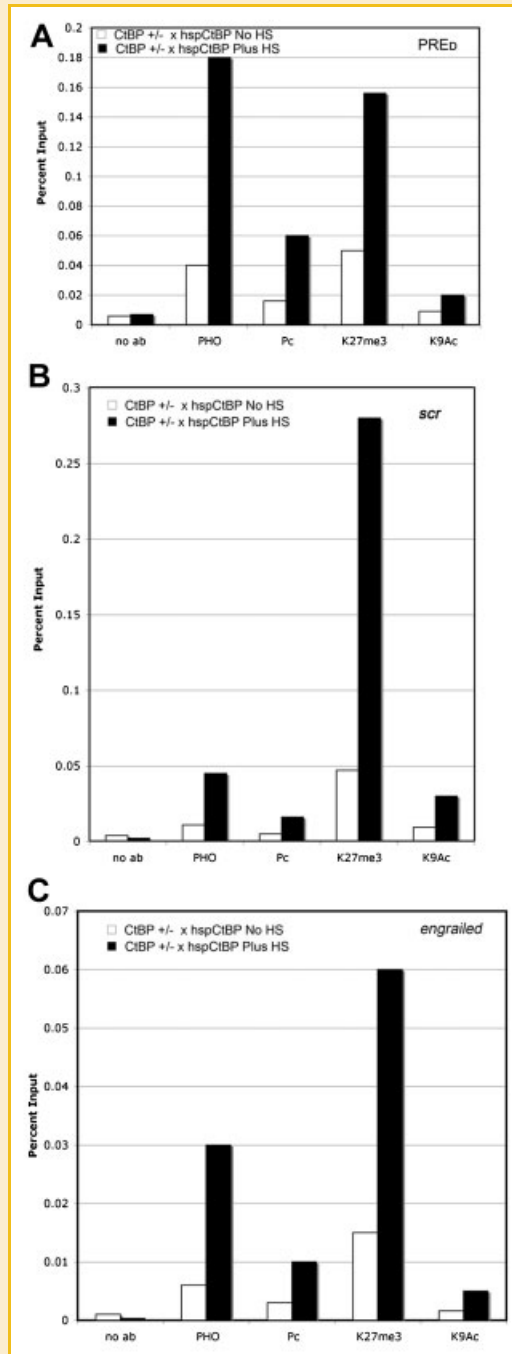

Fig. 5. Expression of a CtBP transgene restores PHO and Pc DNA binding as well as methylation of H3 lysine 27. Wild-type and *ctbp*^{+/−} embryos were harvested at 3 h, either left untreated or heat-shocked to induce CtBP expression, then processed for ChIP assay with antibodies against PHO, Pc, H3 methyl-lysine 27, or H3 acetyl-lysine 9 followed by PCR with primers to the PRE$_D$ (A), *scr* (B), or *engrailed* (C) PRE sequences.

DISCUSSION

CtBP is a well-known corepressor of several DNA binding transcription factors in *Drosophila* (viz. Snail, Hairy, Krüppel, and Knirps) as well as in mammals [Nibu et al., 1998; Poortinga

et al., 1998; Keller et al., 2000; Chinnadurai, 2007]. We show here that loss of CtBP results in elevated expression of intergenic transcripts, reduced PHO DNA binding, and concomitant loss of PcG recruitment. CtBP levels regulate intergenic transcripts across PRE sequences and these transcripts may control PHO DNA binding and subsequent PcG complex recruitment. We previously suggested that CtBP might act as a bridging molecule between YY1/PHO and PcG complexes to target PRC1/2 complexes to specific DNA sequences (PREs) [Srinivasan and Atchison, 2004]. While this remains possible, it does not readily explain the loss of PHO binding to PREs in a $ctbp^{+/-}$ background. Our results here are consistent with a new model in which CtBP represses transcription of intergenic transcripts that interfere with PHO DNA binding. In this model, wild-type levels of CtBP repress transcription of these intergenic transcripts thus allowing PHO DNA binding and subsequent PcG recruitment (Fig. 6). We suggest that reduced PHO binding is a secondary effect of increased intergenic transcripts. The use of intergenic transcripts to locally control PHO binding would allow a subset of PREs to be inactivated even though they have PHO binding sties.

It is well known that CtBP mediates both long-range and short-range transcriptional repression with Hairy, Knirps, Krüppel, and Snail. Hence, it might be well envisioned that a 50% loss of CtBP leads to sub-stoichiometric levels of CtBP resulting in transcriptional derepression with concomitant upregulation of intergenic transcripts across PREs. Our model (Fig. 6) predicts the existence of promoter/enhancer regions flanking PRE sequences that bind to sequence specific transcription factors that mediate their activity in a CtBP-dependent manner. Preliminary analyses of the sequences flanking PRE_D, scr, and en indicate putative binding sites for DNA-binding transcription factors such as Hairy, Knirps, and Snail, all of which are known to recruit CtBP for their transcriptional activity. It would be interesting to investigate whether these flanking regions do act as promoter regions for CtBP-mediated regulation, or whether more complex long-range enhancer activity is responsible for CtBP-mediated transcriptional repression of intergenic transcripts.
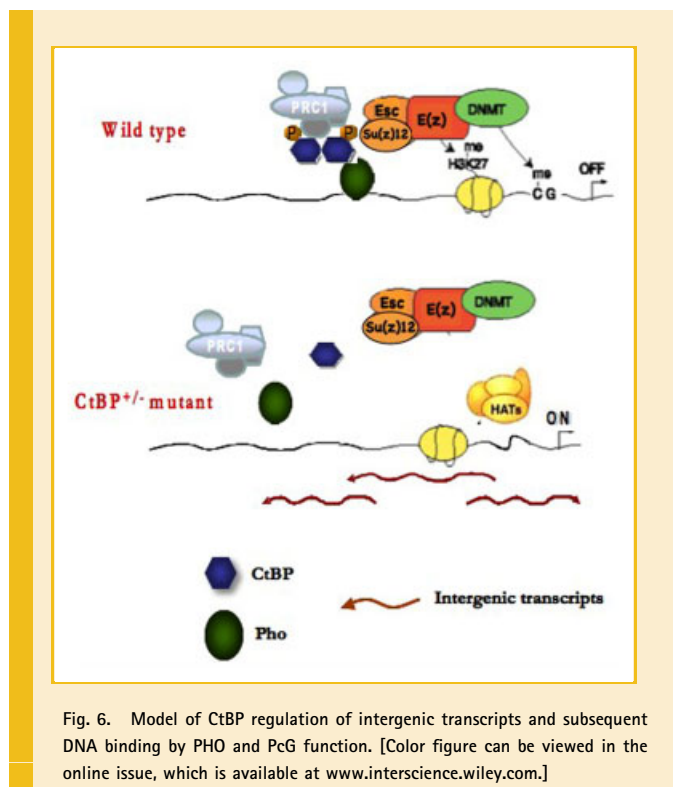
In *Drosophila*, earlier studies using in situ hybridization showed expression of non-coding transcripts across the regulatory domain of *abd-A* and *abd-B* in the same orientation as the *hox* genes [Zhou et al., 1999; Bae et al., 2002; Rank et al., 2002]. Interestingly, these non-coding BX-C transcripts are expressed before *hox* gene transcription leading to the proposition that their early expression may cause the BX-C region to adopt an open chromatin structure leading to the recruitment of activator proteins to regulatory regions [Bae et al., 2002; Hogga and Karch, 2002]. Transcription of ncRNA can regulate gene expression because forced transcription through regulatory sequences results in lost PcG repression [Hogga and Karch, 2002; Rank et al., 2002].

Our studies not only show the presence of short intergenic transcripts across various PRE regions during embryogenesis, but for the first time demonstrate that decreased expression of the corepressor CtBP (in $ctbp^{+/-}$ embryos) can cause induction of intergenic transcripts in vivo. The effects are striking, with reduced CtBP levels resulting in induction of intergenic transcripts, loss of DNA binding by PHO, loss of Polycomb recruitment to the cognate PRE sequences, and reduced trimethylation of histone H3 on lysine 27. When levels of CtBP protein are restored by heat-shock in transgenic flies, these transcripts are downregulated demonstrating that CtBP regulates these transcripts. With elevated CtBP levels and reduced intergenic transcription, PHO DNA binding and PcG recruitment are restored. The temporal expression pattern of intergenic transcripts in the $ctbp^{+/-}$ background yields transcripts peaking at the PRE_D and scr sequences at 3 and 10 h, while transcripts peak at 14 h at the en PRE. This correlates well with the protein expression profile. *Ubx* gene expression peaks in the embryonic stage, whereas *engrailed* expression peaks at later stages of development. Thus, these transcripts may naturally regulate PRE function during development. Transient changes in CtBP expression or post-translational mechanisms may regulate the function of CtBP to mediate transient changes in intergenic transcription levels leading to altered PHO DNA binding and PcG complex recruitment.

It is not yet clear whether the effect of intergenic transcripts on PHO DNA binding is direct or indirect. The act of transcription may directly dislodge PHO from DNA, or the mechanism of reduced PHO binding in response to lowered CtBP levels and elevated intergenic transcripts could be indirect. Histone modifications resulting from transcriptional activity could potentially provide a mechanism for controlling PHO DNA binding [Tie et al., 2009], or lowered CtBP levels might induce expression of genes that control PHO DNA binding in some other way. Additional details of the mechanism of CtBP regulating intergenic transcripts and PcG recruitment to DNA will require further studies.

Fig. 6. Model of CtBP regulation of intergenic transcripts and subsequent DNA binding by PHO and PcG function. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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