

Drosophila* C-terminal Binding Protein Functions as a Context-dependent Transcriptional Co-factor and Interferes with Both Mad and Groucho Transcriptional Repression

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***Drosophila* C-terminal binding protein (dCtBP) and Groucho have been identified as Hairy-interacting proteins required for embryonic segmentation and Hairy-mediated transcriptional repression. While both dCtBP and Groucho are required for proper Hairy function, their properties are very different. As would be expected for a co-repressor, reduced Groucho activity enhances the hairy mutant phenotype. In contrast, reduced dCtBP activity suppresses it. We show here that dCtBP can function as either a co-activator or co-repressor of transcription in a context-dependent manner. The regions of dCtBP required for activation and repression are separable. We find that mSin3A-histone deacetylase complexes are altered in the presence of dCtBP and that dCtBP interferes with both Groucho and Mad transcriptional repression. Similar to CtBP's role in attenuating E1A's oncogenicity, we propose that dCtBP can interfere with corepressor-histone deacetylase complexes, thereby attenuating transcriptional repression. Hairy defines a new class of proteins that requires both CtBP and Groucho co-factors for proper function.**

Transcriptional repression is an important feature of developmental processes, where it is necessary for establishing complex patterns of gene expression (1–4). A rapidly growing subset of repressors appears to act indirectly by recruiting accessory proteins, referred to as co-repressors, that themselves bring about repression (*cf.* Refs. 5 and 6). Examples of such pairs of proteins are the interaction of the Sin3 co-repressor with the Mad basic helix-loop-helix zipper protein and the interaction of the Tup1/Ssn6 co-repressors with several types of DNA-binding proteins (7, 8). The mechanisms whereby these repressor-co-repressor complexes mediate repression are the subject of many ongoing studies. One emerging theme is the recruitment of histone deacetylases (HDACs)¹ to alter local

chromatin structure at promoters; hyperacetylated chromatin is usually associated with active transcriptional states, whereas hypoacetylated chromatin is usually associated with repressed transcriptional states (9, 10). The Mad-Sin3 repressor complex has been shown to recruit HDAC through specific interaction of Sin3 and HDAC (see Ref. 11 and references therein).

Transcriptional repression plays a major role in segmentation gene expression that leads to proper body patterning during early *Drosophila* development (*cf.* Refs. 1 and 4). A number of different transcriptional repressors present in the early *Drosophila* embryo have been shown to encode sequence-specific DNA binding transcription factors that function by recruiting co-repressor proteins. Two putative co-repressors acting during embryogenesis have been identified: Groucho (Gro) and *Drosophila* C-terminal binding protein (dCtBP) (12–14). Both of these factors do not bind DNA themselves but are brought to the DNA through their interaction with sequence-specific DNA binding repressors.

Gro was first demonstrated to function as a co-repressor through its interactions with the *hairy* pair-rule segmentation gene (Ref. 13; reviewed in Refs. 15 and 16). *hairy* functions as a dedicated repressor and holds a key position in the segmentation gene hierarchy, since it is one of the first genes to show a spatially reiterated (metameric) pattern of expression that is central to establishing the proper embryonic body plan (17). Hairy's conserved C-terminal WRPW tetrapeptide motif was shown to be necessary and sufficient for interaction with Gro protein using biochemical, two-hybrid, and genetic approaches (13, 18). Gro represses both basal and activated transcription when recruited to endogenous promoters through protein-protein interaction or when tethered to a heterologous promoter (13, 18). Gro interacts genetically and physically with the *Drosophila* histone deacetylase Rpd3; interaction of Gro with Rpd3 is necessary for efficient Gro-mediated repression, and its activity is sensitive to the HDAC inhibitor, trichostatin A (TSA; Ref. 19). However, the relatively mild phenotypic defects associated with the Rpd3 mutation have led to the suggestion that Gro does not function solely through recruitment of the Rpd3 histone deacetylase (20). In addition to its interaction with Hairy, Gro mediates repression through other classes of DNA-binding transcriptional regulators including Engrailed, Dorsal, Tcf, and Runt (21–25).

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¹ The abbreviations used are: HDAC, histone deacetylase; CtBP, C-terminal binding protein; dCtBP and mCtBP, *Drosophila* and mouse

CtBP, respectively; E(spl), enhancer of split; FLAG, FLAG epitope tag; Gro, Groucho; GST, glutathione *S*-transferase; IVT, *in vitro* translated; MT, Myc epitope tag; Rb, retinoblastoma; SID, Sin3A interaction domain; Tcf, T cell factor; TSA, trichostatin A; aa, amino acids; PCR, polymerase chain reaction.

dCtBP is the *Drosophila* homolog of human CtBP, a 48-kDa cellular phosphoprotein initially shown to interact with the C terminus of the adenovirus E1A oncoprotein and, while bound, disrupts the function of a conserved N-terminal E1A domain in *cis* (26, 27). CtBP homologs have also been reported in mouse (mCtBP1 and mCtBP2), *Xenopus* (XCtBP), and *C. elegans* (CeCtBP), where they have been shown to interact physically with a diverse set of factors (*cf.* Refs. 14, 26, and 28–32). Human CtBP was identified on the basis of its ability to bind the C terminus of E1A using the consensus motif PXLDSX(K/R). While most of the CtBP-interacting proteins identified to date contain this consensus motif, a different motif, PLSLV, has been shown to be required for Hairy and XTcf-3 interaction (14, 28).

CtBP proteins have been shown to function as co-repressors *in vitro*. When mCtBP1 or mCtBP2 proteins were fused to heterologous DNA binding domains and examined in transcriptional tethering assays, these proteins were efficient repressors of transcription (29, 32). In the case of mCtBP1, the repression was shown to be TSA-sensitive, suggesting a role for histone deacetylases (29). Together these observations suggest that CtBP, like Gro, functions utilizing a repression mechanism involving alteration of local chromatin structure.

The different transcriptional repressors shown to function during early *Drosophila* development have been placed in two classes on the basis of their effective range of action. Short range repressors have been proposed to inhibit the core transcription complex or upstream activators and function over relatively short distances (~100 base pairs), while long range repressors have been proposed to silence gene expression over long distances (>1 kilobase pair; Refs. 23 and 33). While the mechanism underlying short range and long range repression is not known, Levine and colleagues (12, 34) have noted that the Hairy and Dorsal long range repressors bind Gro, while many of the short range repressors (Knirps, Krüppel, Snail) bind dCtBP. This observation has led to their proposal that Gro and dCtBP mediate these separate pathways of transcriptional repression (35). However, one enigmatic observation is that Hairy, defined as a long range repressor, binds both Gro and dCtBP (14, 35). While both Gro and dCtBP are required for proper Hairy function, their properties are very different. As would be expected for a co-repressor, reduced maternal Gro activity has been shown to enhance the Hairy mutant phenotype (13). In contrast, reduced doses of maternal dCtBP suppress the Hairy mutant phenotype, suggesting that dCtBP acts in a different manner (14). These differences in Gro and dCtBP properties led us to investigate further the transcriptional properties of dCtBP.

EXPERIMENTAL PROCEDURES

Cell Culture—All cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Hyclone). 293, NIH 3T3, and CV-1 cells were obtained from the Eisenman laboratory. B78 cells were obtained from the Groudine/Weintraub laboratories.

Plasmid Construction

Reporters—The 4×GAL14DLUC reporter system was described previously (36). The pG5LxLuc reporter (Fig. 5A) was constructed by cutting the 4×GAL14DLUC reporter with *Bgl*II, blunting this site with Klenow, followed by an *Eco*RI partial digestion to retrieve a fragment containing the luciferase gene and SV40 poly(A) site. This fragment was then subcloned into the *Sma*I-*Eco*RI sites of pG5E4, a plasmid containing five Gal4 binding sites upstream of the E4 TATA box (37). A pair of oligonucleotides coding for two tandem LexA binding sites was subcloned into the *Bam*HI site located between the Gal4 binding sites and E4 minimal promoter. Construct accuracy was checked by sequencing (top oligo, 5'-GATCGATGTACTGTATGTACATACAGTACGTCGACTC-GAG-3'; bottom oligo, 5'-GATCCTCGAGTCGACGTACTGTATGTAC-ATACAGTACATC-3').

Gal4-DBD Fusions—The Gal4-DBD (aa 1–145) and Gal4-Mad (SID) fusions were described previously (38). To generate the Gal4-Gro fusion, full-length *gro* cDNA was excised from pCite4b⁺ using *Bam*HI-*Bgl*II and then subcloned in the *Bam*HI site of the pSPGal plasmid described previously (38). To generate the Gal4-dCtBP full-length (aa 1–387) and N-T partial protein fusions, each protein fragment was generated by standard PCR using primers containing *Bam*HI sites, cut with *Bam*HI, and subcloned into the *Bam*HI site of pSPGal plasmid described previously (38). The dCtBP pieces (N-T) used are as follows: N, amino acids 1–95; P, aa 1–210; Q, aa 149–387; R, aa 149–210; S, aa 255–387; and T, aa 255–325. The Gal4-dCtBP(H→Q) mutant was generated by PCR site-directed mutagenesis from full-length dCtBP using primers containing the altered nucleotide sequence. Orientation was determined by restriction mapping and sequencing.

Other Transcription Constructs—The E1A clones used in these studies, E1A-12Swt, E1A-Δ2–36 (E1AΔp300), and E1A-YN49/598 (E1AΔRb), were described previously (39). The LexDBD-VP16 fusion construct was described previously (40).

Epitope Tag Constructs—MT-dCtBP was generated by PCR from the dCtBP cDNA (14) with primers containing *Xho*I (5') and *Xba*I (3') sites and then subcloning into the *Xho*I-*Xba*I sites of the pCS2+MT vector (40). MT-mSin3A was described previously (34). FLAG-mSin3A was provided by Y. Shio (Fred Hutchinson Cancer Research Center).

GST Fusions—The wild-type GST-dCtBP fusion was described previously (14). The GST-dCtBP deletion constructs contain deletions of five amino acids (Δ613, deleting aa 205–209; Δ688, deleting aa 230–234; Δ805, deleting aa 269–273; and Δ865, deleting aa 289–293). These deletions were made within the full-length dCtBP cDNA using primers that delete 15 nucleotides corresponding to the deleted five amino acids. The final PCR products were cut with *Bam*HI-*Sma*I and then subcloned into the *Bam*HI-*Sma*I sites of pGex3X.

In Vitro Translation Constructs—All constructs were generated by subcloning cDNA fragments generated by PCR with primers containing restriction enzyme recognition sequences into the pCite 4a⁺ vector (Novagen). The full-length Hairy cDNA (aa 1–343), Hairy C terminus (aa 95–343), and full-length Knirps cDNA were subcloned into pCite using *Eco*RI-*Bgl*II sites. An *Eco*RI-*Bam*HI fragment containing the C terminus 100 amino acids of E1A was subcloned into pCite using *Eco*RI-*Bgl*II sites. Full-length Groucho was subcloned into pCite using *Bam*HI-*Sal*I sites.

Repression Assays

Transfections and luciferase assays were performed by standard procedures (42). In all assays, 1.5 μg of reporter and 3.5 μg of Gal4 plasmids were used per 6-cm dish. Transfections were performed three times in duplicate, and transfection efficiencies were normalized using a co-transfected β-galactosidase plasmid (36, 43).

Immunoprecipitations and Western Blotting

Transfections into 293 cells were performed by standard procedures using 10 μg of plasmid per 10-cm dish (42). Due to lower transfection efficiencies with calcium phosphate, lipofection was used in NIH 3T3 cells in these experiments using SuperFect (Qiagen) according to the manufacturer's instructions. Low stringency immunoprecipitations were performed as described (36, 43). For histone deacetylase assays, low stringency immunoprecipitations were performed, and the precipitates were resuspended in HD buffer (20 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol). For radio-immunoprecipitation experiments, low stringency immunoprecipitations were performed after cells were metabolically labeled for 3 h with [³⁵S]methionine (PerkinElmer Life Sciences).

Immunoprecipitated proteins were resolved on SDS-polyacrylamide gels and examined by autoradiography or immunoblot analysis. Western blots were performed by electrophoretic transfer of polyacrylamide-resolved polypeptides to nitrocellulose membranes (Schleicher and Schuell). Membranes were blocked in PBST (1× phosphate-buffered saline, 0.1% Tween) containing 2% nonfat dry milk. Membranes were probed in PBT (1× phosphate-buffered saline, 0.1% bovine serum albumin, 0.1% Tween), washed four times in PBT, and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Jackson ImmunoResearch). Membranes were then washed four times in PBT and analyzed by ECL (Amersham Pharmacia Biotech).

Antisera used were as follows: anti-HDAC1,2 from E. Seto (University of South Florida), anti-mSin3A from R. Eisenman (Fred Hutchinson Cancer Research Center), anti-FLAG from Y. Shio, and anti-9e10

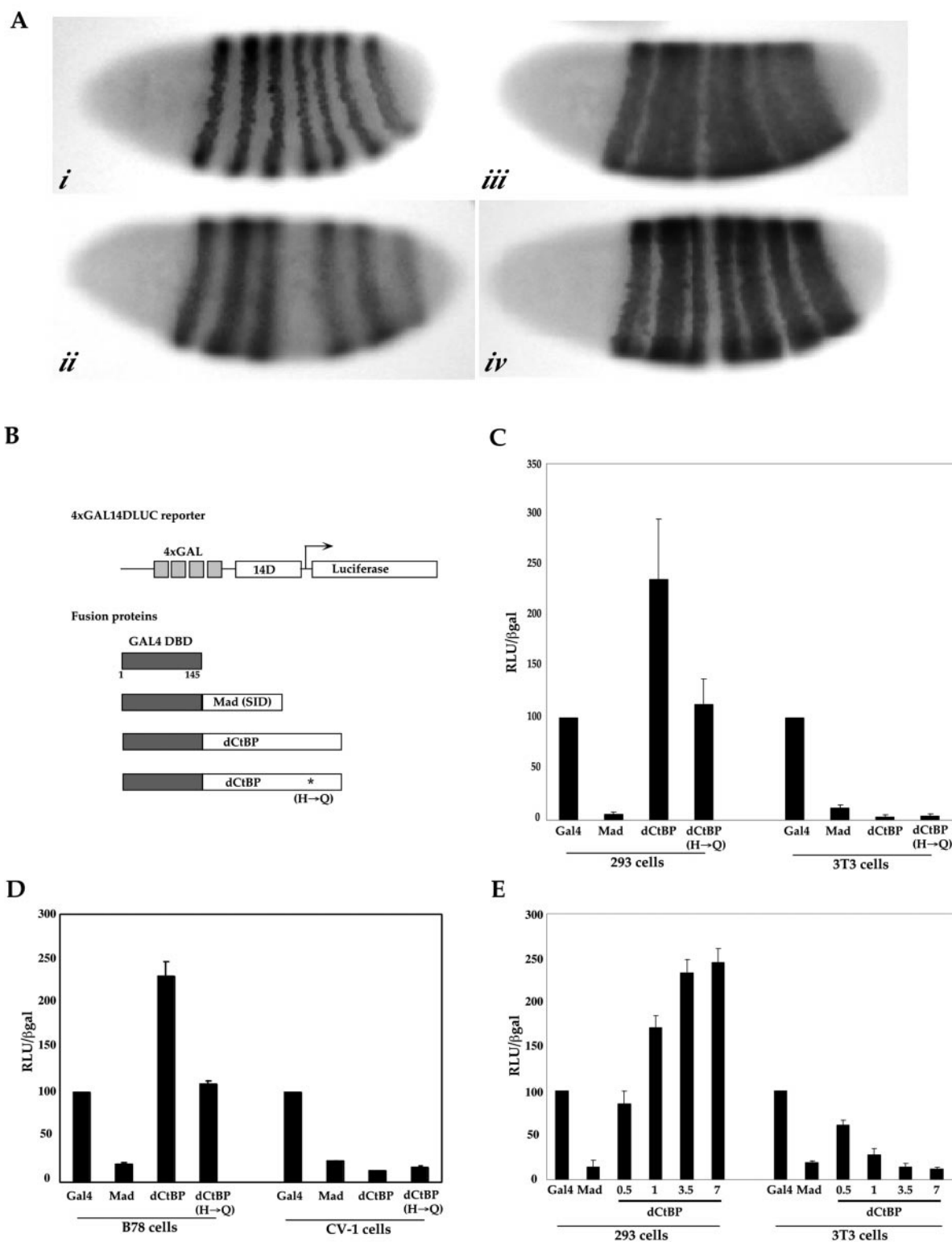


FIG. 1. dCtBP functions in a context-dependent manner. A, dCtBP can enhance or suppress segmentation gene expression. *i-ii*, genetic interactions between *dCtBP* and *knirps*. Heterozygous *kni* males were mated to wild type (*i*; *kni*¹⁰/+ progeny from the following cross: +/+ females × *kni*¹⁰/TM3 males) or heterozygous *dCtBP* (P1590) females (*ii*; *kni*¹⁰/+ progeny from the following cross: P1590/+ females × *kni*¹⁰/TM3 males). Reduction of maternal *dCtBP* results in the loss of repression of Ftz in the interstripe regions between stripes 3 and 6. Note the loss of Ftz stripe 4 and the lack of refinement for stripes 5–7 (compare *ii* with *i*). Similar results have been reported for Even-skipped expression (12). *iii-v*, genetic interactions between *dCtBP* or *groucho* and *hairy*. Ftz expression is shown in an embryo trans-heterozygous for a strong *hairy* allele, *h*^{7H}, and a weak *hairy* allele, *h*^{12C}, showing the intermediate classic pair-rule phenotype (*h*^{7H}/*h*^{12C} progeny from the following cross: *h*^{7H}/TM3 females × *h*^{12C}/TM3 males). The classic pair rule phenotype generated by the *h*^{7H}/*h*^{12C} allelic combination (*iii*) is suppressed (*iv*) when one copy of *dCtBP* (P1590) is removed maternally (*h*^{7H} P1590/*h*^{12C} + progeny from the following cross: *h*^{7H} P1590/TM3 females × *h*¹² +/TM3 males). Note the improved resolution of the Ftz stripes due to increased repression of Ftz in the interstripe regions. B–E, dCtBP can activate or repress transcription. B, schematic diagram of the reporter and expression plasmids used in the following experiments. C and D, 293 and NIH 3T3 cells (C) or B78 and CV-1 cells were transfected with the 4×GAL14DLUC reporter and Gal4 or the GAL4DBD fusion proteins diagrammed in B. Mad represses transcription in all cell types. dCtBP activates transcription weakly (2–3-fold) in 293 cells (C) or B78 cells (D) but strongly represses transcription (20-fold) in NIH 3T3 cells (C) or CV-1 cells (D). Mutation of a conserved catalytic histidine residue (His³¹⁵ → Q) within dCtBP's acid

(anti-Myc epitope tag) from J. Roberts (Fred Hutchinson Cancer Research Center).

Histone Deacetylase Assays

Histone deacetylase assays were performed as described (43).

In Vitro Binding Assays

[³⁵S]methionine-labeled *in vitro* translated proteins were obtained using the Promega TnT T7 quick coupled transcription translation system. Binding assays were performed as described by Hurlin *et al.* (44) except that all fusion proteins were expressed at 30 °C to improve protein solubility. All *in vitro* translated proteins were precleared by incubation for 1 h at 4 °C with equal amounts of GST alone on glutathione-Sepharose beads in L-buffer (phosphate-buffered saline, 1% bovine serum albumin, 0.5% Nonidet P-40 and protease inhibitors).

Embryo Analysis

Flies were cultured and crossed on yeast-cornmeal-molasses-malt extract medium at 25 °C. The alleles used in this study are as described previously (14), except for the *kni*¹⁰/*TM3* allele that was obtained from the Bloomington Stock Center. The *h*^{7H} *P1590(dCtBP)* *gro*^{E47} triple mutant chromosome was generated by standard recombination techniques starting from the *h*^{7H} *P1590* chromosome described previously (14) and *gro*^{E47}/*TM3* (S. Artavanis-Tsakonas). Larval cuticle preparations were prepared and analyzed as described by Wieschaus and Nüsslein-Volhard (45). Embryos were prepared, and immunohistochemical detection of proteins was performed as described previously (46) using anti-*ftz* antisera (from H. Krause) and alkaline phosphatase-coupled secondary antibodies (Jackson Laboratories) visualized with Substrate Kit II reagents (Vector Laboratories, Inc.).

RESULTS

dCtBP Can Enhance or Suppress Segmentation Gene Expression—dCtBP has been linked to a number of early *Drosophila* developmental repressors including the gap gene, *knirps* (*kni*), and the primary pair-rule gene, *hairy* (12, 14). While the prevailing model is that CtBP functions as a co-repressor for these DNA binding factors, genetic interactions between dCtBP and these developmental repressors show that dCtBP can both enhance and repress target gene expression in a transcription factor-dependent manner (12, 14). As expected for a co-repressor, reduction of maternal dCtBP activity enhances the *knirps* mutant phenotype (*ii* in Fig. 1A; Ref. 34). This phenotype is visualized in early embryos in the loss of repression of the downstream target gene *fushi tarazu* (*ftz*) in the posterior interstripe regions. Ftz is normally expressed in seven transverse stripes (see *i* in Fig. 1A). Reduction of maternal dCtBP activity results in the loss of Ftz repression in interstripe 3–6 regions and a subsequent loss of Ftz stripe 4 and broadening of the remaining Ftz stripes (*ii* in Fig. 1A).

In contrast, we find that reduction of maternal dCtBP activity suppresses the *hairy* mutant phenotype (14). *hairy* mutations exhibit loss of alternating segment wide regions (17), such that larva trans-heterozygous for the *h*^{7H} allele and a weaker *hairy* allele, *h*^{12C}, display the classic pair-rule phenotype. This phenotype is also visualized in early embryos in the loss of repression of the downstream target gene *fushi tarazu* (*ftz*) in all interstripe regions and a subsequent fusion of Ftz stripes (*iii* in Fig. 1A). This *hairy* mutant phenotype is suppressed by reducing the maternal *dCtBP* dose, resulting in the restoration of Ftz repression in the interstripe regions (*iv* in Fig. 1A; Ref. 14). The *hairy* mutant phenotype can be enhanced by reducing the maternal dose of the co-repressor *groucho* (see Fig. 7, *G* and *H*; Ref. 13). The opposing effects of dCtBP in combination with *kni* or *hairy* during segmentation suggest that dCtBP is likely to be part of different complexes that carry out distinct functions.

dCtBP Can Activate or Repress Transcription—To characterize the transcriptional properties of dCtBP, we made expression constructs encoding proteins with the Gal4 DNA binding domain fused to wild-type and mutant forms of dCtBP (Fig. 1B) and tested their activity in human 293 cells on the same reporter gene construct used to describe Mad/mSin3A transcriptional repression (4×GAL14DLUC; Refs. 36 and 43). As a positive control for transcriptional repression, we used an expression construct with the Gal4 DNA binding domain fused to the mSin3A-interacting (SID) repression domain of Mad (Gal4-Mad; Ref. 36). Whereas Gal4-Mad effectively represses transcription, Gal4-dCtBP shows transcriptional activation in 293 cells (Fig. 1, *C* and *E*). The activation by Gal4-dCtBP is weak (2–3-fold), similar to that reported for factors such as Myc (reviewed in Ref. 47).

CtBP proteins have homology to D-2-hydroxy acid dehydrogenases, but the significance of this homology is unclear. Rather than having catalytic activity, this homology region may function as a dimerization motif (26). We tested a mutant Gal4-dCtBP protein containing a point mutation in a conserved catalytic histidine residue (His³¹⁵ → Gln) within dCtBP's acid dehydrogenase homology domain to determine if this region of CtBP has activity that might be required for this activation. This mutant form of dCtBP (Gal-dCtBP(H→Q)) was no longer able to activate transcription (Fig. 1C).

While these experiments were in progress, the mCtBP1 and mCtBP2 proteins were reported to be potent repressors of transcription in similar tethering assays using SL2 or NIH 3T3 cells (29, 32). These studies also showed that mCtBP2 containing a similar point mutation in the dehydrogenase conserved catalytic histidine residue (His³²¹ → Ala) repressed transcription as efficiently as the wild-type mCtBP2 protein (32). Since this result was opposite to what we obtained in 293 cells, we examined the effects of our Gal4-dCtBP fusion proteins on the 4×GAL14DLUC reporter in NIH 3T3 cells. Consistent with the published reports for the mCtBP homologues, both our wild-type Gal4-dCtBP and the Gal4-dCtBP(H→Q) mutant efficiently repress transcription in NIH 3T3 cells (Figs. 1, *C* and *E*). We have also examined dCtBP's transcriptional properties in other cell lines; dCtBP activates transcription in B78 mouse melanoma cells (~2–3-fold; Fig. 1D) and represses transcription in CV-1 green monkey kidney cells (~10-fold; Fig. 1D). Equivalent levels of wild-type and mutant proteins were expressed as assayed by Western analysis (data not shown). Activation or repression by the Gal-dCtBP proteins in the various cell types required the presence of the Gal4 DNA binding domains in the reporter plasmid (data not shown). Together, these results suggest that dCtBP activates or represses transcription in a cell type-dependent manner and is consistent with our genetic observations.

dCtBP Domains Required for Activation and Repression Are Distinct—Because the dCtBP histidine mutation affected dCtBP's ability to activate but not repress transcription, it suggested that different regions of the dCtBP protein may be required for these activities. To map the regions of the dCtBP protein required to activate or repress transcription, we generated a series of dCtBP protein fragments fused in frame to the Gal4 DNA binding domain (Fig. 2A) and tested them in 293 and NIH 3T3 cells on the 4×GAL14DLUC reporter described above. The regions of dCtBP required for these transcriptional activities do not overlap. The smallest region of dCtBP identi-

dehydrogenase domain prevented dCtBP from activating transcription in 293 or B78 cells but had no effect on dCtBP's ability to repress transcription in NIH 3T3 or CV-1 cells. The values shown (relative luciferase units (RLU)) are the average and S.D. of three experiments performed in duplicate and standardized to a co-transfected β-galactosidase plasmid (*βgal*), with the Gal4 vector alone set to 100. *E*, dCtBP does not squelch transcription. 293 or NIH 3T3 cells were transfected with increasing amounts (in micrograms) of GAL4DBD-dCtBP.

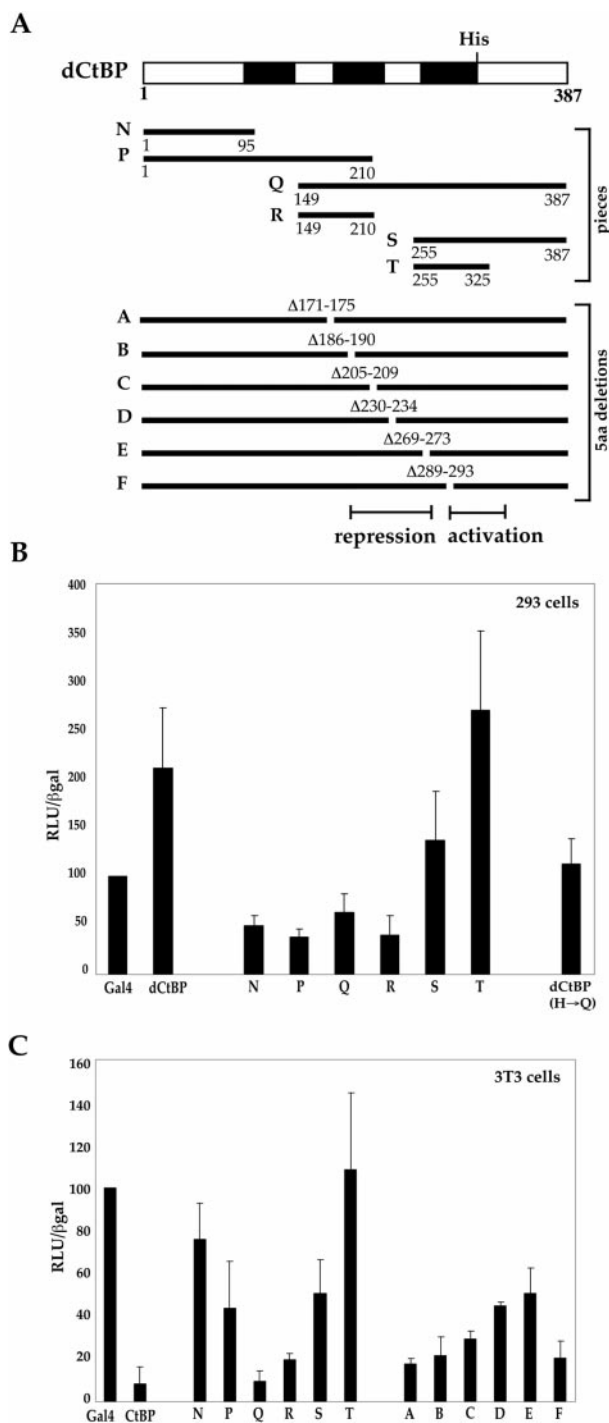


FIG. 2. Distinct dCtBP domains are required for activation or repression. *A*, schematic diagram of the dCtBP protein pieces and the five-amino acid deletion proteins fused to the GAL4DBD and used in the following mapping experiments. The three acid dehydrogenase homology domains in dCtBP are shaded in black, and the position of the conserved catalytic histidine residue is indicated. The smallest domains required for transcriptional activation or repression are indicated. *B* and *C*, different nonoverlapping regions of the dCtBP protein are required for it to function as a transcriptional activator or repressor in these assays. 293 (*B*) or NIH 3T3 (*C*) cells were transfected with the 4 \times GAL14DLUC reporter and Gal4, GAL4DBD-dCtBP fusion proteins, or the GAL4DBD-dCtBP deletion proteins diagrammed in Fig. 1*B*.

fied for efficient activation in 293 cells maps to amino acids 255–325 (fragment T in Fig. 2*B*). While fragment T is sufficient for activation, the larger fragments Q and S, which also contain this region show little or no activation activity, presumably due to the presence of other regulatory regions (*i.e.* fragment Q also

contains the repression domain; see below). This activation region includes the conserved putative catalytic histidine residue that when mutated (dCtBP(H \rightarrow Q)) abrogates dCtBP activation in 293 cells. By examining the dCtBP protein fragments described above and a series of full-length GAL4-dCtBP constructs containing five-amino acid internal deletion mutations, the region of dCtBP identified for efficient repression in NIH 3T3 cells maps to amino acids 190–273 (Fig. 2, *A* and *C*). Western analysis and immunofluorescence were done to confirm that all dCtBP protein fragments were similarly expressed and exhibit proper subcellular localization (data not shown).

E1A Alters dCtBP's Transcriptional Properties—293 cells constitutively express E1A (48). Since CtBP was originally identified as an E1A-binding protein, we examined the effect of E1A on dCtBP transcriptional activity in 293 and NIH 3T3 cells. The high levels of E1A present in 293 cells could titrate out the Gal4-dCtBP, not allowing it to function efficiently as a co-repressor in 293 cells. In this case, expressing additional E1A should have no effect in 293 cells and would be expected to disrupt dCtBP's ability to repress transcription in NIH 3T3 cells. We examined the effect of co-expressing E1A and dCtBP in both 293 and NIH 3T3 cells. Expression of E1A (12S) alone had little effect on our reporter system in either cell type (Fig. 3). Consistent with E1A titrating dCtBP, co-expression of E1A turned dCtBP from a strong repressor (20-fold) into a weak activator (1.5-fold) in NIH 3T3 cells (Fig. 3*B*). However, co-expression of E1A also turned dCtBP from a weak activator (3-fold) to a strong activator (14-fold) in 293 cells (Fig. 3*A*), suggesting that the E1A present in 293 cells is not titrating dCtBP. The E1A (13S) isoform was indistinguishable from the 12S isoform in these studies (data not shown).

CtBP has been proposed to regulate E1A-mediated transformation by modulating CR1-dependent transactivation (26). CR1 is one of the E1A N-terminal conserved domains that mediates association of E1A with transcriptional regulators such as Rb, p130, and p300 (reviewed in Ref. 49). To see if an intact CR1 is required for E1A function in this assay, we examined two E1A N-terminal deletion/substitution mutations shown to affect p300 or Rb binding (Δ p300 and Δ Rb, respectively; Ref. 39; see "Experimental Procedures") but that have intact dCtBP binding sites. Both of these mutations severely affected E1A's ability to convert dCtBP from a repressor to an activator in NIH 3T3 cells (Fig. 3*B*). Only one of these, the E1A(Δ p300) mutation, affected the ability of E1A to enhance dCtBP activation in 293 cells (Fig. 3*A*). dCtBP's ability to activate transcription in the presence of the E1A(Δ Rb) mutation was indistinguishable from wild-type E1A (Fig. 3*A*). E1A clearly has an effect on dCtBP's transcriptional properties in these cell lines. However, the higher levels of E1A present in 293 cells alone cannot be responsible for the cell type differences in dCtBP's transcriptional properties, consistent with the observation that dCtBP activates transcription in other cell lines (B78 cells; Fig. 1*D*) not transformed with E1A. We have examined a number of likely candidate proteins that may mediate these transcriptional differences based on their properties described in the literature (*i.e.* histone acetyltransferases, p30, ku80), but these have been present in the immunoprecipitates (data not shown). Further studies are under way to identify these factors.

dCtBP Is Not Associated with HDAC Activity in 293 or NIH 3T3 Cells—Recent studies have implicated the recruitment of histone deacetylases to repressor/co-repressor complexes (for reviews see Refs. 11 and 50–53). We examined low stringency dCtBP immunoprecipitates from 293 or NIH 3T3 cells for HDAC activity. We generated a Myc epitope-tagged (MT-) dCtBP protein expression construct and assayed for HDAC

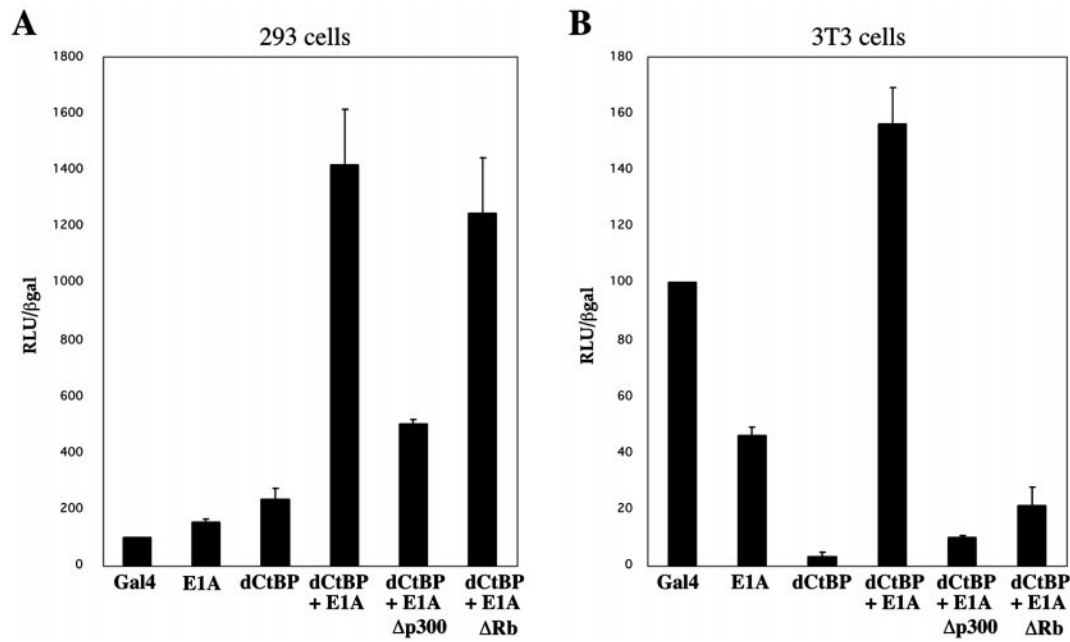


FIG. 3. E1A alters dCtBP's transcriptional properties. Co-transfection with E1A makes dCtBP a better transcriptional activator in 293 cells (A) and turns dCtBP from a repressor into an activator in NIH 3T3 cells (B). 293 or NIH 3T3 cells were transfected with the 4×GAL14DLUC reporter and Gal4 or GAL4DBD-dCtBP with and without E1A. Two E1A mutations were also examined in this assay. E1AΔp300 is a deletion of amino acids 2–36 that has been shown to affect the binding of p300. E1AΔRb is a point mutation (Tyr⁴⁹ → N) reported to disrupt binding of the retinoblastoma protein. Disrupting E1A activity through these mutations interfered with its ability to turn dCtBP from a repressor into an activator (B). The E1AΔp300 mutation reduced E1A's effects on dCtBP transcriptional activation in 293 cells, where the E1AΔRb mutation was indistinguishable from wild-type E1A activity.

activity following low stringency immunoprecipitation with antibodies recognizing the Myc epitope tag (9e10; Ref. 54). The vector alone was used as a negative control (40), and MT-mSin3A served as a positive control (36) for HDAC activity. Whereas the MT-mSin3A positive control showed HDAC activity in both 293 and NIH 3T3 cells, we did not detect HDAC activity in MT-dCtBP immunoprecipitates in either cell type (Fig. 4A), despite dCtBP's ability to activate or repress transcription in these cells. Consistent with these results, we do not detect an interaction between dCtBP and HDAC in GST pull-down assays (data not shown). In addition, while HDAC1 and HDAC2 proteins can be detected by Western analysis of MT-mSin3A immunoprecipitates (Fig. 4C, lane 2), they are not detectable in MT-dCtBP immunoprecipitates (Fig. 4C, lane 3).

dCtBP Interferes with mSin3A-associated HDAC Activity—We next examined whether or not dCtBP could affect HDAC activity recruited by MT-mSin3A. Because dCtBP and mSin3A do not interact physically in directed yeast two-hybrid assays, GST pull-down assays (data not shown), or low stringency immunoprecipitations (Fig. 4D), we expected independent MT-dCtBP and MT-mSin3A complexes. We were surprised to find that when MT-dCtBP and MT-mSin3A were co-transfected, no HDAC activity was recovered in the 9e10 (MT-) low stringency immunoprecipitates (Fig. 4, A and B). Consistent with the lack of HDAC activity, HDAC protein could not be detected by Western analysis of the 9e10 low stringency immunoprecipitates (Fig. 4C). Strong HDAC activity and HDAC protein are present in the MT-mSin3A alone low stringency immunoprecipitates from 293 cells (Fig. 4, A–C). This loss of MT-mSin3A-associated HDAC activity is due to the presence of MT-dCtBP. When the amount of MT-dCtBP co-transfected compared with MT-mSin3A was reduced by 10- or 100-fold, HDAC activity was restored (Fig. 4B) and HDAC protein could again be detected in the resulting 9e10 low stringency immunoprecipitates (Fig. 4C). The loss of MT-mSin3A-associated HDAC activity in the presence of MT-dCtBP requires that the dCtBP and mSin3A proteins be co-transfected. When lysates

from individual MT-dCtBP and MT-mSin3A transfections were mixed prior to the HDAC activity assay, full HDAC activity associated with MT-mSin3A alone was observed (data not shown). The loss of MT-mSin3A-associated HDAC activity in the presence of MT-dCtBP is cell type-dependent; MT-mSin3A-associated HDAC activity, although reduced, was still detectable in the presence of MT-dCtBP in NIH 3T3 cells where dCtBP functions as a repressor rather than as an activator of transcription (Fig. 4A).

MT-mSin3A Is Not Recognized by the 9e10 Antibody in the Presence of dCtBP—To further characterize the MT-dCtBP and MT-mSin3A complexes formed in the individually and co-transfected cells, immunoprecipitations were performed from [³⁵S]methionine-labeled 293 cells using the monoclonal 9e10 antibodies or polyclonal antibodies recognizing mSin3A. While MT-mSin3A was efficiently expressed and immunoprecipitated by the 9e10 antibody when transfected alone, it was not detectable in immunoprecipitates from cells co-transfected with MT-dCtBP (Fig. 4D). The inability to detect mSin3A protein when co-transfected with dCtBP is not an epiphenomenon associated with the Myc tag, since co-transfection of dCtBP with a FLAG-tagged mSin3A yields similar results (Fig. 4E, top panel). mSin3A is expressed at equivalent levels in these lysates (bottom panel in Fig. 4E and data not shown) and can be immunoprecipitated (Fig. 4D) or recognized in the FLAG plus 9e10 immunoprecipitates (second panel in Fig. 4E) using polyclonal antibodies recognizing the mSin3A protein directly. The MT-mSin3A Myc epitope tag is not recognized in the presence of co-transfected MT-dCtBP, despite its presence in the lysates. Together, our results suggest that MT-mSin3A is forming a different complex (lacking HDAC) or adopts a different conformation in the presence of dCtBP.

dCtBP Interferes with Mad Transcriptional Repression—To determine the functional significance of dCtBP interference of mSin3A-HDAC complexes, we examined the effect of dCtBP expression on Mad transcriptional repression in 293 and NIH 3T3 cells using the pG5LxLuc reporter (Fig. 5A; see “Experi-

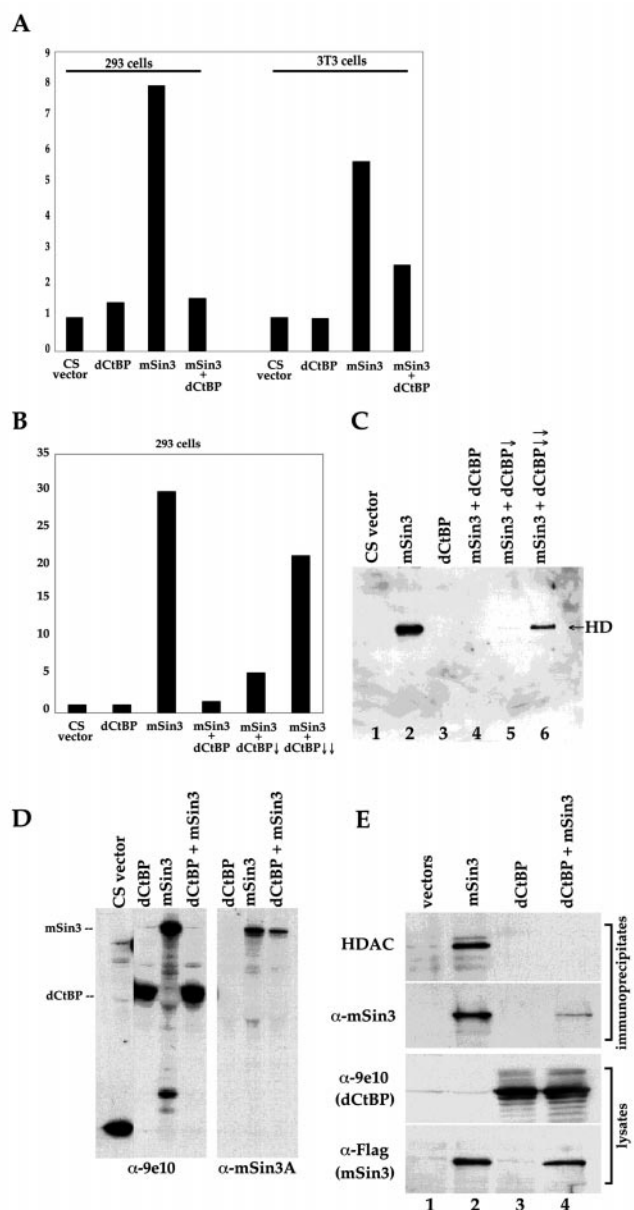


FIG. 4. dCtBP interferes with mSin3A-HDAC complexes. *A*, dCtBP is not associated with HDAC activity in 293 or NIH 3T3 cells. Histone deacetylase activity was assayed from low stringency 9e10 (anti-Myc epitope tag) immunoprecipitates of 293 or NIH 3T3 cells transfected with MT-vector alone, MT-dCtBP, or MT-mSin3A. The values shown (relative HDAC activity) are the average of the amount of released [3 H]acetic acid (a measure of histone deacetylase activity), measured in triplicate, with the CS2 expression vector alone set to 1. *B*, the loss of MT-mSin3A-associated HDAC activity is due to the presence of MT-dCtBP. The amount of MT-dCtBP transfected compared with MT-mSin3A was reduced by 10-fold (*dCtBP* \downarrow) or 100-fold (*dCtBP* $\downarrow\downarrow$). *C*, Western analysis of 9e10 immunoprecipitates from *B* probed with antibodies recognizing HDAC1. The presence of HDAC1 correlates with HDAC activity (compare *B* and *C*). Similar results were obtained with antibodies recognizing HDAC2 (not shown). *D*, MT-mSin3A is not recognized by the 9e10 (MT-) antibody in the presence of MT-dCtBP. Low stringency immunoprecipitations were performed from [35 S]methionine-labeled 293 cells transfected with MT-vector, MT-dCtBP, or MT-mSin3A or co-transfected with MT-dCtBP and MT-mSin3A, using 9e10 (MT-) monoclonal antibodies or polyclonal antibodies recognizing mSin3A. *E*, 293 cells were transfected with empty MT- plus FLAG vectors, MT-dCtBP, FLAG-mSin3A, or MT-dCtBP plus FLAG-mSin3A. Western analysis of 9e10 (MT-) plus FLAG low stringency immunoprecipitates probed with antibodies recognizing HDAC1/2 (*top panel*) or polyclonal antibodies recognizing mSin3A (*second panel*) is shown. Cell lysates (before immunoprecipitation) probed with anti-9e10 (to recognize MT-dCtBP; *third panel*) or anti-FLAG (to recognize FLAG-mSin3A; *bottom panel*) show that these proteins are expressed.

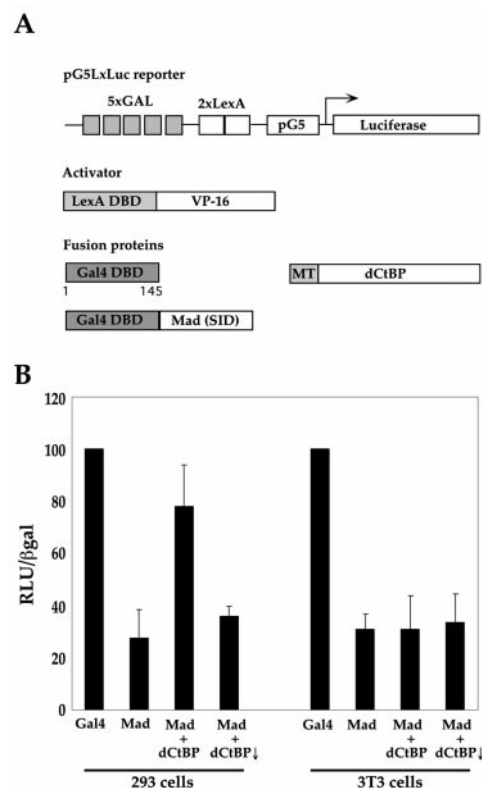


FIG. 5. dCtBP interferes with Mad transcriptional repression. *A*, schematic diagram of the reporter and expression plasmids used in the following experiments. *B*, 293 or NIH 3T3 cells were transfected with the pG5LxLuc reporter, LexA-VP16 activator plasmid, and Gal4 or the GAL4DBD-Mad (SID) fusion protein diagrammed in *A*. The amount of MT-dCtBP transfected compared with Mad was also reduced by 10-fold (*dCtBP* \downarrow).

mental Procedures"). In this reporter system, VP16 strongly activates transcription, and co-transfection of the Gal4-Mad protein represses this activation 10-fold (Fig. 5*B*). When MT-dCtBP is co-transfected with Gal4-Mad, Mad is no longer able to repress transcription in 293 cells where dCtBP activates transcription (Fig. 5*B*). The severity of the inhibition is proportional to the amount of MT-dCtBP expression plasmid co-transfected compared with Gal-Mad (Fig. 5*B*). Co-transfection of MT-dCtBP and Gal4-Mad in NIH 3T3 cells where dCtBP represses transcription had no effect on Mad transcriptional repression (Fig. 5*B*). Since endogenous mSin3 protein is recruited by Gal4-Mad, this functional assay confirms our *in vitro* results that dCtBP inhibits the function of mSin3-HDAC complexes.

dCtBP Interferes with Gro Transcriptional Repression—Since dCtBP interferes with mSin3A-HDAC complexes, dCtBP could also interfere with Gro-HDAC complexes during early *Drosophila* development. We examined the effect of dCtBP expression on Gro transcriptional repression in the pG5LxLuc reporter system described above. When MT-dCtBP is co-transfected with Gal4-Gro, Gro's ability to repress transcription is significantly reduced in 293 cells, where dCtBP activates transcription (Fig. 6*A*). Similar to dCtBP's effects on Mad, the severity of the Gro inhibition is proportional to the amount of MT-dCtBP expression plasmid co-transfected, and MT-dCtBP/Gal4-Gro co-transfection in NIH 3T3 cells (where dCtBP represses transcription) had no effect on Gro transcriptional repression (Fig. 6*A*).

dCtBP and Gro Can Bind Hairy Simultaneously in Vitro—We examined whether dCtBP and Gro can associate with Hairy simultaneously such that dCtBP could interfere with Gro-HDAC by examining their interactions in GST pull-

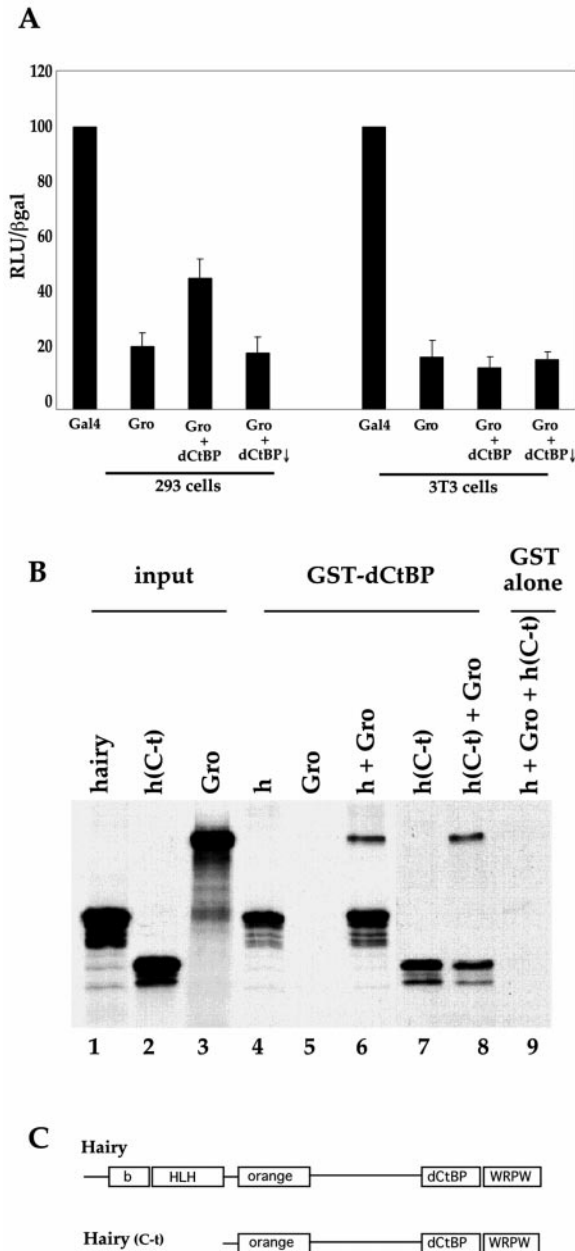


FIG. 6. dCtBP interferes with Gro transcriptional repression. A, 293 or NIH 3T3 cells were transfected with the pG5LxLuc reporter, Lex-VP16 activator plasmid, and Gal4 or the GAL4DBD-Gro fusion protein. The amount of MT-dCtBP transfected compared with Gro was also reduced by 10-fold (*dCtBP*↓). B–C, dCtBP and Groucho can bind Hairy simultaneously *in vitro*. B, ³⁵S-labeled Hairy full-length protein (*input*, lane 1), a truncated Hairy protein (*input*, lane 2), and Groucho protein (*input*, lane 3) do not bind to GST alone (lane 9). While Groucho does not bind to GST-dCtBP (lane 5), it is retained when incubated in the presence of full-length Hairy (lane 6) or a truncated form of Hairy missing its basic helix-loop-helix dimerization motif (lane 8). C, schematic diagram of the full-length and truncated Hairy proteins used in the experiment described above.

down assays (Fig. 6B). When tested alone, *in vitro* translated Hairy (IVT-Hairy), but not IVT-Gro, is bound by GST-dCtBP. However, when IVT-Hairy and IVT-Gro are both present, IVT-Gro can now be detected in a GST-dCtBP pull-down assay. To rule out the possibility that the IVT-Hairy is homodimerizing in this assay through Hairy's basic helix-loop-helix domain with one of the Hairy subunits binding to GST-dCtBP and the other binding to IVT-Gro, we repeated the assay with a truncated form of IVT-Hairy. Hairy (C-term) does not contain Hairy's basic helix-loop-helix dimerization motif (Fig. 6C) and

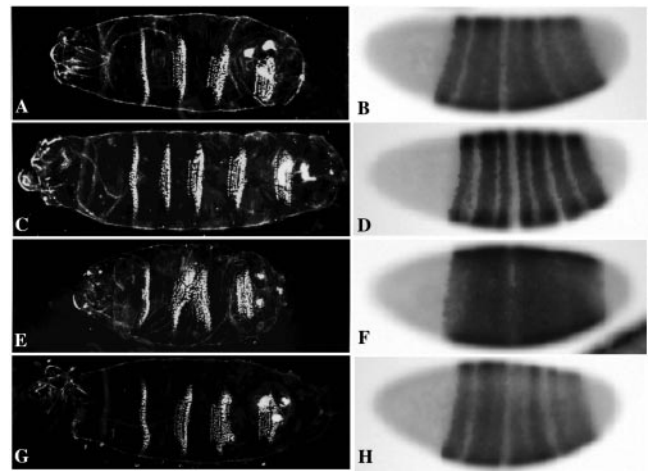


FIG. 7. Genetic interactions among hairy, groucho, and dCtBP. A and B, cuticle phenotype of a larva (A) and Ftz expression in an embryo (B) trans-heterozygous for a strong hairy allele, *h*^{7H}, and a weak hairy allele, *h*^{12C}, showing the intermediate classic pair-rule phenotype (*h*^{7H}/*h*^{12C} progeny from the following cross: *h*^{7H}*TM3* females × *h*^{12C}/*TM3* males). The classic pair rule phenotype generated by the *h*^{7H}/*h*^{12C} allelic combination is suppressed (C, D) when one copy of dCtBP (P1590) is removed maternally (*h*^{7H} P1590/*h*^{12C} + progeny from the following cross: *h*^{7H} P1590/*TM3* females × *h*^{12C} +/*TM3* males) and enhanced (E, F) when one copy of Gro is removed maternally (*h*^{12C} *gro*^{E47}/*h*^{7H} + progeny from the following cross: *h*^{12C} *gro*^{E47}/*TM3* females × *h*^{7H} +/*TM3* males). G and H, dCtBP and Gro act antagonistically. Enhancement of the *h*^{7H}/*h*^{12C} pair rule phenotype when one copy of *gro* is removed maternally is attenuated when one copy of dCtBP is simultaneously removed maternally (*h*^{7H} P1590 *gro*^{E47}/*h*^{12C} + + progeny from the following cross: *h*^{7H} P1590 *gro*^{E47}/*TM3* females × *h*^{12C} + +/*TM3* males). Anterior is to the left.

does not dimerize with itself or full-length Hairy (data not shown). IVT-Gro protein can also be detected when using the truncated IVT-Hairy protein and GST-dCtBP, suggesting that both dCtBP and Groucho can bind simultaneously to Hairy *in vitro*.

dCtBP Interferes with Gro Function *In Vivo*—To determine the functional significance of dCtBP interference of Gro transcriptional repression *in vitro*, we examined the effect of simultaneously reducing the dose of dCtBP and *gro* on Hairy function *in vivo*. hairy mutations exhibit loss of alternating segment wide regions (17), such that larva trans-heterozygous for the *h*^{7H} allele and a weaker hairy allele, *h*^{12C}, display the classic pair-rule phenotype (Fig. 7A) as a result of the loss of repression of hairy's downstream target, Ftz, in the interstripe regions (Figs. 1A and 7B). This hairy mutant phenotype is suppressed by reducing the dCtBP dose maternally (Figs. 1A and 7, C and D; Ref. 14), where it is enhanced by reducing the *gro* dose maternally (Figs. 1A and 7, E and F; Ref. 13). Consistent with dCtBP's interference with Gro transcriptional repression, reducing the dose of dCtBP maternally reverses the enhancing effect of reduced *gro* dose on the hairy mutant phenotype (Fig. 7G) as well as restoring Ftz expression (Fig. 7H).

DISCUSSION

dCtBP Can Activate or Repress Transcription in a Context-dependent Manner—The dCtBP and mCtBP proteins have been identified as interacting proteins for a number of sequence-specific DNA binding developmental repressor proteins. When the mCtBP proteins were initially examined in transcriptional tethering assays, they were potent repressors of transcription, leading to the view that CtBP functions as a co-repressor for all of these factors (29, 32). While we find that dCtBP acts as a co-repressor under the same conditions reported for the mCtBP proteins, we have also identified cell types in which dCtBP behaves as a weak activator of transcrip-

tion. Similar to dCtBP having opposite effects in our transcriptional tethering assays, dCtBP appears to display opposing functions in *Drosophila* embryos as well. Whereas reduction of maternal dCtBP activity enhances the *knirps* and *Krüppel* mutant phenotypes (Fig. 1A; Ref. 34), we find that it suppresses the *hairy* mutant phenotype (Figs. 1A and 7, C and D; Ref. 14). These differences are reflected in the altered expression of the downstream segmentation gene, *ftz*. Our results suggest that dCtBP could be part of different multiprotein complexes that carry out distinct functions.

Since E1A is constitutively expressed in 293 cells (48) and CtBP was initially identified as an E1A-interacting protein, we suspected that E1A may account for the difference in dCtBP's transcriptional properties; the E1A present in 293 cells could effectively titrate the transfected dCtBP, preventing it from functioning as a repressor. While E1A does have an effect on dCtBP transcriptional properties in these assays and is representative of the type of factors that must be differentially recruited in these different cell types, it does not account for the opposing behaviors of dCtBP in all of the cell types. Co-transfecting E1A was not expected to affect dCtBP activity in 293 cells, where it is already present at high levels; however, E1A makes dCtBP a substantially more potent activator in these cells. Co-transfection with E1A also turned dCtBP from a potent repressor into a weak activator in NIH 3T3 cells. Thus, E1A is clearly able to affect dCtBP's transcriptional properties by creating conditions that favor dCtBP playing a role in transcriptional activation, perhaps by shifting the balance of factors present in the cells to favor particular multiprotein complex compositions. The presence of high levels of E1A cannot wholly account for the fact that dCtBP can function as an activator. We also find that dCtBP can activate transcription in non-E1A-transformed cells, such as the B78 mouse melanoma cells (Fig. 1D). As well, mutations in the N terminus of E1A that affect its function, but not its ability to bind to dCtBP, are reduced or no longer able to turn dCtBP from a repressor to an activator in NIH 3T3 cells or to increase its activation capability in 293 cells. In these cases, the E1A protein is unlikely to be titrating away dCtBP but must be actively involved in the transcription complexes. Finally, E1A is not present in *Drosophila* embryos, where transcription factor-dependent differences are observed.

Consistent with dCtBP being part of different complexes with distinct transcriptional properties, we find that the regions of the dCtBP protein required for its activation and repression functions are separable. Thus, these cell type-dependent differences are probably due to differences in protein interactions. That CtBP has been shown to associate with a large number of diverse proteins opens up many possible scenarios whereby dCtBP could be involved in regulatory mechanisms including competition for protein interaction and complex formation.

dCtBP Does Not Appear to Utilize HDACs for Repression—Many repressor-co-repressor complexes have been hypothesized to recruit HDACs and repress transcription by altering local chromatin structure. CtBP proteins have also been postulated to utilize such a mechanism for repressing transcription. In favor of this view, human CtBP1 has been suggested to interact with histone deacetylase both *in vitro* and *in vivo* (55). In addition, Criqui-Filipe *et al.* (29) have shown that mCtBP1 or the mCtBP binding domain of mNet1 fused to the Gal4 DNA binding domain represses transcription and is sensitive to TSA.

However, a growing number of observations suggest that recruitment and use of HDACs is not the primary mechanism of CtBP repression. The mild phenotypic defects associated with the *Drosophila* Rpd3 mutation suggest that this histone

deacetylase does not represent a major pathway of repression in the early embryo (20). The SV40, thymidine kinase, and adenovirus major late promoters have been shown to be sensitive to repression by CtBP, but they are insensitive to TSA (30, 56). Recent work has also implicated CtBP in histone deacetylase-independent E2F/Rb-mediated repression through its interaction with CtIP (57). While dCtBP functions as a potent repressor in NIH 3T3 cells, we do not detect HDAC protein or activity in dCtBP low stringency immunoprecipitates from these cells, also favoring an HDAC-independent mechanism of repression.

dCtBP-Groucho Interactions—While dCtBP does not appear to function in a complex with HDAC, dCtBP's presence inhibits the function of mSin3-HDAC complexes. Since dCtBP interferes with HDAC recruitment or activity, it raises the possibility that such interference may be a general feature of dCtBP function and that dCtBP could interfere with other HDAC complexes such as that proposed to form with Gro. dCtBP and Gro are both required for Hairy-mediated repression, but they appear to play very different roles in this process (13, 14). Indeed, Hairy may be the founding member of a class of proteins, including XTcf-3 and E(spl)mδ/C, that requires both co-factors. It is interesting to note that for proteins in this class, CtBP does not function as expected for a co-repressor. Reduced maternal dCtBP activity suppresses rather than enhances the *hairy* mutant phenotype, and alteration of the XCtBP binding sites for XTcf-3 does not show strong repression of *siamois* (14, 28). It is also interesting to note that proteins in this class interact with CtBP using a non-PXDLSX(K/R) motif; Hairy and XTcf-3 have been shown to use the PLSLV motif, whereas E(spl)mδ/C uses PVNLA (14, 28).

dCtBP could be functioning with members of the Hairy class in a manner similar to that initially proposed for its interaction with E1A. The N-terminal half of E1A is sufficient for cooperative transformation with the *ras* oncogene through its interactions with various cellular proteins (reviewed in Refs. 49, 58, and 59). While the C-terminal half of E1A is dispensable for its cooperative transformation, when removed, a "supertransforming" phenotype is observed. CtBP was identified as the factor binding to the C terminus of E1A that attenuates its oncogenicity (*i.e.* behaves as a tumor suppressor; Refs. 26, 49, and 60). dCtBP may perform a similar function in *Drosophila* by binding to factors such as Hairy and attenuating their repressor activity. dCtBP does not appear to alter Hairy's ability to interact with other proteins such as Gro, but it may attenuate Hairy-mediated repression by not allowing Gro to form as potent a repression complex. Both dCtBP and Gro have been shown to affect several distinct nonoverlapping processes in early development. dCtBP may be recruited at different times during these early developmental stages to attenuate Gro's ability to be a potent repressor. Such a mechanism may be required to provide specificity when the early *Drosophila* embryo develops essentially as a closed system running on maternally provided proteins until transcriptional regulation can be employed with the start of zygotic transcription in the embryo.

CtBP and Acid Dehydrogenases—CtBP proteins contain regions of strong homology to a family of D-isoform 2-hydroxy acid dehydrogenases (26). Acid dehydrogenases are considered to be enzymes primarily involved in intermediary metabolism. Since tumor cells differ from their untransformed counterparts in their growth rate and energy metabolism requirements, dehydrogenases may affect transformation and tumorigenesis (26, 61). When tested, the mCtBP and human CtBP proteins did not exhibit dehydrogenase or NAD binding activity, leading to the suggestion that a structural (dimerization) rather than an enzymatic motif has been conserved (26, 32). Consistent with this

view, dCtBP has been shown to dimerize (14) and a point mutation in the mCtBP2 dehydrogenase conserved catalytic histidine residue (His³²¹ → Ala) represses transcription as efficiently as the wild-type mCtBP2 protein (32).

We find that a similar point mutation in the dCtBP conserved catalytic histidine residue (His³¹⁵ → Gln) disrupts its ability to activate, but not repress, transcription in Gal4 tethering assays. At present, we are not able to distinguish if dCtBP has dehydrogenase activity required for some of its functions (*i.e.* activation) or whether the H315Q mutation disrupts dCtBP's interaction with itself or another protein. However, one intriguing possibility arises from the observation that acid dehydrogenases can affect the rate of specific reductive acylation reactions (*i.e.* acetylation, butyration; Ref. 62) that have been implicated in the control of gene expression. This observation leaves open the possibility that CtBP proteins possess functional dehydrogenase activity that could alter or compete with deacetylase activity. One exciting possibility would be that CtBP encodes dehydrogenase activity that can attenuate HDAC activity. In 293 cells, where dCtBP acts as a transcriptional activator, it could possess dehydrogenase activity that disrupts mSin3-associated HDAC activity. A similar situation may apply in cases where proteins use both the Gro and dCtBP co-factors. In the early fly embryo, where Hairy recruits Gro and presumably Gro-HDAC complexes, dCtBP may be recruited to temporarily attenuate Gro activity, providing temporal regulation or specificity to otherwise ubiquitously distributed maternal Gro activity. Unraveling the complex interactions biochemically and matching them to their developmental roles will be crucial to understanding the mechanisms in which these proteins are involved.

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