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Drosophila CtBP: a Hairy-interacting protein required for embryonic segmentation and Hairy-mediated transcriptional repression

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***hairy* is a *Drosophila* pair-rule segmentation gene that functions genetically as a repressor. To isolate protein components of Hairy-mediated repression, we used a yeast interaction screen and identified a Hairy-interacting protein, the *Drosophila* homolog of the human C-terminal-binding protein (CtBP). Human CtBP is a cellular phosphoprotein that interacts with the C-terminus of the adenovirus E1a oncoprotein and functions as a tumor suppressor. dCtBP also interacts with E1a in a directed yeast two-hybrid assay. We show that dCtBP interacts specifically and directly with a small, previously uncharacterized C-terminal region of Hairy. dCtBP activity appears to be specific to Hairy of the Hairy/Enhancer of split [E(spl)]/Dpn basic helix–loop–helix protein class. We identified a P-element insertion within the dCtBP transcription unit that fails to complement alleles of a known locus, *l(3)87De*. We demonstrate that dCtBP is essential for proper embryonic segmentation by analyzing embryos lacking maternal dCtBP activity. While Hairy is probably not the only segmentation gene interacting with dCtBP, we show dose-sensitive genetic interactions between dCtBP and *hairy* mutations.**

Keywords: C-terminal-binding protein/Hairy/*Drosophila*/segmentation/transcriptional repression

Introduction

Transcriptional repression is an important feature of developmental processes (Herschbach and Johnson, 1993; Gray *et al.*, 1995, Gray and Levine, 1996; Ip and Hemavathy, 1997). The early development of the *Drosophila* embryo is marked by its progressive subdivision into increasingly more precise spatial domains achieved through the actions of a hierarchy of maternal and zygotic segmentation genes (maternal→gap→pair-rule→segment polarity; reviewed in Ingham, 1988; Pankratz and Jäckle, 1993). The proper spatial and temporal expression of these genes requires the coordinated functions of both transcriptional activators and repressors. One such repressor, *hairy* (*h*), is needed for proper embryonic segmentation. Its expression in stripes serves to establish the reiterated (metameric) pattern underlying the basic embryonic body plan, as well as the adult bristle pattern during larval/pupal stages (Ingham *et al.*, 1985). During embryonic segmentation, *h*, along with *even-skipped* (*eve*) and *runt*, are referred to as primary pair-rule genes: they respond directly to gap gene cues and affect each other's expression (Carroll and Scott, 1986; Harding *et al.*, 1986; Howard and Ingham, 1986; Ingham and Gergen, 1988). In addition, *h* behaves genetically as a negative regulator of a downstream (secondary) pair-rule gene, *fushi tarazu* (*ftz*; Carroll and Scott, 1986; Howard and Ingham, 1986). Consistent with *h*'s role as a primary repressor of *ftz* expression, *ftz* stripes are expanded in *h* mutant embryos and extinguished when *h* is ectopically expressed from the heat-shock promoter (Carroll and Scott, 1986; Howard and Ingham, 1986; Ish-Horowicz and Pinchin, 1987). During larval development, *h* behaves genetically as a negative regulator of *achaete* (*ac*; Botas *et al.*, 1982). *h* is required to suppress ectopic bristle production on various adult cuticular structures including the wing and the notum (Moscoso del Prado and Garcia-Bellido, 1984a,b). More recently, Hairy was shown to be expressed ahead of the morphogenetic furrow in the eye imaginal disc and to affect furrow progression when removed in conjunction with another helix–loop–helix (HLH) repressor protein, Extramacrochaetae (Emc; Brown *et al.*, 1991, 1995).

h encodes a nuclear protein with an HLH domain (Hooper *et al.*, 1989; Rushlow *et al.*, 1989) which belongs to a subclass of repressor bHLH proteins including the structurally related *Drosophila* proteins encoded by *deadpan* (*dpn*; Bier *et al.*, 1992) and seven members of the *Enhancer of split* complex [*E(spl)-C*; E(spl)m3, -m5, -m7, -m8, -mβ, -mγ and -mδ; Klämbt *et al.*, 1989; Delidakis and Artavanis-Tsakonas, 1992; Knust *et al.*, 1992], as well as several vertebrate homologs (Sasai *et al.*, 1992; Feder *et al.*, 1993, 1994; Ishibashi *et al.*, 1993). These proteins are genetically required throughout development as transcriptional repressors of genes necessary for processes such as sex determination, segmentation and neurogenesis. Members of the Hairy/E(spl) class share several regions of homology. They have a conserved HLH domain, required for protein dimerization, that is preceded by a basic region, required for DNA binding (Murre *et al.*, 1989a,b). An intact bHLH domain is required for Hairy function during segmentation and when ectopically expressed during sex determination (Wainwright and Ish-Horowicz, 1992; Dawson *et al.*, 1995). In addition, fly Hairy protein, fly E(spl)m8 protein and mammalian HES proteins have been shown to bind N-box sequences (CACNAG) as homodimers *in vitro* (Sasai *et al.*, 1992; Tietze *et al.*, 1992; Oellers *et al.*, 1994; Ohsako *et al.*, 1994; Van Doren *et al.*, 1994). Hairy protein has also been demonstrated to mediate repression of the *achaete* bHLH gene *in vivo*, while the related Dpn protein was shown in transient transfection assays to bind DNA within the *Sex-lethal* (*Sxl*) promoter in a sequence-specific manner

and repress reporter gene expression (Ohsako *et al.*, 1994; Van Doren *et al.*, 1994; Hoshijima *et al.*, 1995; Fisher *et al.*, 1996).

The Hairy/E(spl) proteins are also characterized by two other conserved domains, the Orange domain and the C-terminal conserved tetrapeptide WRPW. Functional studies have shown that both of these domains, as well as the bHLH domain, are needed for the proper function of these proteins (Wainwright and Ish-Horowicz, 1992; Oellers *et al.*, 1994; Dawson *et al.*, 1995). The Orange domain contributes to functional specificity among Hairy/E(spl) proteins (Dawson *et al.*, 1995; Giebel and Campos-Ortega, 1997). The WRPW motif has been shown to be necessary and sufficient for the recruitment of Groucho, a WD repeat-containing protein that is not able to bind DNA on its own but, when brought to an endogenous or heterologous promoter, serves as a strong repressor of transcription (Paroush *et al.*, 1994; Fisher *et al.*, 1996; Parkhurst, 1998). Replacement of the Hairy WRPW motif with the transcriptional activation domain of VP16 results in rapid induction of transcription of N-box-containing promoters (Jiménez *et al.*, 1996). These results together have led to the prevailing view that Hairy functions as a promoter-bound repressor: an intact bHLH region is required for Hairy to bind to specific DNA sites where it then recruits the Groucho co-repressor protein.

However, recruitment of Groucho does not account for all of Hairy's repressor properties. We find that Hairy can function genetically as a repressor in the absence of the WRPW motif, and presumably the Groucho co-repressor (Dawson *et al.*, 1995). In particular, the Orange domain has been shown to be required for proper function of Hairy and E(spl)m8 (Dawson *et al.*, 1995; Giebel and Campos-Ortega, 1997). Our results from mapping functional domains of the Hairy protein suggest that Hairy is involved in separable repression mechanisms: repression in some cases requiring the bHLH and Orange domains, and that in other cases requires the bHLH and C-terminal WRPW motif (Dawson *et al.*, 1995). Thus, Hairy may function modularly, with the scope and specificity of its interactions dependent upon the proteins recruited to its various conserved domains. Based on the expectation that Hairy works as part of a multiprotein complex, we used the yeast two-hybrid protein interaction system and identified a Hairy-interacting protein, the *Drosophila* homolog of human C-terminal-binding protein (CtBP), that interacts with a small, previously uncharacterized C-terminal region of Hairy.

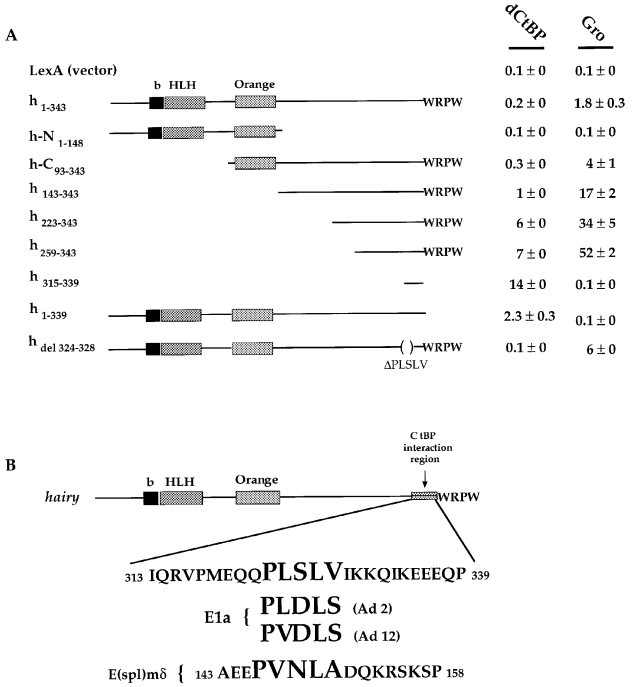

Fig. 1. Mapping the dCtBP interaction domain in Hairy. (**A**) Fusion proteins containing partial Hairy protein mutants were used to map the interaction domain with dCtBP (*h–C28*) in directed yeast interactions to a 25 amino acid region just N-terminal to the WRPW motif. Deletion of five amino acids within the context of full-length Hairy abolishes interaction with dCtBP. The relative values of β-galactosidase activity for each interaction of Hairy with dCtBP or Groucho (as a control) are listed on the right. (**B**) Protein sequence of the 25 amino acid region of Hairy and the 16 amino acid region of E(spl)mδ that is sufficient for interaction with dCtBP are shown. The consensus CtBP-binding sites for two adenovirus types are shown along with the closest match in Hairy and E(spl)mδ.

Results

Hairy and dCtBP interact specifically in yeast and in vitro

To target protein interactions with specific conserved regions of the Hairy protein, we carried out a two-hybrid screen using a LexA-tagged Hairy partial protein bait. This strategy also allowed us to circumvent the reporter system repression that we encountered when using full-length Hairy protein as a bait. We screened a VP16-tagged *Drosophila* library constructed from 0–4 h embryonic mRNAs with a bait that encodes the Hairy Orange domain through to the C-terminus (*h–C*, amino acids 93–343; Figure 1A) and identified a positive clone (*h–C28*) represented by a single cDNA fusion (see Materials and methods). In directed yeast two-hybrid assays, *h–C28* interacts weakly with full-length Hairy, but more strongly with Hairy partial proteins and with one other member of Hairy-class bHLH proteins, E(spl)mδ (Table I; Figure 1A). It does not interact with Dpn and interacts poorly, if at all, with E(spl)m3, -m5, -m8, -mβ and -mγ. In addition, *h–C28* does not show interaction with proteins from other HLH classes (i.e. Scute, Emc; Table I).

We mapped the region of Hairy required for interaction with *h–C28* using a series of Hairy deletions and partial proteins fused to LexA (Figure 1). *h–C28* interacts strongly with a 25 amino sequence immediately upstream of, but not including, the C-terminal WRPW motif (Figure 1B). This identifies a previously undefined protein interaction domain within Hairy. We also find that dCtBP interacts with itself (Table I).

We sequenced the cDNA insert, and a sequence database search revealed 63% sequence identity over the entire *h–C28* clone compared with the human CtBP, a 48 kDa phosphoprotein (Figure 2). Based on the high degree and extent of this homology, we conclude that *h–C28* encodes the *Drosophila* homolog of the human CtBP protein, and we will refer to it as dCtBP. Human CtBP was identified as a protein that binds the C-terminus of the adenovirus

E1a oncoprotein (Boyd *et al.*, 1993; Schaeper *et al.*, 1995). Because of the high sequence conservation from human to *Drosophila* CtBP, we expected dCtBP also to interact with E1a. dCtBP indeed interacts strongly with the C-terminus of the Ad2 E1a protein in a directed two-hybrid assay (Table I). Full-length E1a fused to LexA is lethal to yeast cells and could not be tested. Point mutations within a six amino acid motif in the E1a C-terminus, PXDLSX, eliminate or attenuate CtBP binding (Schaeper *et al.*, 1995). We searched for a similar sequence within the sufficient 25 amino acid interaction region of Hairy, and identified the five amino acid PLSLV sequence (Figure 1B). Deletion of these five amino acids (ΔPLSLV) from full-length Hairy abolishes interaction with dCtBP while still retaining Hairy's ability to interact with other proteins, including Groucho which binds to the adjacent WRPW sequence (Figure 1A). We also mapped the dCtBP interaction domain for E(spl)mδ. Figure 1B shows the 16 amino acids (143–158) sufficient for this interaction. Deletion of five amino acids similar to the Hairy consensus from full-length E(spl)mδ (ΔPVNLA) abolishes interaction with dCtBP, while deletion of an adjacent five amino acids (ΔRSKSP) has no effect.

We also used an *in vitro* binding assay to examine the interaction specificity between dCtBP and Hairy. dCtBP fused to glutathione *S*-transferase (GST–dCtBP) was expressed in bacteria and immobilized on glutathione–Sepharose beads. dCtBP was then tested for its ability to bind ^{35}S-labeled full-length Hairy or Hairy proteins with mutated dCtBP (ΔPLSLV) or Groucho (ΔWRPW) binding domains. Consistent with the yeast interactions, dCtBP specifically pulls down the full-length Hairy and HairyΔWRPW fusion protein, but its ability to bind the HairyΔPLSLV protein is severely reduced (Figure 3).

Table I. dCtBP specifically interacts with Hairy and E(spl)mδ bHLH family members in directed yeast two-hybrid assays (β-galactosidase activity in diploid yeast strains)

Baits	Prey			
	VP16	dCtBP	Scute	Groucho
LexA	0.2	0.1	0.2	0.1
Hairy				
$full_{1–343}$	0.1	0.2	0.1	1.8 ± 0.3
h-$N_{1–148}$	0.1	0.1	–	0.1
h-$C_{93–343}$	0.1	0.3	–	4 ± 1
$h_{314–339}$	0.1	14 ± 0	–	0.1
Dpn	0.1	0.1	0.1	23 ± 1
E(spl)				
m3	0.2	0.2	8.0 ± 0.2	34 ± 6
m4	0.1	0.1	3.4 ± 0.4	58 ± 10
m5	0.1	0.1	0.1	58 ± 15
m8	0.1	0.2	0.1	67 ± 11
mβ/A	0.5	0.8	8 ± 1	55 ± 11
mγ/B	0.4	0.7	6.0 ± 0.3	15 ± 0
mδ/C	0.1	8 ± 1	0.1	13 ± 1
Emc	0.1	0.1	71 ± 3	0.1
E1a (C-term.)	0.1	18 ± 4	0.1	0.1
dCtBP	0.2	31 ± 6	0.2	0.1

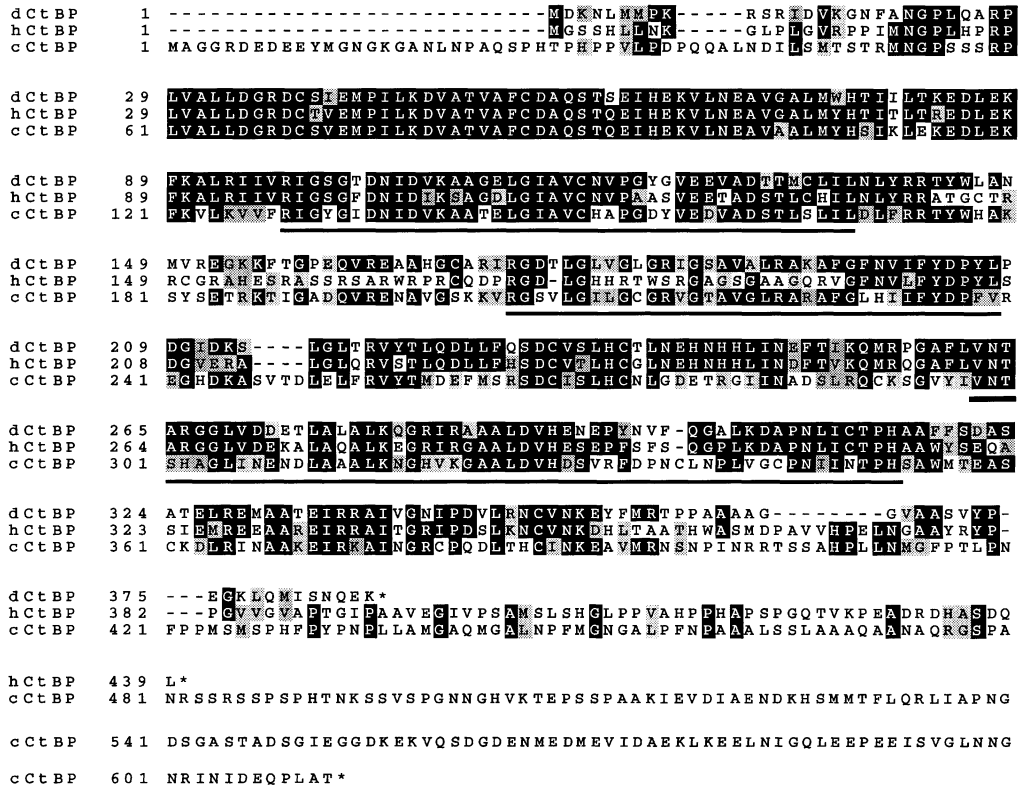

Fig. 2. Amino acid sequence of *Drosophila* CtBP compared with its human and *Caenorhabditis elegans* homologs. The *Drosophila* sequence derived from the original cDNA identified in the two-hybrid screen is shown. Amino acid identities are indicated by black boxes and similarities are indicated by gray boxes. Sequences were aligned using CLUSTALW and analyzed with BOXSHADE. This cDNA encodes a protein isoform that is shorter than the human or worm clones. The underlined regions denote regions of homology between dCtBP and D-isoform 2-hydroxy acid dehydrogenases.

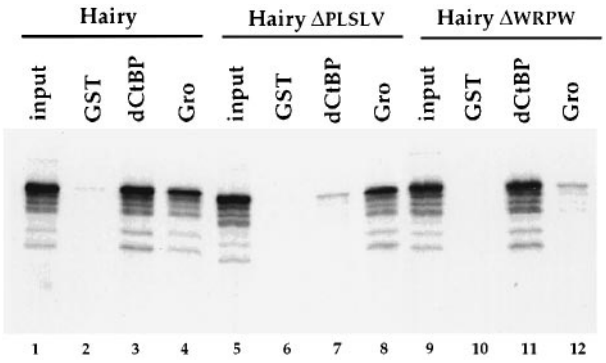

Fig. 3. *In vitro* interaction of Hairy with dCtBP. ^{35}S-labeled full-length Hairy protein (input, lane 1) binds to GST–dCtBP (lane 3) and GST–Gro (lane 4), but not to GST alone (lane 2). Hairy protein lacking the dCtBP-binding domain (HairyΔPLSLV; input, lane 4) no longer binds efficiently to GST–dCtBP (lane 7), but retains its ability to bind GST–Gro (lane 8). Hairy protein lacking the Groucho-binding domain (HairyΔWRPW; input, lane 9) is bound by GST–dCtBP (lane 11), but not efficiently by GST–Gro (lane 12) or GST alone (lane 10).

dCtBP encodes several mRNAs that are dynamically expressed

Developmental Northern analysis of wild-type flies using the dCtBP two-hybrid insert as a probe shows that three major transcripts (2.5, 2.7 and 4.0 kb) are expressed dynamically throughout all stages of development, whereas an additional 3.5 kb transcript was detected predominantly in adult females and embryos stages (Figure 4A). dCtBP transcript levels increase both early during oogenesis and embryogenesis, and later in pre-pupae stages.

By screening early embryonic cDNA libraries with the two-hybrid cDNA insert as a probe (see Materials and methods), we have identified four different cDNA classes to date (including one corresponding to the cDNA represented by the original two-hybrid clone). Each of these four classes encodes the first 376 amino acids of dCtBP then alternatively splice such that the most C-terminal amino acids (5–83 amino acids), as well as the 3′ non-coding regions, are different (with the two-hybrid cDNA having an additional 10 amino acids). We have not yet identified cDNAs corresponding to all transcripts identified by Northern analysis. However, a dCtBP subclone containing only the ATG to the junction where the sequence divergence occurs (amino acids 1–376) retains full interaction with Hairy, indicating that all protein isoforms should interact with Hairy. Therefore, the remainder of our analysis uses the cDNA isoform identified in the two-hybrid screen.

We examined the spatial expression of dCtBP in ovaries and whole mount embryos. dCtBP mRNA is detected in the germarium and in the nurse cells throughout the early oogenic stages, peaking in stage 10 nurse cells (Figure 4B and C). These transcripts are later dumped into the oocyte where they are distributed ubiquitously (Figure 4C). The maternal transcript persists throughout the early embryonic stages (Figure 4D and E). Transcript levels drop during gastrulation and are maintained at this lower level throughout the remainder of embryogenesis (data not shown).

Characterization of a P-element insertion within the dCtBP transcription unit

Using the two-hybrid cDNA insert as a probe, dCtBP was localized to the 87D 5–9 region on polytene chromosomes

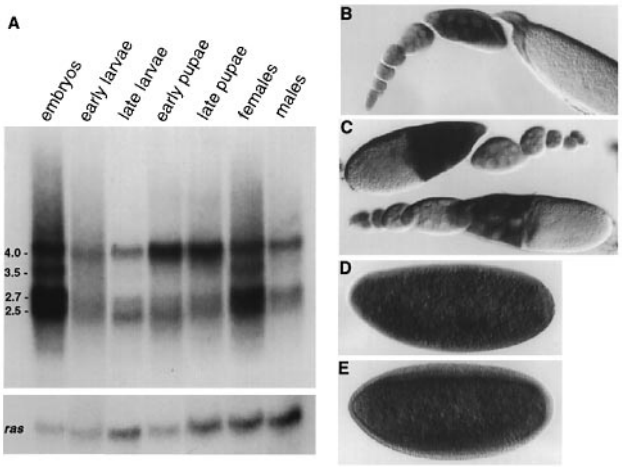

Fig. 4. Spatial and temporal expression of dCtBP during *Drosophila* development. (**A**) Developmental Northern analysis of wild-type mRNAs, using the dCtBP cDNA clone identified in the two-hybrid screen as a probe, shows that dCtBP is expressed at all developmental stages, with increased accumulation during oogenesis, early embryogenesis and pre-pupal stages when imaginal discs are developing. In addition, a 3.5 kb transcript is detected predominantly in adult females and 0–24 h embryos. *ras* was used as a loading control (Mozer *et al.*, 1985). The relative stages and developmental times are listed for each lane. Spatial expression of dCtBP in wild-type oogenesis (B and C) and embryogenesis (D and E). dCtBP expression is detected ubiquitously in the germarium and early oogenic stages (**B**), and is highly expressed in nurse cells by stage 10 (**C**). This transcript is dumped into the oocyte and is detected ubiquitously in early (**D**) and cellular blastoderm stage (**E**) embryos.

(data not shown), and the genomic locus was cloned and mapped (Figure 5A; see Materials and methods). By Southern analysis, we found that the dCtBP cDNA recognizes genomic fragments over an ~10 kb region (highlighted on the genomic map in Figure 5A). We identified a single P-element-induced mutation (P1590; from the Bloomington Stock collection; Spradling *et al.*, 1995) mapping to the cytological location for dCtBP. Rescue and mapping of the DNA flanking the P1590 P-element shows that the 1590 P-element is inserted within the dCtBP transcription unit (Figure 5A; see Materials and methods).

The P1590 strain carries a homozygous lethal insertion, with the homozygotes dying as pharate adults. When dissected from their pupal cases, P1590 homozygotes exhibit duplicated and ectopic bristles (macrochaetes) on the notum and scutellum (data not shown). The P1590 strain also exhibits a strong maternal effect phenotype. It was on the basis of its maternal requirement that we originally identified the P-1590 allele in a screen for maternal-effect lethals (S.Dawson, M.Meyer and S.M. Parkhurst, unpublished). In this screen, a change of function mutation in an RNA polymerase II subunit, *wimp*, was used to reduce, but not eliminate, P-1590 maternal contribution (Parkhurst and Ish-Horowicz, 1991). Embryos derived from mothers *trans*-heterozygous for *wimp* and the P1590 allele die, and cuticle preparations of these embryos show segmentation defects, ranging from pairwise fusions of adjacent denticle bands to more widespread denticle fusions (Figure 5C).

To confirm that the P-element and hence the disruption of dCtBP is responsible for the mutant phenotype, the P1590 P element was excised as described by Török

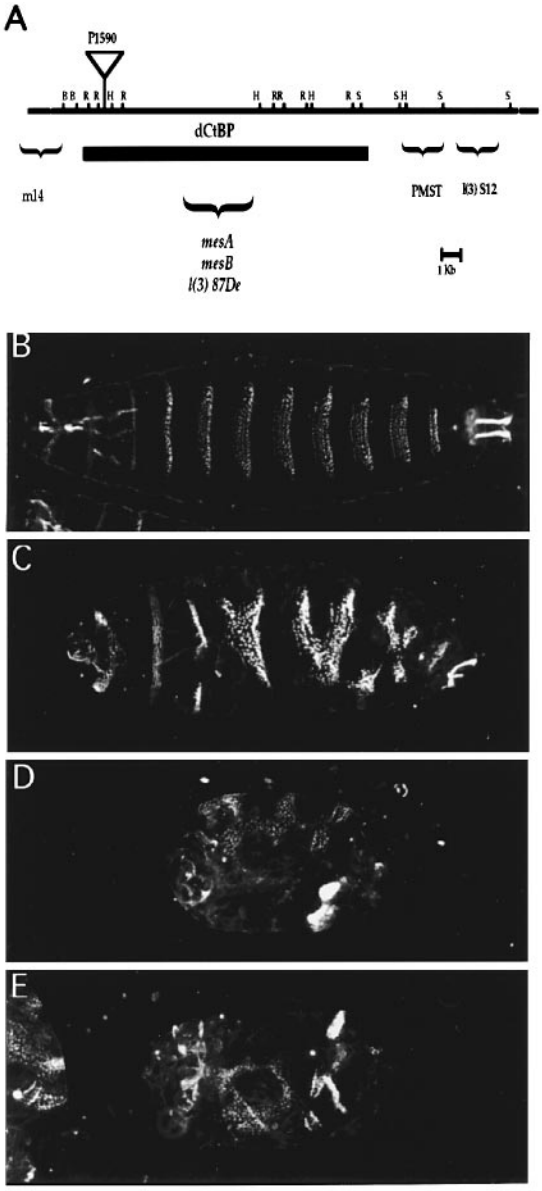

Fig. 5. dCtBP genomic stucture and P1590/dCtBP mutant cuticle phenotypes. (**A**) Genomic organization of dCtBP. Restriction map of the 87D region encoding dCtBP and neighboring transcripts. The P1590 P-element insertion site is depicted (triangle). The extent of the dCtBP transcription unit based on hybridization of cDNA to genomic DNA is shown as a dark bar below the molecular map. Saturation mutagenesis in the region has identified three lethal complementation groups in the region covered molecularly by dCtBP: *messyA* (*mesA*), *messyB* (*mesB*) and *l(3)87De*. The location of neighboring transcription units as confirmed by Northern analysis is shown. All *Bam*HI (B), *Eco*RI (R), *Hin*dIII (H) and *Sal*I (S) sites are shown. (B–E) Maternal dCtBP is required for embryonic segmentation. Cuticle phenotypes of embryos lacking maternal dCtBP (**C–E**) compared with wild-type (**B**). (C) Cuticle phenotype of a heterozygous P1590 larva derived from a *wimp*/+ mother showing pairwise fusions of the ventral denticle bands. (D–E) Larval cuticles from germline mosaics generated with the P1590 allele, showing a consistent, but severely disrupted pattern. Anterior is to the left.

et al. (1993). One of the eight generated revertants was homozygous viable, a precise molecular excision by Southern analysis (data not shown), and, when tested with *wimp*, failed to give a mutant phenotype, verifying that the P-element is responsible for the observed mutant phenotype. In addition, complementation of the lethality

Table II. P1590 encodes dCtBP and fails to complement alleles of a known locus, *l(3)87De*

(**A**) P1590 fails to complement alleles of *l(3)87De*

Genotype of		Progeny	
♀ parent	♂ parent	P1590 *mes* or *87De*	P1590 or *mes* balancer
P1590/TM3[a]	$mesA^1$/MRS	157	241
P1590/TM3	$mesA^{13}$/MKRS	143	283
P1590/TM3	$mesB^4$/MRS	120	208
P1590/TM3	$mesB^5$/MRS	162	266
P1590/TM3	$87De^1$/MKRS	0	232
P1590/TM3	$87De^{10}$/MKRS	0	277

(**B**) Revertants of P1590 complement alleles of *l(3)87De*

♀ parent	♂ parent	*87De*	*87De* or P1590
		P1590 or rev4	balancer
$87De^1$/MKRS	P1590/TM3	0	178
$87De^1$/MKRS	$P1590^{rev4}$/ $P1590^{rev4}$	97	121

[a]TM3, MRS and MKRS are balancer chromosomes.

of *l(3)87De* alleles is obtained in crosses between flies from the P1590 revertant line and flies carrying *l(3)87De* alleles (see Table IIB and below).

l(3R)87De corresponds to dCtBP/P1590

The 87D cytological region has been mapped extensively, both molecularly and genetically (Hilliker *et al.*, 1980; Bender *et al.*, 1983; Spierer *et al.*, 1983; Bossy *et al.*, 1984; Gausz *et al.*, 1986). By both Southern and Northern analysis, we mapped the molecular limits of dCtBP (and the P-element insertion site) and correlated our maps with the published map of the region (Figure 5A; Bender *et al.*, 1983; Bossy *et al.*, 1984). Saturation mutagenesis throughout 87D identified three lethal complementation groups, *messyA* (*mesA*), *messyB* (*mesB*) and *l(3)87De*, mapping within the physical limits of the dCtBP transcription unit. All three of these complementation groups have both distinct and overlapping phenotypes (Hilliker *et al.*, 1980; Lindsley and Zimm, 1992). *mesA* is characterized by semi-lethal alleles that show extra head and thoracic bristles. *mesB* is also characterized by semi-lethal alleles associated with an outstretched wing phenotype, as well as exhibiting duplicated bristles reminiscent of the *mesA* alleles but at a lower penetrance. Escapers of *l(3)87De* alleles are reported to have phenotypes resembling those of *mesB*. Complementation analysis between P1590 and alleles representing these loci show that P1590 fails to complement *l(3)87De* (Table II). While P1590 complements both *mesA* and *mesB* alleles, the resulting trans-heterozygous adults display phenotypes similar to those described for the *mes* alleles alone. Based on the molecular complexity of the dCtBP mRNAs, the molecular limits of the dCtBP locus and the phenotypes of the various *mes* allelic combinations, it is likely that all three of these loci are pseudoalleles of dCtBP/P1590.

Maternal dCtBP is required for proper embryonic segmentation

Since *wimp* reduces, but does not eliminate maternal function, we examined loss of dCtBP/P1590 function in germ-

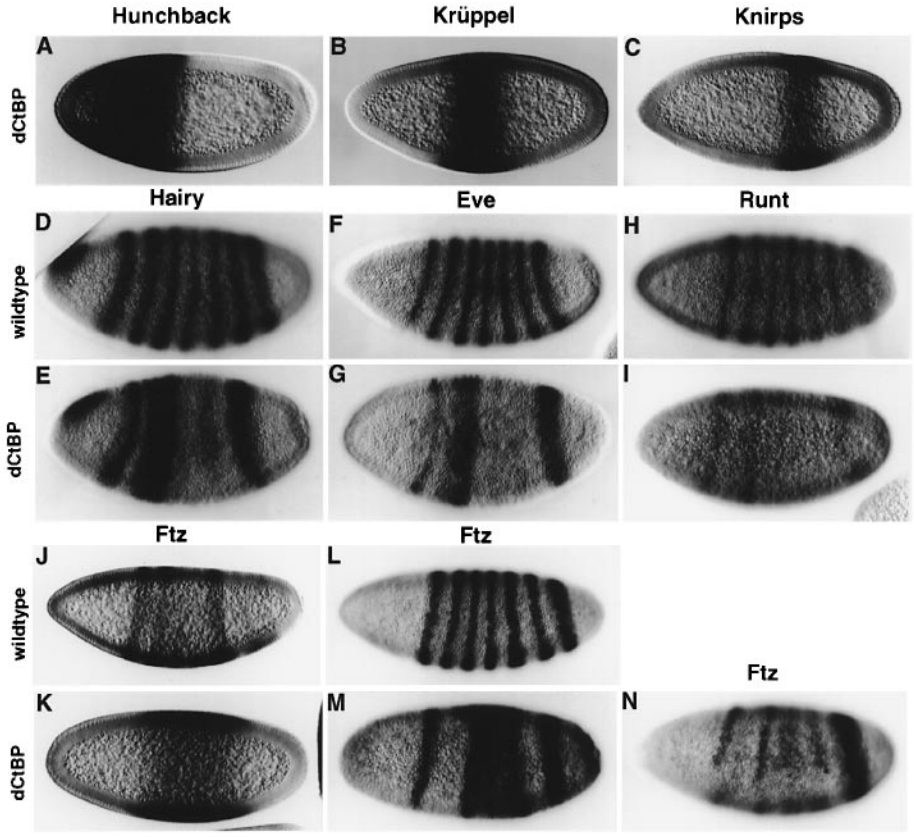

Fig. 6. Pair-rule gene proteins, but not gap gene proteins, are disrupted in embryos lacking maternal dCtBP. Wild-type (D, F, H, J and L) or embryos lacking maternal dCtBP (A–C, E, G, I, K, M and N) were stained for segmentation gene proteins. Gap gene expression is normal for Hunchback (**A**), Krüppel (**B**) and Knirps (**C**). Primary pair-rule gene expression shows stripe-specific repression for Hairy (**E**), Eve (**G**) and Runt (**I**) compared with wild-type (**D**, **F** and **H**, respectively). Ftz stripes initially are derepressed (expanded) in early cellular blastoderm embryos lacking maternal dCtBP (**K**) compared with wild-type (**J**). This broad band of Ftz expression later resolves such that some of the stripes become distinct (**M**, compared with wild-type in **L**). The ventral-most region of embryos beginning at the cellular blastoderm stage fails to express segmentation gene protein. Ftz stripes fail to encircle the embryo (**N**). Anterior is to the left and dorsal is up.

line clones using the FLP–DFS technique. The FLP–DFS system incorporates the presence of a dominant female sterile (DFS) mutation, ovo^{D1}, and the FLP–FRT yeast site-specific recombination system to create germline-specific mosaics (Golic, 1991; Chou and Perrimon, 1992; Chou *et al.*, 1993). A P1590 FRT82B chromosome had been generated previously as part of a screen using the FLP–DFS technique to look for maternal phenotypes in zygotic single P-element-induced mutations (Perrimon *et al.*, 1996). Embryos derived from germline clones generated with this chromosome were reported to have segmentation defects resulting in pair-wise fusions, as well as large holes in the ventral cuticle (Perrimon *et al.*, 1996). Using this P1590 FRT82B stock, we typically obtain more severe cuticle disruptions than previously reported: we consistently observe embryos that are significantly shorter than wild-type, with either 'lawns' of denticles on the ventral cuticle or severely fused or missing denticle bands (Figure 5D and E).

Loss of maternal dCtBP disrupts segmentation

If the segmentation defects observed in embryos lacking maternal dCtBP are due to its interaction with Hairy, we would expect disruptions in patterning similar to those found in *hairy* mutations or loss of maternal Groucho (Carroll and Scott, 1986; Howard and Ingham, 1986; Paroush *et al.*, 1994). In particular, we would expect the expression of the other primary pair-rule genes to be disrupted and *ftz* expression to be derepressed. Consistent with this, we find that Ftz stripes are expanded in embryos lacking maternal dCtBP (Figure 6K). However, this broad band of expression later resolves into stripe-specific *ftz* repression, with stripes 2, 4, 5 and 6 predominantly affected (Figure 6M). We also observed aberrant expression of the primary pair-rule gene proteins, Eve and Runt, as well as of Hairy itself (Figure 6E, G and I). Since the primary pair-rule genes respond directly to gap gene cues, we also examined gap gene expression in embryos lacking maternal dCtBP. Expression of the three gap genes examined, Hunchback, Krüppel and Knirps, appears normal in these embryos (Figure 6A–C).

In addition to its effects on anterior–posterior patterning, embryos lacking maternal dCtBP also show disruptions of dorsoventral patterning. Beginning with the expression of the pair-rule genes, a lack of segmentation gene expression is detected on the ventral surface (Figure 6N).

Loss of maternal dCtBP does not result in a neurogenic phenotype or disrupt Sex-lethal expression

The Hairy-interacting protein Groucho interacts with all members of the Hairy/E(spl)/Dpn class of bHLH proteins through their characteristic C-terminal WRPW motif. As predicted, loss of the Groucho gene product maternally

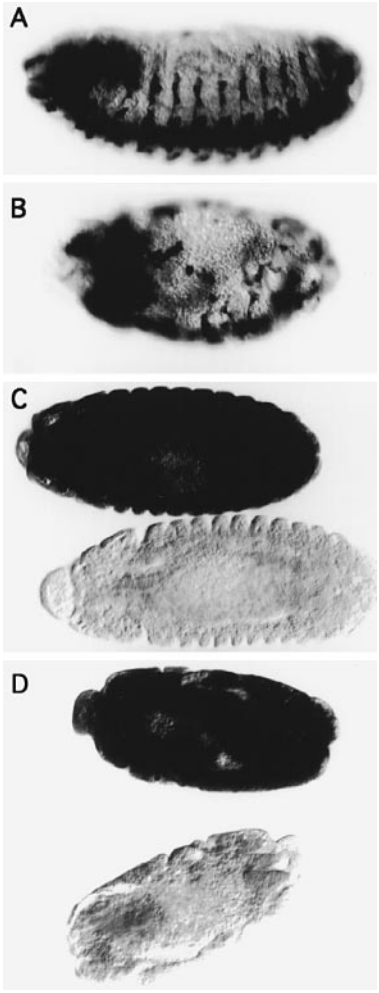

Fig. 7. Embryos lacking maternal dCtBP do not exhibit neurogenic hyperplasia or affect Sex-lethal expression. While the pattern of mAb22C10 (neurogenic marker) staining is disrupted, there is no increase in the number of positive cells in embryos lacking maternal dCtBP (**B**) compared with wild-type (**A**). Similarly, while morphology is disrupted by late embryogenesis, sex detemination as assayed by staining with Sex-lethal antibodies is normal in embryos lacking maternal dCtBP (**D**) compared with wild-type (**C**). (Antibodies generated against Sex-lethal stain all cells in females, but no cells in males.)

results in defects in sex determination, segmentation and neurogenesis, processes that are affected by the different members of this repressor bHLH protein class. We postulated that not all of these processes would necessarily be affected by maternal removal of dCtBP, since it only interacts with Hairy and one of the E(spl) proteins.

Mutations within the *E(spl)* locus and embryos lacking maternal Groucho protein display a neurogenic phenotype: hyperplasia of the nervous system with the concomitant loss of epidermis. While dCtBP interacts with only one of the E(spl) bHLH proteins, E(spl)mδ, we examined neurogenesis in embryos from P1590 germline clones. We used the mAb22C10 antibody which recognizes both central and peripheral nervous system structures to assay any alterations in neural development (Fujita *et al.*, 1982). We do not observe an overall increase in mAb22C10-positive cells (Figure 7B); however, proper patterning of the embryonic nervous system has been disrupted, probably due to earlier effects on segmentation.

dCtBP does not interact in yeast with either Dpn or E(spl)m3, the two bHLH proteins of the Hairy class having a role in sex determination. We examined expression of Sxl protein in embryos from P1590 germline clones and, contrary to ubiquitous Sxl expression in all Groucho mutant embryos (Paroush *et al.*, 1994), Sxl expression was not affected in embryos lacking maternal dCtBP (Figure 7D).

Maternal reduction of dCtBP specifically alters the Hairy segmentation phenotype

In addition to the disruption of patterning in P1590 germline clones, we examined dCtBP/P1590 for genetic interaction with *h*. *h* mutations result in a range of cuticle phenotypes from loss or fusion of adjacent denticle bands to a fusion of most of the segments ('lawn' phenotype), with the most common phenotype called the classic pair-rule phenotype that results from the loss of alternating segment-wide regions (Ingham *et al.*, 1985). Larvae homozygous for a strong *h* allele, h^{7H}, display the extreme 'lawn' phenotype (Figure 8C), whereas larva trans-heterozygous for the h^{7H} allele and a weaker *h* allele, h^{12C}, display the classic pair-rule phenotype (Figure 8A). We initially used this h^{7H}/h^{12C} allelic combination to examine if reducing the dCtBP dose maternally would suppress or enhance the intermediate pair-rule phenotype. P1590 was genetically recombined onto a chromosome containing the h^{7H} allele. Reducing the dose of dCtBP maternally results in the suppression of the h^{7H}/h^{12C} mutant cuticle phenotype (Figure 8B; Table III). Likewise, reducing the dose of dCtBP maternally in the severe h^{7H} background suppresses the extreme lawn phenotype. We did not observe any alterations in viability or phenotype of any progeny classes when P1590 was *trans*-heterozygous with h^{7H}, or when the h^{7H} *P1590* recombinant chromosome was crossed to wild-type (Table III).

Discussion

Using a yeast two-hybrid interaction screen to isolate protein components of Hairy-mediated repression, we identified a Hairy-interacting protein encoded by the *Drosophila* homolog of the human CtBP gene. We defined a 25 amino acid region adjacent to the WRPW motif in Hairy as both necessary and sufficient for interaction with dCtBP. We have identified a P-element insertion within the dCtBP transcription unit, and show that it fails to complement alleles of the *l(3)87De* locus. We further show that dCtBP is essential for proper embryonic segmentation by analyzing embryos lacking maternal dCtBP activity. While Hairy is probably not the only segmentation gene interacting with dCtBP, we show dose-sensitive genetic interactions between dCtBP and *h* mutations. dCtBP appears to be specific to Hairy of the Hairy/E(spl)/Dpn HLH protein class. Consistent with its interactions in the two-hybrid system, we show that processes affected by other members of this repressor class are not similarly affected by the maternal absence of dCtBP.

dCtBP is required for embryonic segmentation

We have highlighted a role for dCtBP in embryonic segmentation. Reduction of maternal dCtBP in a *wimp* background or loss of maternal dCtBP in germline mosaics results in severe segmentation defects visualized by cuticle

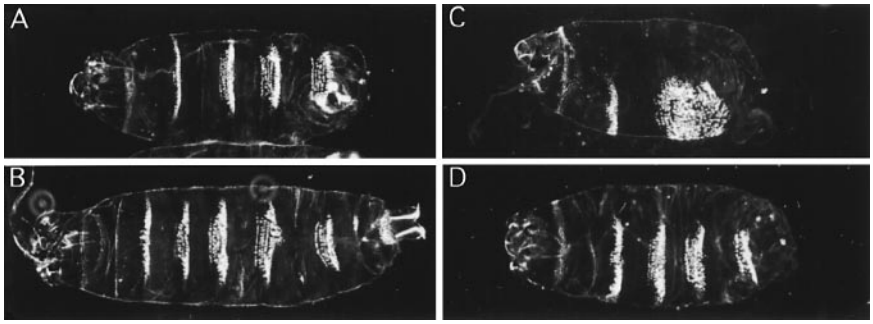

Fig. 8. Genetic interactions between P1590/dCtBP and *hairy*. (**A**) Cuticle phenotype of a larva *trans*-heterozygous for a strong *hairy* allele, h^{7H}, and a weak *hairy* allele, h^{12C}, showing the intermediate classic pair-rule phenotype (h^{7H}/h^{12C} progeny from the cross: $h^{7H}/TM3$ ♀×h^{12C}/TM3 ♂). (**B**) The classic pair-rule phenotype generated by the h^{7H}/h^{12C} allelic combination is suppressed when one copy of dCtBP is removed maternally (h^{7H} *P1590*/h^{12C} + progeny from the cross: h^{7H} *P1590*/*TM3* ♀×h^{12} +/*TM3* ♂). (**C**) Cuticle phenotype of a larva homozygous for a strong *hairy* allele, h^{7H}/h^{7H}, showing severe fusions of the ventral denticles or a 'lawn' phenotype. (**D**) The severe lawn phenotype generated by the h^{7H}/h^{7H} allelic combination is likewise suppressed when one copy of dCtBP is removed maternally (h^{7H} *P1590*/h^{7H} + progeny from the cross: h^{7H} *P1590*/*TM3* ♀×h^{7H} +/*TM3* ♂.) Anterior is to the left.

preparations or *engrailed* staining (not shown). Expression of the primary pair-rule genes, *eve* and *runt*, as well as expression of *h* itself, is disrupted in embryos lacking maternal dCtBP, whereas the level and spatial positioning of gap gene expression (Hunchback, Krüppel and Knirps) appears normal. The primary pair-rule genes are required to establish each other's expression as well as to direct the striped expression of downstream secondary pair-rule genes, such as *ftz*. Similar to what is seen in *h* mutant embryos, Ftz stripes are expanded throughout the trunk region in embryos lacking maternal dCtBP. In contrast to *h* mutations, this broad band of Ftz expression resolves partially into stripes.

The physical interaction with Hairy and the genetic interaction with *h* indicate a role for dCtBP in Hairy-mediated repression. However, the phenotypes generated with loss of maternal dCtBP activity and the suppression rather than enhancement of Hairy phenotypes suggest that dCtBP is probably interacting with additional proteins during segmentation. We currently are examining genetic and physical interactions of dCtBP with gap gene proteins and protein products from loci other than those we have examined here.

No mutants exist with a molecular lesion in the dCtBP-binding domain of Hairy to date. Of the 11 existing ethyl methanelsulfonate-induced *h* mutations, only four result in missense mutations, uncovering a functional requirement for the basic, HLH and WRPW motifs for proper Hairy function during segmentation (Wainwright and Ish-Horowicz, 1992). However, disruption of the dCtBP-binding domain in the context of full-length Hairy abolishes their interaction in directed yeast two-hybrid assays. Also, reduction of the maternal contribution of dCtBP affects the severity of the segmentation phenotype in *h* mutations.

dCtBP may play further roles in embryonic patterning. Interestingly, we find that dCtBP embryos also show disruptions of the dorsoventral axis (Figure 6N). The stripes of pair-rule expression still present in embryos lacking maternal dCtBP do not completely encircle the embryo, but are missing from the ventral region. Lack of ventral expression is the normal case for Hairy stripe 1 (Hooper *et al.*, 1989), but not for the other stripes. Also, embryos lacking maternal Groucho exhibit defects in both the dorsoventral and terminal pathways (Paroush *et al.*, 1994). Dorsoventral patterning genes such as *twist* and *rhomboid* are thought to require bHLH proteins for their activation, and show an anterior–posterior stripe refinement of their expression patterns (Jiang *et al.*, 1991; Bier *et al.*, 1992; Ip *et al.*, 1992). A possible role for Hairy in repression of such genes was also suggested by Barolo and Levine (1997) using heterologous promoters containing *twist* and *rhomboid* enhancers. dCtBP may participate in dorsoventral gene repression by inhibition through an as yet unexplored function of Hairy, through a specific function of E(spl)mδ or through an unidentified gene that interacts with dCtBP.

Table III. Maternal reduction of P1590/dCtBP suppresses the *hairy* mutant phenotype.

(**A**) P1590/dCtBP and *hairy* interact genetically

Genotype of		Percent of progeny with segmentation defects:
♀ parent	♂ parent	
h^{7H}/TM3[a]	h^{7H}/TM3	28 ± 5
h^{7H} P1590/TM3	h^{7H}/TM3	62 ± 8
h^{7H}/TM3	h^{7H} P1590/TM3	29 ± 7
h^{7H}/TM3	h^{12C}/TM3	20 ± 4
h^{7H} P1590/TM3	h^{12C}/TM3	53 ± 7
h^{7H} P1590/TM3	+/+	6 ± 4
+/+	h^{7H} P1590/TM3	4 ± 2
P1590/TM3	h^{7H}/TM3	6 ± 3
h^{7H}/TM3	P1590/TM3	3 ± 3

(**B**) Distribution of phenotypic severity

Genotype of		Percent of progeny with phenotype[b]		
♀ parent	♂ parent	Lawn	Pair-rule	Mild
h^{7H}/TM3	h^{7H}/TM3	74	26	0
h^{7H} P1590/TM3	h^{7H}/TM3	12	88	0
h^{7H}/TM3	h^{7H} P1590/TM3	92	8	0
h^{7H}/TM3	h^{12C}/TM3	2	98	0
h^{7H} P1590/TM3	h^{12C}/TM3	0	3	97

[a]TM3 is a balancer chromosome.
[b]Phenotype severity is equivalent to that shown in Figure 8C for lawns, A–D for pair-rule, and B for mild. Compare with wild-type shown in Figure 5B.

dCtBP interacts with members of the Hairy/E(spl) class of bHLH repressor proteins

The interaction between dCtBP and Hairy appears to be specific to Hairy of the Hairy/E(spl)/Dpn class of repressor HLH proteins. While dCtBP also interacts with E(spl)mδ, and we might expect to see a neurogenic phenotype due to a disruption of E(spl)mδ function, embryos lacking maternal dCtBP do not show a classic neurogenic phenotype. There is not a large increase in cells expressing neurogenic markers; however, we cannot rule out subtle neural patterning defects.

However, dCtBP may interact with E(spl)mδ later in development. The zygotic phenotype associated with dCtBP mutations is late pupal lethality, with the pharate adults showing duplication of macrochaetes on the head, notum and scutellum. This may be mediated by interaction with E(spl)mδ, as the *E(spl)* genes may affect the number and positioning of macrochaetes, whereas Hairy normally affects only the microchaetes (Ingham *et al.*, 1988; de Celis *et al.*, 1996).

dCtBP is a homolog of a human protein involved in suppression of adenovirus oncogenicity

dCtBP itself is highly homologous to the human CtBP, a 48 kDa cellular phosphoprotein identified on the basis of its ability to bind the C-terminus of E1a. In support of the structure–function conservation with human CtBP, dCtBP interacts strongly and shows synthetic lethality with the C-terminus of the E1a protein in yeast. The smallest region required for CtBP–E1a interaction maps to 14 amino acids, within which is a consensus six amino acid motif, PXDLSX. Within the 25 amino acid interaction domain necessary and sufficient for dCtBP interaction with Hairy, is the motif PLSLV. Within the 16 amino acid interaction domain necessary and sufficient for dCtBP interaction with E(spl)mδ, is the motif PVNLA. Deletion of these five amino acids (ΔPLSLV or ΔPVNLA) within the context of full-length Hairy or E(spl)mδ results in a protein that no longer interacts with dCtBP, even though these mutant proteins retain their ability to interact with other Hairy-interacting proteins (G.Poortinga and S.M. Parkhurst, unpublished) and with Groucho, through its more C-terminal WRPW motif, in directed yeast two-hybrid assays.

Both human and *Drosophila* CtBP proteins contain regions of strong homology to a family of D-isoform 2-hydroxy acid dehydrogenases, but the significance of this homology is unclear. The human clone has been examined for dehydrogenase or NAD-binding activity, but none was detected (Schaeper *et al.*, 1995). Rather than functioning catalytically, Schaeper *et al.* (1995) suggest that these regions may serve a structural role, i.e. as dimerization domains. Consistent with this possibility, dCtBP interacts with itself in the yeast two-hybrid assay (Table I).

The N-terminal half of E1a is sufficient for cooperative transformation with the *ras* oncogene through its interactions with various cellular proteins (reviewed in Chinnadurai, 1992; Moran, 1993; Mymryk, 1996). While the C-terminal half of E1a is dispensible for cooperative transformation, when removed, a 'super-transforming' phenotype is observed. Mapping the C-terminal domain of E1a required for this tumor suppressor activity led to the identification of CtBP (Boyd *et al.*, 1993; Schaeper *et al.*, 1995; Mymryk, 1996). Using a heterologous system where a Gal4–E1a fusion protein is bound to a reporter gene promoter, transcriptional activation by N-terminal portions of the E1a protein can only take place in the absence of CtBP binding. Additionally, prevention of transcriptional activation did not occur if CtBP was provided *in trans*, suggesting a mechanism whereby CtBP binds E1a and, while bound, disrupts the activity of other domains of the E1a protein *in cis* (Sollerbrant *et al.*, 1996).

Hairy requires multiple domains for repression, and one intriguing possibility is that, once bound to Hairy, dCtBP may regulate Hairy's function by interacting with other regions of Hairy, or other Hairy-binding proteins. While dCtBP does not interact with activating HLH proteins (sequestering) or the Groucho co-repressor (recruiting), it does interact specifically with one of the other Hairy-interacting proteins identified in our yeast interaction screen (G.Poortinga and S.M.Parkhurst, unpublished). It will be interesting to determine if the function as well as the mechanism of human CtBP is conserved in *Drosophila*. Since E1a is a viral protein and unlikely to be the endogenous target for CtBP, understanding the role of dCtBP should help to define the endogenous interacting partners for its human homolog.

dCtBP function and Hairy-mediated transcriptional repression

Transcriptional repression by Hairy requires site-specific DNA binding and a direct interaction with the Groucho co-repressor protein via Hairy's C-terminal WRPW motif. A model whereby Hairy simply tethers Groucho to a target promoter is not sufficient to account for all Hairy-attributed functions during development. Indeed, there are likely to be additional components, both direct and indirect, that may be required to establish (i.e. recruit Hairy), maintain and specifically regulate Hairy repression. While dCtBP and Groucho do not interact with each other in a directed two-hybrid assay, they may interact genetically, either synergistically by enhancing different aspects of Hairy repression or they may be antagonistic. While the dCtBP-binding region of the Hairy protein has not been characterized previously, nor do any of the small collection of missense *h* mutations map here, some repression capabilities have been attributed previously to a small region of Hairy including the necessary 25 amino acids but excluding the WRPW (Fisher *et al.*, 1996).

Three major classes of models for transcriptional repression have been proposed: (i) repressors prevent activators from binding DNA ('competition'); (ii) repressors and activators bind to DNA at independent sites, but the repressors interfere with interaction between the activators and the general transcriptional machinery ('quenching'); and (iii) repressors and activators bind to DNA at independent sites, with the repressors interacting (directly) with the general transcriptional machinery ('direct repression'; Herschbach and Johnson, 1993; Gray *et al.*, 1995). Existing evidence makes the first class of models ('competition') unlikely: in particular, Hairy-binding N-boxes and activator-binding E-boxes are non-overlapping at the *Sxl* promoter (Hoshijima *et al.*, 1996). In addition, Hairy appears to be a promoter-bound repressor (Fisher *et al.*, 1996; Jiménez *et al.*, 1996). The existing data do not distinguish

between the other two classes of models: whether Hairy interferes with activators or directly with the basal transcriptional machinery. It is possible that Hairy can participate in both quenching and direct repression mechanisms at different developmental times depending upon the presence and affinities of other interacting proteins recruited to the complexes. Characterization of the remaining Hairy-interacting proteins recovered from our yeast interaction screen should help to determine the scope of interactions possible for Hairy.

Barolo and Levine (1997) have postulated a mechanism for Hairy involving long range repression, not dependent on adjacent activator-binding sites. This may imply a role for Hairy in chromatin structure, where Hairy may have an overall effect on promoter DNA topology. A similar transcriptional repressor system recently was shown to affect histone acetylation (for example, see Roth, 1995; Laherty *et al.*, 1997). In this case, Max–Mad bHLHZip heterodimers bind to DNA and recruit the co-repressor mSin3. mSin3 in turn recruits histone deacetylase that subsequently affects chromatin structure.

While examining the interactions among members of the Hairy/E(spl)/Dpn family in directed yeast two-hybrid assays, we found evidence of partner preferences (Alifragis *et al.*, 1997). We showed that the seven E(spl) proteins can form both homo- and heterodimers with distinct preferences. The different affinities in partner interactions suggest distinct functions for the E(spl) proteins that previously were considered redundant, as well as additional levels of control for repression *in vivo*. The conserved Orange domain contributes to this specificity, maybe by recruiting adaptor proteins. Whereas Groucho interacts with all members of the Hairy/E(spl)/Dpn family, dCtBP interacts primarily with Hairy and E(spl)mδ and, therefore, could provide additional specificity. We have delineated functional specificity amongst the Hairy/E(spl) repressor proteins by identifying a Hairy-specific interacting protein whose *in vivo* function strongly suggests that it has a role in Hairy-mediated repression.

The evidence to date suggests that Hairy can mediate qualitatively different types of repression, perhaps due to the recruitment of different co-proteins forming complexes with different specificities. Since Hairy is probably involved in multiple protein–protein interactions, it will be important to know the scope of interactions that can occur among these proteins and the identity of these proteins, as well as Hairy targets, in order to determine the molecular events leading to transcriptional repression.

Materials and methods

Fly stocks

Flies were cultured and crossed on yeast–cornmeal–molasses–malt extract medium at 25°C. The alleles used in this study are: P[ry^+, *l(3)03463*], ry^{506}/TM3, ry^{RK} (referred to as P1590; from Bloomington Stock Center); $RpII140^{wimp}$, *rucuca*/TM3 (Parkhurst and Ish-Horowicz, 1991); kar^2 $mesA^1$/MRS (J.Gausz, Bowling Green Stock Center); $mesA^{13}$/MKRS (A.Hilliker); kar^2 $mesB^4$/MRS (Bowling Green Stock Center); $mesB^5$/MKRS (A.Hilliker); kar^2 $l(3)87De^1$/MKRS (A.Hilliker); $l(3)87De^{10}$/MKRS (J.Gausz, Bowling Green Stock Center); h^{7H}, *rucuca*/TM3 (D.Ish-Horowicz); h^{12C} *st e*/TM3 (D.Ish-Horowicz); P1590-FRT82B/TM3 (N.Perrimon); FRT82B-ovo^{D1}/TM3 (Bloomington Stock Center); and *y w* hs-FLP22; TM3/CxD (Bloomington Stock Center). Details of these strains can be found in Lindsley and Zimm (1992). A *h dCtBP* double mutant chromosome was generated by standard recombination techniques starting with the h^{7H}, *rucuca* and P1590 chromosomes.

FLP–DFS analysis

Production of germline mosaics using the autosomal FLP–DFS technique was done as described by Perrimon *et al.* (1996). The P1590-FRT82B line was tested for allelism with the original P1590 allele and with *l(3)87De* alleles, where it failed to complement.

Embryo analysis

Embryos were prepared and immunohistochemical detection of proteins was performed as described previously (Parkhurst *et al.*, 1990) using alkaline phosphatase-coupled secondary antibodies (Jackson Labs) visualized with Substrate Kit II reagents (Vector Labs, Inc.). Antisera used were as follows: anti-*hb* from D.Tautz; anti-*Kr*, anti-*kni* and anti-*h* from S.Carroll; anti-*eve* and anti-*en* from N.Patel and C.Goodman; anti-*ftz* from H.Krause; anti-*runt* from P.Gergen; anti-22C10 from E.Giniger; and anti-*Sxl* from D.Bopp.

Immunohistochemical whole-mount *in situ* hybridization was performed according to the protocol of Tautz and Pfeifle (1989). Digoxigenin-substituted probes were obtained by PCR amplification with primers to the 3′ end of the two-hybrid cDNA insert.

Larval cuticle preparations were prepared and analyzed as described by Wieschaus and Nüsslein-Volhard (1986).

Plasmid construction

All LexA and VP16 fusion constructs were made as described in Alifragis *et al.* (1997) with the exception that LexA–h-C28 was constructed by subcloning the VP16 library *Bam*HI cDNA fragment into the *Bam*HI site of pBTM116. The orientation of the insert subsequently was confirmed by sequencing.

Drosophila embryonic yeast two-hybrid library construction

Oligo(dT)-selected mRNA was prepared from 0–4 h embryos as described previously (Mozer *et al.*, 1985). Random-primed cDNA was generated using the Timesaver cDNA synthesis kit (Pharmacia) then ligated to linkers containing both *Not*I (internal) and *Bam*HI (external) sites. The cDNAs were cloned as *Bam*HI fragments into the yeast 2μ vector, f1-VP16 (Hollenberg *et al.*, 1995). The library complexity is ~2.0×10^6, with an average insert size of 1 kb.

Yeast two-hybrid screen

The two-hybrid screen was performed as described previously (Vojtek *et al.*, 1993; Hollenberg *et al.*, 1995). Two different Hairy partial protein baits, *h–N* (amino acids 1–148, containing the bHLH and Orange domains) and *h–C* (amino acids 93–343, containing the Orange–WRPW domains) were used since full-length Hairy protein represses the two-hybrid reporter system when used as a bait (Figure 1A). His^+LacZ^+ clones were recovered then subjected to directed two-hybrid interaction analysis with a number of related and unrelated proteins. From roughly one library complexity screened, 29 positive Hairy-interacting clones were identified, representing nine genes. A single dCtBP clone was isolated using the *h–C* bait. The characterization of the remaining clones will be described elsewhere.

Yeast quantitative β-galactosidase activity assays

The mating protocol and β-galactosidase liquid assays were performed as described in Ausubel *et al.* (1995). The β-galactosidase units were calculated using the formula: $(OD_{420}\times1000)/(OD_{600}\times$reaction time in min).

In vitro interactions of GST fusion proteins

The Hairy cDNA (amino acids 1–343) was subcloned into the pCite vector *Eco*RI–*Bgl*II sites and expressed from the T7 promoter using the Promega TnT *in vitro* expression kit ([^{35}S]methionine-labeled). The dCtBP two-hybrid insert was subcloned into the *Bam*HI site of pGEX-3X. This cDNA begins with the predicted ATG and continues 180 bp past the predicted stop codon. The binding assay was performed as described by Hurlin *et al.* (1995), except that the GST–dCtBP fusion protein was expressed at 30°C to improve protein solubility. The [^{35}S]methionine-labeled *in vitro* translated Hairy and Hairy mutant proteins were pre-cleared 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 and 0.5% NP-40).

Genomic characterization

Genomic DNA flanking the P1590 insertion site was carried out as described by Cooley *et al.* (1988). The two-hybrid cDNA insert was used to screen an EMBL3 *melanogaster* genomic library (R.Blackman). Restriction and Southern analysis were used to characterize and compare the DNA flanking P1590 and of the overlapping genomic phage clones.

We also obtained genomic DNA phage clones that were isolated previously in a chromosomal walk through the rosy–ACE region (Bender *et al.*, 1983) and, by Southern analysis, we positively identified phage subclone 2849 as hybridizing to dCtBP-specific probes.

The two-hybrid cDNA insert was also used to screen an ovary library (Stroumbakis *et al.*, 1994) and several early embryonic cDNA libraries (Novagen, Inc.).

DNA sequencing was carried out manually using Sequenase (United States Biochemical) or with Taq DyeDeoxy terminator AutoSequencing (Applied Biosystems).

Northern analysis

Developmentally staged RNAs were prepared as described previously (Mozer *et al.*, 1985). Northern production and hybridization were as described (Mozer *et al.*, 1985), using 5 μg of poly(A)$^+$-selected mRNA per lane and Magnagraph membrane (Micro Separations Inc.). *Dras* is expressed ubiquitously during development and was used as a loading control (Mozer *et al.*, 1985).

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