Negative regulation of *Ultrabithorax* expression by *engrailed* is required for proper specification of wing development in *Drosophila melanogaster*

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Abstract

In both vertebrates and invertebrates, homeotic selector genes confer morphological differences along the antero-posterior axis. However, insect wing development is independent of all homeotic gene functions, reflecting the ground plan of an ancestral pterygote, which bore wings on all segments. Dipteran insects such as *Drosophila* are characterized by a pair of wings in the mesothoracic segment. In all other segments, wing development is essentially repressed by different homeotic genes, although in the metathorax they are modified into a pair of halteres. This necessitates that during development all homeotic genes are to be maintained in a repressed state in wing imaginal discs. In this report we show that (i) the function of the segment polarity gene *engrailed* (*en*) is critical to keep the homeotic selector gene *Ultrabithorax* (*Ubx*) repressed in wing imaginal discs, (ii) normal levels of En in the posterior compartment of haltere discs, however, are not enough to completely repress *Ubx*, and (iii) the repression of *Ubx* by *en* is independent of Hedgehog signalling through which the long-range signalling of *en* is mediated during wing development. Finally we provide evidence for a possible mechanism by which *en* represses *Ubx*. On the basis of these results we propose that *en* has acquired two independent functions during the evolution of dorsal appendages. In addition to its well-known function of conferring posterior fate and inducing long-range signalling to pattern the developing appendages, it maintains wing fate by keeping *Ubx* repressed.

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Introduction

Homeotic selector genes of the bithorax (BX-C) and Antennapedia (ANTP-C) complexes play critical roles in the elaboration of segmental identities along the antero-posterior axis (A/P) of the fruitfly *Drosophila melanogaster* (Lewis 1978; Kaufman *et al.* 1980). The structure and function of these genes are highly conserved across a wide range of animals, including humans (reviewed in McGinnis and Krumlauf 1992). They encode homeodomain-containing DNA-binding proteins and function as transcriptional regulators of downstream target genes. Genetic studies, principally with the *Ultrabithorax* (*Ubx*) gene of BX-C (Morata and Garcia-Bellido 1976) and the *Antennapedia* (*Antp*) gene of ANTP-C (Struhl 1982) have demonstrated a cell-autonomous requirement for selector gene function throughout development. Although normal development of every segment requires one or more homeotic gene functions, wing development in *Drosophila* is shown to be independent of all homeotic genes (Carroll *et al.* 1995). In the first thoracic segment T1 and in all abdominal segments wing development is totally repressed by different homeotic genes (Lewis 1978; Carroll *et al.* 1995), while in T3 wings are modified by *Ubx* into a pair of small balancing organs, the halteres (Lewis 1978).

Interestingly, *Antp* and *Ubx* are expressed in the embryonic parasegments overlapping T2, although later during development they are not functional in wing imaginal discs (Struhl 1982; Carroll *et al.* 1995). In the embryo, *Ubx* mainly specifies the structures derived from the parasegments 5 and 6 (PS5 and PS6). These parasegments

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correspond to the posterior compartment of T2 (T2p), the anterior and posterior compartments of T3 (T3a and T3p), and the anterior compartment of first abdominal segment (A1a). Loss of *Ubx* function results in the transformation of not only T3 and A1 segments to T2, but also T2p to T1p (Lewis 1978). Its expression in T2p is regulated by two of its enhancer elements, namely *abx* and *pbx* (Muller and Bienz 1991; Castelli-Gair *et al.* 1992). In the wing disc, a derivative of T2, *Ubx* is expressed only in the peripodial membrane (White and Wilcox 1985) and loss of *Ubx* has no effect on wing development (Struhl 1982). Thus, *Ubx* function in T2p, known as its *postprothorax* (*ppx*) function, is exclusively required during early embryonic development (Morata and Kerridge 1981; Casanova *et al.* 1985). The ectopic expression of *Ubx* induced in the mesothorax by *Cbx* mutations or by other genetic methods result in wing-to-haltere transformation (Lewis 1982; Cabrera *et al.* 1985; White and Akam 1985; Casares *et al.* 1997). Thus, it is evident that repression of *Ubx* in the wing disc is necessary for proper specification of wing fate, although little is known regarding how *Ubx* is repressed in the developing wing during post-embryonic stages.

The cell-autonomous nature of homeotic gene function assigns importance to the precise activation of homeotic gene expression within well-defined boundaries and the maintenance of the same. These genes are expressed in the early embryo in response to the A/P positional information provided by the segmentation genes, in particular the gap genes (reviewed in Akam 1987). The products of these segmentation genes are available transiently only during early development and thus the precise maintenance of their expression domains requires other factor/s. The members of *Polycomb (Pc-G)* group of genes have been proposed to be the silencers of homeotic genes, keeping them repressed in the segments anterior to their normal domain of function (Lewis 1978; Duncan 1982; Simon *et al.* 1992). However, the *ppx* function of *Ubx* in T2p during early development, mediated by *abx* and *pbx* regulatory elements, is independent of *Pc-G* function (Muller and Bienz 1991). This suggests that *Pc-G* function alone is not sufficient to keep *Ubx* repressed during development and other genes may be involved in this process.

The segment polarity genes in *Drosophila* divide embryonic segments into precisely defined regions, the anterior and posterior compartments (Garcia-Bellido *et al.* 1973; Garcia-Bellido 1975; Morata and Lawrence 1975). The *en* gene, which encodes a homeodomain-containing DNA-binding protein, functions as a 'selector gene' (Garcia-Bellido 1975) and confers posterior identity to cells. It also prevents them from crossing the compartment boundary and mixing with anterior cells (Garcia-Bellido and Santamaria 1972; Morata and Lawrence 1975; Lawrence and Morata 1976; Lawrence and Struhl 1982). In dorsal (wing and haltere) and ventral (leg) limb primordia, apart from conferring posterior fate it activates long-range signals through the signalling protein Hedgehog (Hh) to pattern limb growth and development (Simmonds *et al.* 1995; Tabata *et al.* 1995; Zecca *et al.* 1995). Interestingly, results of two earlier studies have presented genetic evidence for repression of *Ubx* function by *en* during wing development (Eberlein and Russell 1983; Emerald and Roy 1997). Wing-to-haltere transformations have been observed in certain heteroallelic combinations of *en* (Eberlein and Russell 1983). In an earlier report, we have shown that ectopic expression of En in the anterior compartment of haltere disc induces haltere-to-wing transformation, suggesting repression of Ubx function by ectopic En (Emerald and Roy 1997). These earlier studies imply negative interaction between *en* and *Ubx*, either common downstream targets activated by one and repressed by the other or downregulation of *Ubx* expression by *en*. The latter possibility, in turn, raises an important question: how does *Ubx* escape repression by *en* in the posterior compartment of haltere discs?

Here we present results of a detailed study on the exact nature of interaction between *en* and *Ubx*. We show that *Ubx* is normally repressed by *en* in the posterior compartment of wing imaginal discs. However, in haltere discs, *Ubx* escapes this repressor activity owing to stronger positive regulation by other segmentation genes than the negative regulation by *en*. Not only did ectopic expression of En in the anterior compartment of haltere discs result in haltere-to-wing transformation, an increase in En levels in the posterior compartment also induced such transformation. The degree of transformation is further enhanced by decreasing the levels of Ubx and suppressed by increasing the levels of Ubx. We also show that repression of *Ubx* by *en* is independent of Hedgehog (Hh) signalling, which is the pathway through which *en* mediates the long-range signalling and coordinates wing development. In addition, our results suggest that *Ubx* is a direct target of *en*. We discuss the implications of these results for the evolution of wing development.

Materials and methods

Genetics: The Canton-S strain of *Drosophila melanogaster* was used as the wild-type strain. All the alleles of *Ubx* and *en* are described in Lindsley and Zimm (1992). The flp-out technique (Struhl and Basler 1993) was used to ectopically express En and Hh in the haltere discs. To induce En-expressing mitotic clones, f^{36a} *hsp*70-*FLP* (Zecca *et al.* 1995) female flies were crossed to $Tub\alpha1 > f^{+} > en / CyO$ (Zecca *et al.* 1995) male flies. Similarly, to induce mitotic clones expressing Hh, *y hsp*70-*FLP* female flies were crossed to *y*; $Tub\alpha1 > y^{+} > hh\,(ry^{+})$; *ry* (Struhl and Basler 1993) male flies. The progeny of both the crosses were heat-shocked at various developmental stages at 37°C for 1 h. The heat-shocked progeny were allowed to develop until eclosion at 25 ± 1°C. For inducing $Tub\alpha1 > en$ clones in Ubx^{1} and Cbx^{Hm} heterozygous backgrounds, f^{36a} *hsp*70-*FLP* female

flies were crossed to $Tub\alpha1 > f^{+} > en/CyO; Ubx^{1}/TM6\ Tb$ and $Tub\alpha1 > f^{+} > en/CyO; Cbx^{Hm}/TM6\ Tb$ male flies, respectively, and the progeny were treated as above.

Somatic clones for the null allele en^{E} were generated by crossing *w*; P[FRT42] $Df(2R)en^{E}/CyO$ male flies to *w hsp*70-*FLP*; P[FRT42] P[$arm-lacZ$]51*D* female flies. To remove both *en* and *inv*, P[FRT]42 $inv^{30}en^{9.6}/CyO$ male flies were crossed to either *y hsp70-FLP;* P[FRT42] *M*(2*R*) / *CyO* or *y hsp70-FLP* P[FRT42]πM female flies. The progeny of both the crosses were heat-shocked at various developmental stages at 37°C for 1 h. The heat-shocked progeny were allowed to develop until eclosion at 25 ± 1°C. Removal of En (in somatic clones) was confirmed by staining wing imaginal discs of late third-instar larvae either with monoclonal anti-β-galactosidase or anti-En antibodies. In all the experiments, maximum number of somatic clones were obtained in the animals heat-shocked during late first instar and early second instar stages.

The wings and halteres of adult flies of the desired genotype were boiled in 10% KOH, dehydrated through ascending grades of alcohol, cleared in clove oil, dissected, and mounted in Zeiss mounting medium.

Immunohistochemisry: Imaginal discs were processed for anti-Ubx, anti-Antp, anti-En, anti-β-galactosidase (all monoclonal) antibody staining essentially as described earlier (Patel *et al.* 1989). Anti-Ubx antibodies were obtained from R. White (University of Cambridge, UK), Anti-Antp was from D. Brower (University of Arizona, Tucson, USA), anti-En antibodies were from P. O'Farrell (University of California, San Francisco, USA), and anti-β-galactosidase was purchased from Sigma, USA.

Results

Derepression of Ubx in the wing disc in certain heteroallelic combinations of en

Although early during embryogenesis *Ubx* expression is manifested and maintained in T2p, it is neither expressed nor required in the posterior compartment of wing imaginal discs (Struhl 1982). This suggests that *Ubx* is repressed in T2p during postembryonic development. Eberlein and Russell (1983) have reported that two of the heteroallelic combinations of *en*, viz. $Df(2R)en^{30}/Df(2R)en^{28}$ and $Df(2R)en^{28}/en^{1}$, show wing-to-haltere transformations. To determine if *en* has a role in silencing *Ubx* during wing development, we analysed different heteroallelic combinations of *en* for wing-to-haltere homeotic transformation (table 1). In addition to the two heteroallelic combinations reported by Eberlein and Russell (1983), $Df(2R)en^{30}/Df(2R)en^{X31}$ also exhibited wing-to-haltere transformation. In this genotypic combination, nearly 5% of the flies ($n = 234$) exhibited wing-to-haltere homeotic transformation (figure 1). Such wing-to-haltere transformations are normally caused by gain-of-function mutations in *Ubx*, namely *Contrabithorax* (*Cbx*) mutations, which cause ectopic expression of Ubx in wing imaginal discs (Morata 1975; Lewis 1982; Cabrera *et al.* 1985; White and Akam 1985). The wing-to-haltere transformation observed in $Df(2R)en^{30}/Df(2R)en^{X31}$ flies could also be due to ectopic expression of Ubx. To verify this possibility we analysed

Table 1. Different heteroallelic combinations of *en* examined for wing-to-haltere homeotic transformation.

Genotype	Phenotype
1. $Df(2R)en^{X31}/en^{IIB86}$	Embryonic lethal
2. $Df(2R)en^{X31}/en^{E}$	Embryonic lethal
3. $Df(2R)en^{X31}/en^{9.6}inv^{30}$	Embryonic lethal
4. en^{1}/en^{1}	Engrailed wing phenotype
5. $Df(2R)en^{X31}/en^{1}$	Engrailed wing phenotype
6. $en^{IIB86}/Df(2R)en^{30}$	Engrailed wing phenotype
7. $en^{1}/en^{9.6}inv^{30}$	Engrailed wing phenotype
8. en^{1}/en^{E}	Engrailed wing phenotype
9. $Df(2R)en^{X31}/Df(2R)en^{30}$	Engrailed wing phenotype and wing-to-haltere transformation
10. $Df(2R)en^{30}/Df(2R)en^{28}$	Engrailed wing phenotype and wing-to-haltere transformation
11. $en^{1}/Df(2R)en^{28}$	Engrailed wing phenotype and wing-to-haltere transformation

Homeotic transformation observed in $Df(2R)en^{30}/Df(2R)en^{28}$ and $en^{1}/Df(2R)en^{28}$ combinations has been reported earlier by Eberlein and Russell (1983).

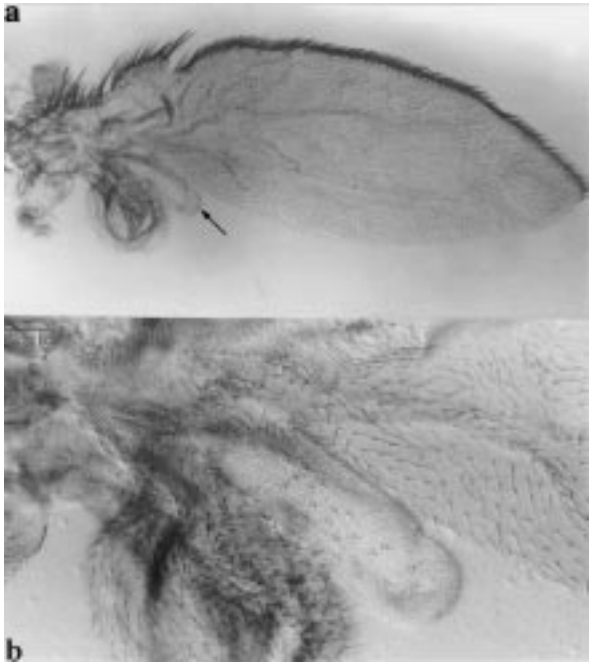

Figure 1. Homeotic transformation in *en* alleles. (a) $Df(2R)en^{30}/Df(2R)en^{X31}$ wing. Note that a part of the wing blade is transformed to haltere (arrow). (b) The transformed region of the wing in (a) shown at higher magnification. Note the development of sensilla trichoidea.

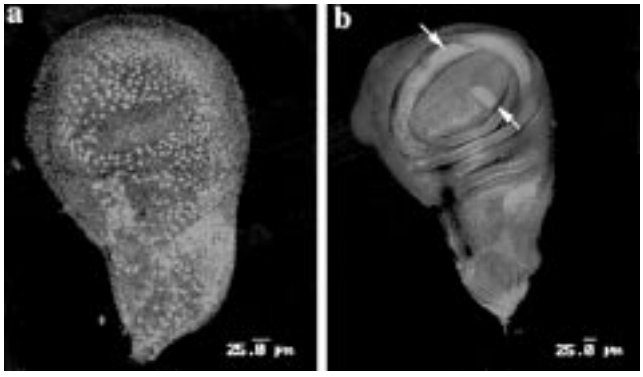

Figure 2. Reduction in *en* function derepresses *Ubx* expression in wing discs. *Ubx* expression was detected by immunofluorescence labelling using Texas Red as the fluoroprobe. (a) In wild-type wing discs, *Ubx* is expressed only in the peripodial membrane. (b) $Df(2R)en^{30}/Df(2R)en^{X31}$ wing disc. Note the appearance of Ubx protein in a small cluster of cells in the posterior compartment (arrows). In both the discs, anterior is to the left.

wing imaginal discs from $Df(2R)en^{30}/Df(2R)en^{X31}$ larvae for *Ubx* expression. We indeed observed the presence of Ubx protein in those larvae (figure 2b), suggesting that loss/reduction in *en* function results in the derepression of Ubx.

In addition, we analysed the expression of *Antp* in the heteroallelic combination $Df(2R)en^{30}/Df(2R)en^{X31}$, which shows wing-to-haltere transformation. We did not see any change (qualitative or quantitative) in the pattern of *Antp* expression in wing discs (data not shown).

Extensive studies have been carried out by generating somatic clones of embryonic lethal alleles to unravel the function of *en* during wing development (Kornberg 1981; Lawrence and Struhl 1982; Hidalgo 1994; Sanicola *et al.* 1995; Zecca *et al.* 1995). We repeated these experiments by generating somatic clones of en^{E} to remove *en* alone and $en^{9.6}\,inv^{30}$ to remove both *en* and *invected* (*inv*) in developing wing discs. We did observe pattern duplications identical to the ones reported earlier, but not the homeotic transformation of wing cells to haltere cells (data not shown). Considering the unambiguous phenotypes of heteroallelic combinations of *en* described above, it was intriguing that none of the studies employing *en* null alleles has revealed wing-to-haltere transformations. The allelic specificity of these phenotypes is ruled out as three different combinations of *en* alleles displayed similar wing-to-haltere transformations.

Overexpression of En represses Ubx expression in the haltere disc

We further examined the role of *en* in modulating *Ubx* expression in haltere discs. We had earlier shown that ectopic expression of En in the anterior compartment of haltere discs results in haltere-to-wing transformation (Emerald and Roy 1997). If *en* can repress *Ubx* in both wing and haltere discs, how does *Ubx* escape repression by *en* in the posterior compartment of haltere discs? Since *Ubx* is maximally expressed in PS6, i.e. T3p and A1a, its positive regulation in the posterior compartment of haltere discs may override its negative regulation by *en*. In such a scenario, changes in the levels of En and/or Ubx would offset the balance and may result in haltere-to-wing transformations. We increased the levels of En in haltere discs by clonally inducing ectopic En expression from a constitutive promoter using the flp-out technique (Zecca *et al.* 1995). The $Tub\alpha1>en$ clones were identified in the adult flies by the presence of *forked* (f) bristles as a marker (Emerald and Roy 1997). $Tub\alpha1>en$ clones causing ectopic expression of En in haltere discs displayed haltere-to-wing transformation irrespective of whether they were in anterior or posterior compartment (figure 3, b–d). The phenotype was cell-autonomous $(n > 400)$. When $Tub\alpha1>en$ clones were induced in Ubx^{1} heterozygous background, we observed enhanced haltere-to-wing transformations: transformed halteres displayed larger number of margin-specific bristles ($n = 60$, figure 3e).

In this set of experiments, however, there is a possibility of En and Ubx (both being homeodomain-containing transcription factors) competing for the same binding sites and in the process Ubx getting competed out. We therefore examined haltere discs for *Ubx* expression following ectopic expression of En. Haltere discs carrying $Tub\alpha1>en$ clones showed loss of *Ubx* expression within the limits of the clones, i.e. wherever En is ectopically expressed (figure 4c). We further confirmed these results by ectopically expressing En with the help of GAL4–UAS system (Brand and Perrimon 1993). En was overexpressed in the haltere margin with the help of *vg*–GAL4 driver (Simmonds *et al.* 1995) and UAS–*en* construct (Tabata *et al.* 1995). Haltere imaginal discs from these larvae showed reduction in the levels of Ubx in the D/V boundary and adult flies exhibited haltere-to-wing transformations in both anterior and posterior margins (data not shown).

Taken together, these results not only suggest that *en* is necessary to keep *Ubx* repressed in wing imaginal discs, but also indicate that the normal levels of En in the haltere disc are not sufficient to repress *Ubx* expression. However, small increase in En levels is enough to upset the balance and cause downregulation of Ubx, leading to haltere-to-wing transformation.

Cbx mutations abolish en-mediated repression of Ubx

Dominant mutations in the *Ubx* gene partially transform the second thoracic segment to the third thoracic segment (*Cbx* phenotype) owing to the ectopic expression of Ubx in the former segment (Morata 1975; Lewis 1982; Cabrera *et al.* 1985; White and Akam 1985; Botas *et al.* 1988; Castelli-Gair *et al.* 1990). One such gain-of-function allele of *Ubx*, *Haltere mimic* (Cbx^{Hm}), displays complete transformation of wing to haltere (figure 5a); thus adult flies have four

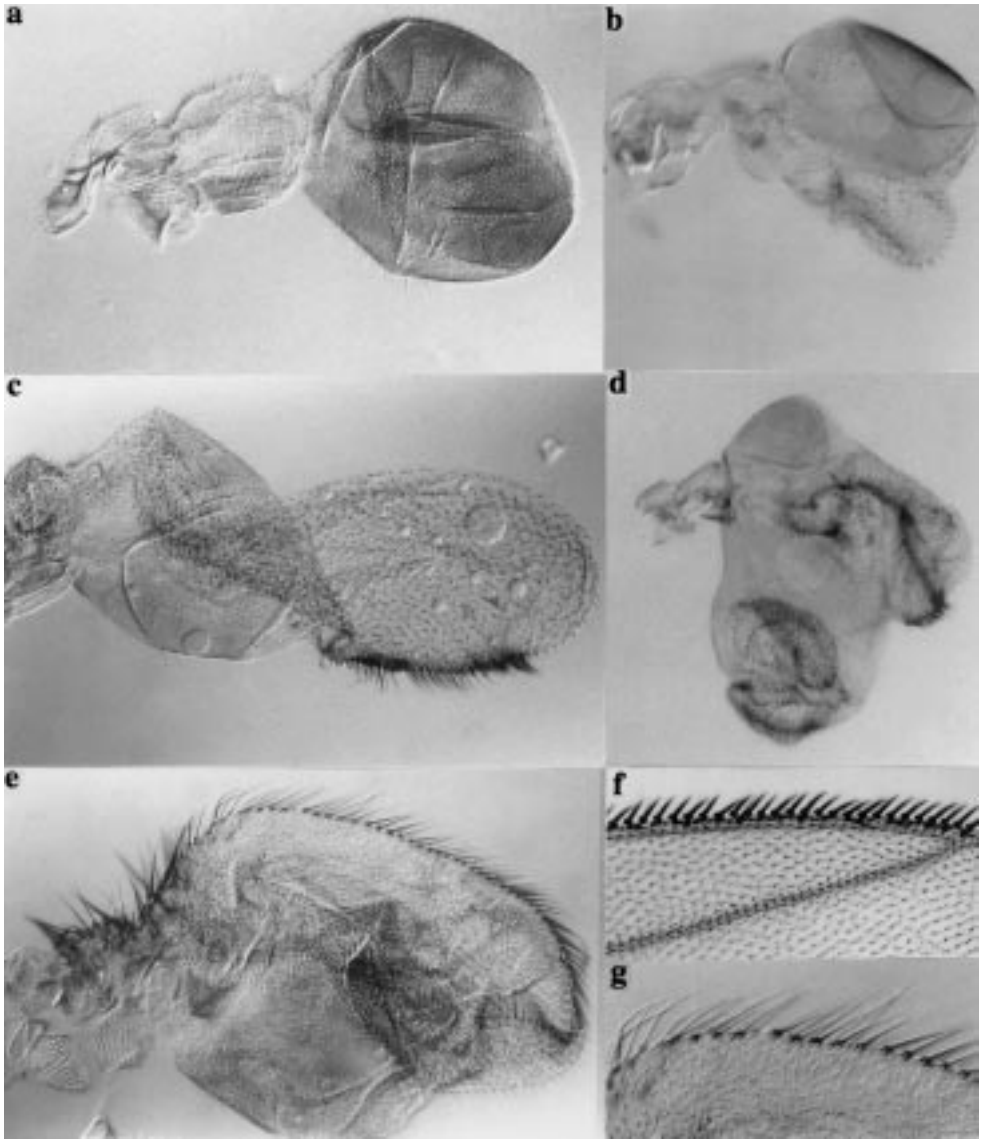

Figure 3. Haltere-to-wing transformation by ectopic En. (a) Wild-type haltere. (b–d) Haltere-to-wing transformation by ectopic En in both anterior (c, d) and posterior (b, d) compartments. (e) Enhancement of haltere-to-wing transformation in Ubx^{1}/+ background. Note that the margin is completely transformed. (f) f^{36a} wing at higher magnification to show anterior margin. (g) Anterior margin of the haltere shown in (e) at higher magnification. In all the halteres (a–e) in this figure, anterior margin is to the top.

halteres and no wings. If *en* functions upstream to *Ubx*, T2 halteres of Cbx^{Hm} mutant flies may respond differently to *Tubα1 > en* clones compared to wild-type T3 halteres (figures 3, b–d). To examine this possibility we induced *Tubα1 > en* clones in Cbx^{Hm} heterozygous background and compared the phenotypes between T2 and T3 halteres of the same fly. We observed that ectopic expression of En in Cbx^{Hm} background did not induce homeotic transformation in either T2 or T3 halteres; however, both the halteres displayed pattern duplications (figure 5, b&c). The absence of any homeotic transformation in the T3 halteres of Cbx^{Hm} flies by ectopic En suggests that Cbx^{Hm} mutations deregulate *Ubx* expression in both T2 and T3 dorsal discs. Certain *Cbx* mutations are known to activate in *trans* (by transvection) the wild-type *Ubx* allele present on the homologous chromosome (Castelli-Gair *et al.* 1990; Casares *et al.* 1997). The very high efficiency of *Tubα1 > en* clones to induce haltere-to-wing transformation would therefore be useful to study such transvection effects of *Ubx* alleles. The similarity in the phenotypes induced by ectopic En in Cbx^{Hm} halteres (figure 5, b & d) and by ectopic Hh in normal halteres (see below; figure 5d) suggests that the *en*-mediated genetic pathway that generates positional signals to pattern imaginal discs is not altered in Cbx^{Hm} background.

Repression of Ubx expression by en is not mediated by hh

Previous studies have shown that in wing imaginal discs the organizing function of *en* is mediated through *hh*,

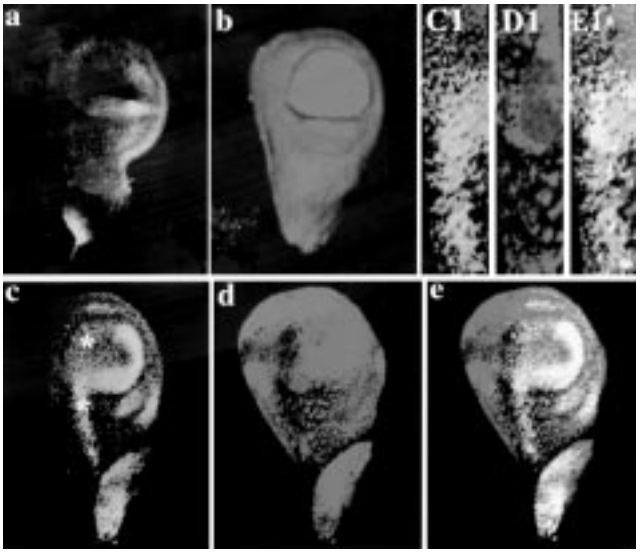

Figure 4. Ectopic En represses *Ubx* expression in the haltere disc. (a, b) Wild-type haltere discs stained for En (a) and Ubx (b). (c, d) Haltere disc after inducing *Tubα1 > en* clones and stained for both En (c, label: FITC) and Ubx (d, label: Texas red). In (e) the two labels are superimposed. Note the repression of Ubx in the anterior compartment due to ectopic En (asterisks). (C1–E1) Higher magnification of the region marked by asterisks in (e).

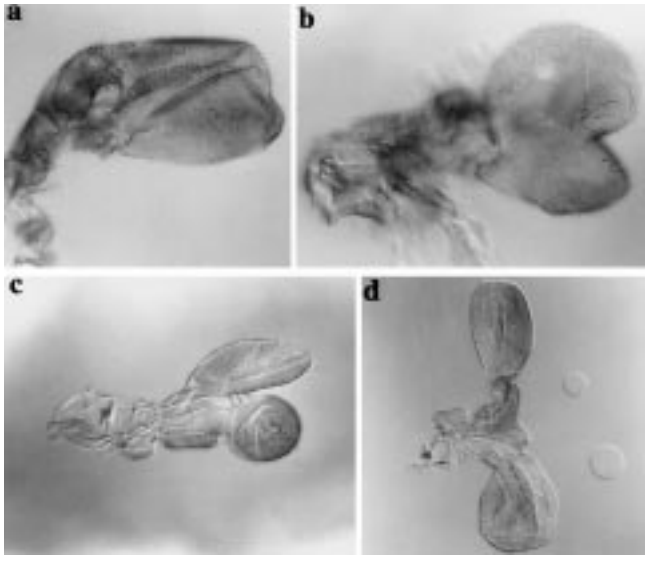

Figure 5. Negative regulation of *Ubx* by *en* is suppressed by *Cbx* and is not mediated through *hh*. (a) Cbx^{Hm} wing showing transformation towards haltere. (b, c) Pattern duplication in T2 (b) and T3 (c) halteres by ectopic En in Cbx^{Hm} background. Note the absence of any haltere-to-wing transformation. (d) Pattern duplication (along the antero-posterior axis) in the haltere (in wild-type background) by ectopic Hh. All the halteres in this figure are at the same magnification and the anterior margin is to the top.

essentially a non-cell-autonomous phenomenon (Tabata *et al.* 1992; Tabata and Kornberg 1994; Zecca *et al.* 1995). However, the haltere-to-wing transformations induced by ectopic En were cell-autonomous, suggesting an *hh*-independent pathway to repress *Ubx*. To further test whether *en*-mediated haltere-to-wing homeotic transformation is mediated through Hh signalling or not, we ectopically expressed Hh in haltere discs using the flp-out technique (Basler and Struhl 1994). The rationale behind this experiment was, if En-induced homeotic transformations of haltere were mediated through Hh, ectopic expression of Hh would also induce haltere-to-wing homeosis. However, ectopic expression of Hh did not result in any homeotic transformation (figure 5d), although it induced pattern duplication of halteres in agreement with its role in pattern formation along the A/P axis. This suggests that *en*-mediated repression of *Ubx* is independent of Hh signalling. This is further supported by the fact that none of the loss-of-function alleles of *hh* (we have examined three allelic combinations) is known to display wing-to-haltere transformation.

Is Ubx a direct target of en?

Although the En protein has been well characterized and is shown to be a homeodomain-containing transcription factor, very little is known about its downstream target genes. The results presented above, however, do not provide enough evidence to show that *en* directly represses *Ubx* by acting on its *cis* regulatory elements. TAATAATAA, TAAATTAAT (Desplan *et al.* 1988; Gould *et al.* 1990) and TCAATTAAAT (Serrano *et al.* 1995) are the consensus binding motifs for En, the core sequence being TAAT. Recently, TAATTA has also been shown to be a core sequence to which En binds with high specificity (Draganescu and Tullius 1998). We searched both the strands of the entire *Ubx* gene, which has been completely sequenced (Martin *et al.* 1995), for these En binding sites. Expected numbers were calculated on the basis of the third-order Markov chain theory (Lewis *et al.* 1995). In this method, the probability of occurrence of a given sequence is conditional upon the probability of obtaining the three bases that immediately precede it. An estimate of the conditional probability of obtaining, for example, an A after the trinucleotide TAA was obtained as the ratio of the total number of TAAA tetranucleotides to the total number of TAA trinucleotides observed in the entire *Ubx* sequence. Thus, the probability of obtaining the sequence TAAATTAAT would be the product of individual probabilities of obtaining TAAA, AAAT, AATT, ATTA, TTAA and TAAT.

Ubx is a very large gene with complex arrangement of regulatory regions. The full-length gene is approximately 150 kbp long and the transcriptional unit itself is around 77 kbp. We found 33 probable En binding motifs in the *Ubx* sequence (table 2), out of which 23 are in the transcribed region (figure 6). There are as many as 10 potential En binding sites clustered around the *bx* region enhancer (figure 6), raising the possibility that En may be binding to one or more of these *Ubx* sites to regulate its expression. These sites are different from the three *hunchback* (*hb*) binding sites present in the *bx* region enhancer (Qian *et al.* 1991).

Table 2. Comparison of observed (O) and expected (E) numbers of the three different En binding motifs and the consensus core sequence in the entire *Ubx* sequence (146 kbp) and in the *bx* enhancer region (12.3 kbp).

	Entire *Ubx* sequence			*bx* enhancer region		
Motif	Observed	Expected*	*P***	Observed	Expected*	*P***
TAATTA	236	160.93	0.0001	22	21.04	NS
TAATAATAA	15	6.48	0.001	3	1.10	0.02
TAAATTAAT	14	12.8	NS	7	1.40	0.0001
TCAATTAAAT	4	2.26	NS	0	0.15	-

Both forward and reverse strands of DNA were searched for En binding motifs. Note that the clustering of TAATAATAA and TAAATTAAT motifs in *bx* enhancer region is statistically highly significant, although the distribution of only TAATAATAA is significant for the entire *Ubx* sequence.

*Expected numbers were calculated on the basis of the third-order Markov chain theory (Lewis *et al.* 1995).

**Probability that the observed number or a larger number exceeds expected number based on the cumulative Poisson distribution. NS, Not significant ($P > 0.05$).

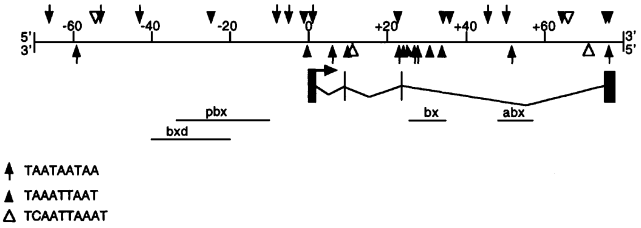

Figure 6. Distribution of the putative En binding sites within the *Ubx* sequence. The map of the complete *Ubx* gene with prominent regulatory regions and introns and exons is shown. The observed En binding sites are shown for both the strands. Note the clustering of En binding sites around the *bx* enhancer region.

However, molecular experiments such as DNA footprinting are to be carried out to confirm the binding of En protein to these sites.

Discussion

Three classes of zygotically expressed segmentation genes, viz. gap, pair rule and segment polarity genes, act in a hierarchical fashion to divide the *Drosophila* embryo into segments and further divide each segment into two compartments (for a review see Akam 1987). Once the segments are formed, homeotic selector genes function in each segment to generate segmental diversity along the A/P axis. Embryos lacking segment polarity genes are not properly segmented but they have morphological differences along the A/P axis. Conversely, embryos lacking all the homeotic genes still form normal, although identical, segments. Therefore, it was presumed that segment polarity genes and homeotic genes do not interact during development. The only exception to this comes from the experiments on the genetic interactions between the segment polarity gene *en* and the homeotic gene *Ubx* during embryonic development (Martinez-Arias and White 1988; Mann 1994). It was shown that En represses *Ubx* expression in the posterior compartment of PS6 and therefore the posterior compartment of PS6 contains lower levels of Ubx than the anterior compartment (Martinez-Arias and White 1988; Mann 1994). It had also been suggested that this modulation of Ubx in PS6 is required for the maintenance of the morphology of PS6 as it leads to the proper expression of the *Ubx* target gene *Distalless* (Mann 1994).

Wing development in *Drosophila* is shown to be independent of homeotic gene function. In T1 and in all abdominal segments wing development is repressed by different homeotic genes (Carroll *et al.* 1995). The expression patterns and mutant phenotypes of *Ubx* suggest that segmental identity is controlled at the level of compartments or parasegments in embryos (Martinez-Arias and Lawrence 1985). In the embryo, T2p is the anteriormost compartment to express *Ubx*. However, *Ubx* expression in this compartment is later repressed to facilitate wing development. Members of *Pc-G* genes are global silencers of homeotic genes, keeping them repressed in the segments anterior to their normal domain of expression (Lewis 1978; Duncan 1982; Simon *et al.* 1992), which cannot account for the localized repression of *Ubx* in the wing imaginal disc (Muller and Bienz 1991). In this context, we examined the nature of interactions between *en* and *Ubx* during wing development. We provide genetic and molecular evidence to show that *en* functions as a repressor of *Ubx* during wing development. Reduction in *en* function results in the derepression of *Ubx* in the wing disc (figure 2), leading to wing-to-haltere transformations. Conversely, ectopic expression of En in the anterior compartment of haltere discs results in the repression of *Ubx* (figure 4) and thereby induces haltere-to-wing transformations (figure 3).

The choice of *en* for this function during evolution raises the problem of how normal levels of *Ubx* expression are maintained during haltere development in T3. We have more than one line of evidence to suggest that the negative regulation of *Ubx* by *en* is dependent on the levels of En protein product. First, small increases in the levels of En in the posterior compartment of haltere discs induce haltere-to-wing transformations (figure 3). Second, the T2 and T3 halteres of *Cbx* mutants do not show any homeotic

transformation in response to ectopic En (figure 5, b & c). This can be attributed to stronger positive regulation by other segmentation genes than the negative regulation by *en*, thereby rendering ectopic-En-mediated repression inefficient. Finally, enhanced transformation phenotype is observed when ectopic expression of En is induced in *Ubx* heterozygous background, which again suggests that relative degrees of positive and negative regulation play the critical role.

Cbx alleles push the boundary of *Ubx* expression to the anterior by one parasegment (Lewis 1982; Cabrera *et al.* 1985; White and Akam 1985; Botas *et al.* 1988; Castelli-Gair *et al.* 1990), which results in wing-to-haltere transformation. This is due to the break points in the upstream regulatory region uncoupling the *cis*-acting suppressors and activators (mainly *abx* and *pbx*) of *Ubx* expression (Castelli-Gair *et al.* 1992). These studies have further suggested that the function of *Ubx* repression in the wing disc depends on the preceding action of embryonic repressors (Muller and Bienz 1991; Castelli-Gair *et al.* 1992). The absence of haltere-to-wing homeosis by ectopic En in Cbx^{Hm} background suggests that En cannot override the function of other *trans*-acting factors (such as the products of *hb, tailless, fushi tarazu*; White and Lehman 1986; Qian *et al.* 1991) functioning in the embryo to repress *Ubx* expression in PS4 and PS5. In other words, *en* regulation of *Ubx* expression in T2 is localized to wing disc and is effective only during postembryonic stages.

The presence of several En binding motifs within the *Ubx* gene (table 2 and figure 6) and the modulation of *Ubx* expression in the background of both loss of function and gain of function of *en* suggest direct interaction between the two genes. In addition, negative regulation of *Ubx* expression in both wing and haltere disc is independent of *hh* function (figure 5d), through which the nonautonomous, long-range signalling of *en* are mediated. At this stage one could argue that Cbx^{Hm} mutations disrupt En binding sites in the *Ubx* gene, resulting in ectopic Ubx in wing discs and thus cause wing-to-haltere transformation. This, however, is ruled out since Cbx^{Hm} mutation causes wing-to-haltere transformations in both anterior and posterior compartments. In addition, the putative En binding sites are clustered mostly in the *bx* region (figure 6), whereas Cbx^{Hm} deletion is localized to the *pbx* region of the *Ubx* gene (Lindsley and Zimm 1992).

At this stage, one cannot completely rule out the possibility that *Ubx* repression by *en* is mediated by another, *hh*-independent pathway. Wing-to-haltere transformations have been reported for many loss-of-function alleles of *polyhomeotic* (*ph*), a member of polycomb group of genes (Dura *et al.* 1985, 1987). During early embryonic development, *en* is negatively regulated by *ph* (Dura and Ingham 1988), whereas in late stages *ph* is positively regulated by *en* and probably it is a direct target of *en* (Serrano *et al.* 1995). Although further study is needed to confirm this, it is possible that *en* activates *ph*, which in turn represses *Ubx* in the posterior compartment of the wing disc.

However, only certain heteroallelic combinations of *en* show wing-to-haltere transformations, but not the somatic clones of *en* null alleles. There have been several examples of such complexities in gene functions. For example, it has been recently shown that misexpression of *teashirt* (*tsh*) induces ectopic eyes, although *tsh* alleles do not have any visible eye phenotype under normal conditions (Pan and Rubin 1998; Bhojwani *et al.* 1997). Nonetheless, a potential role for *tsh* in eye development was first suggested by the observation that in a certain sensitized genetic background *tsh* mutations exhibit reduced-eye phenotype (Bhojwani *et al.* 1997). Embryonic and postembryonic development in higher eukaryotes is characterized by the complexity of regulation of individual genes and complex genetic pathways. Not surprisingly, a majority of the genes in eukaryotes do not exhibit easily assayable loss-of-function phenotypes (Miklos and Rubin 1996). Our studies thus provide further support to the view that gain-of-function genetics provides useful information on gene regulatory networks when loss-of-function phenotypes are not conclusive enough (Pan and Rubin 1998).

Conclusions

Although homeotic gene expression patterns are well defined and established in the early embryo, modulations of their expression pattern do occur during later stages of development. There have been instances of new genetic mechanisms causing these local changes in the homeotic gene expression patterns. For example, *tsh* negatively regulates *Antp* expression in the eye-antennal discs (Bhojwani *et al.* 1997), although it is downstream to *Antp* in the embryonic epidermis and mesoderm (McCormick *et al.* 1995). In this report, we have shown localized modulation of *Ubx* expression by *en* in the wing disc during postembryonic development. However, we have observed normal patterning of wing and haltere discs in *Cbx* background, although *en* can no longer repress *Ubx* expression in those wing discs. In addition, we have shown that negative regulation of *Ubx* by *en* is not mediated by *hh*. These results suggest that the two roles of *en*, i.e. the repression of *Ubx* expression to maintain wing fate and the induction of long-range signals to pattern developing fields, have been independently acquired during evolution.

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