Published in final edited form as:
Dev Biol. 2007 August 15; 308(2): 621–631.

The function and regulation of *Ultrabithorax* in the legs of *Drosophila melanogaster*

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Abstract

Alterations in *Hox* gene expression patterns have been implicated in both large and small-scale morphological evolution. An improved understanding of these changes requires a detailed understanding of *Hox* gene *cis*-regulatory function and evolution. *cis*-regulatory evolution of the *Hox* gene *Ultrabithorax* (*Ubx*) has been shown to contribute to evolution of trichome patterns on the posterior second femur (T2p) of *Drosophila* species. As a step toward determining how this function of *Ubx* has evolved, we performed a series of experiments to clarify the role of *Ubx* in patterning femurs and to identify the *cis*-regulatory regions of *Ubx* that drive expression in T2p. We first performed clonal analysis to further define *Ubx* function in patterning bristle and trichome patterns in the legs. We found that low levels of *Ubx* expression are sufficient to repress an eighth bristle row on the posterior second and third femurs, whereas higher levels of expression are required to promote the development and migration of other bristles on the third femur and to repress trichomes. We then tested the hypothesis that the evolutionary difference in T2p trichome patterns due to *Ubx* was caused by a change in the global *cis*-regulation of *Ubx* expression. We found no evidence to support this view, suggesting that the evolved difference in *Ubx* function reflects evolution of a leg-specific enhancer. We then searched for the regulatory regions of the *Ubx* locus that drive expression in the second and third femur by assaying all existing regulatory mutations of the *Ubx* locus and new deficiencies in the large intron of *Ubx* that we generated by P-element-induced male recombination. We found that two enhancer regions previously known to regulate *Ubx* expression in the legs, *abx* and *pbx*, are required for *Ubx* expression in the third femur, but that they do not contribute to pupal expression of *Ubx* in the second femur. This analysis allowed us to rule out at least 100kb of DNA in and around the *Ubx* locus as containing a T2p-specific enhancer. We then surveyed an additional approximately 30kb using enhancer constructs. None of these enhancer constructs produced an expression pattern similar to *Ubx* expression in T2p. Thus, after surveying over 95% of the *Ubx* locus, we have not been able to localize a T2p-specific enhancer. While the enhancer could reside within the small regions we have not surveyed, it is also possible that the enhancer is structurally complex and/or acts only within its native genomic context.

Keywords

cis-regulation; evolution; *Drosophila*; leg; trichome; *Ultrabithorax*

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INTRODUCTION

Although it has been inferred that much of developmental evolution occurs by changes in *cis*-regulatory regions, in only a few cases have the individual regulatory changes been identified (Gompel et al., 2005;Wang and Chamberlin, 2004). We have previously shown that the detailed pattern of trichomes on the legs of *D. melanogaster* is evolving rapidly and that some of this evolutionary change is attributable to *cis*-regulatory evolution at the *Ubx* locus (Stern, 1998). A stronger test of this hypothesis, and a more comprehensive understanding of how *Ubx* has evolved to alter body plans (Averof and Patel, 1997;Mahfooz et al., 2004), requires a detailed understanding of *Ubx cis*-regulatory structure and evolution.

The three pairs of legs of the adult *Drosophila melanogaster* differ in size and shape and in the distribution of different types of bristles and trichomes (Hannah-Alava, 1958). These differences are generated, ultimately, by the action of the homeotic genes *Sex Combs Reduced* (*Scr*) and *Ultrabithorax* (*Ubx*). *Scr* expression in the prothoracic legs (hereafter T1) is required to define specific features of these legs, such as the sex combs in males, lateral rows of bristles on the anterior tibia, and large bristles on the posterior femur (Struhl, 1982). *Ubx* function is required to define features of the metathoracic (T3) legs (Casanova et al., 1985;Kerridge and Morata, 1982;Morata and Kerridge, 1981;Struhl, 1982). *Ubx* clones induced at approximately four hours of development, however, cause a transformation of posterior femurs of both T2 and T3 towards T1, a phenotype known as a *postprothorax* (*ppx*) transformation (Casanova et al., 1985;Kerridge and Morata, 1982;Morata and Kerridge, 1981). This effect is due to a loss of *Scr* repression by *Ubx* in the posterior of T2 and T3 early in development (Little et al., 1990;Struhl, 1982). Although loss of function clones of *Ubx* and *Scr* induced after embryogenesis have no obvious effects on mesothoracic (T2) bristle patterns (Kerridge and Morata, 1982;Morata and Garcia-Bellido, 1976;Morata and Kerridge, 1981;Struhl, 1982), *Ubx* is required to repress the differentiation of trichomes in a proximal patch of cuticle on the posterior femur of T2 during metamorphosis (Stern, 1998). The size of this patch of smooth or naked cuticle has evolved amongst *Drosophila* species, with certain strains of *D. melanogaster* exhibiting a smaller patch relative to *D. simulans* (Stern, 1998).

Here we present results from a series of experiments that examine the role of *Ubx* in patterning legs and that seek to identify sequences responsible for differences in *Ubx* expression that account for an evolved leg phenotype between *Drosophila* species. We expanded on previous studies and found that in addition to the well characterized functions of *Ubx* in patterning T3, *Ubx* function is required to determine the fate and behavior of several cell types in T2 and T3 at multiple times during development. Some functions require high levels of *Ubx* expression, whereas only low levels of *Ubx* expression are required for other functions. Analysis of *Ubx* mutations identifies two previously characterized enhancers as required for *Ubx* expression in the third femur. To determine how *Ubx* leg expression has evolved, we first tested for, but found no evidence that, global *cis*-regulation of *Ubx* expression has evolved between *D. melanogaster* and *D. simulans*. We also found no direct evidence, either through analysis of mutants or analysis of enhancer constructs, for a discrete enhancer responsible for *Ubx* expression in the second femur. These results point toward complex regulation of *Ubx* function in T2.

MATERIALS AND METHODS

Ubx clones

Clonal analysis using a null allele of *Ubx* was performed to provide a detailed characterization of the role of *Ubx* in patterning trichomes and bristles on T2 and T3 legs. The *Minute* technique (Morata and Ripoll, 1975) was utilized to generate large territories of tissue homozygous for

a *Ubx* null allele. With this technique, clones that carry two wild-type alleles of *Minute*$^{+}$ overproliferate relative to neighboring cells that are heterozygous for a null allele of *Minute*. (Unless otherwise specified, null alleles are indicated generically with a superscript minus sign and wild-type alleles with a superscript plus sign. For example, clones carrying two wild-type copies of *Minute* are referred to as *Minute*$^{+}$ clones.) Virgin females with the genotype f^{36a}; f^{+}*87D M*(*3*)*95A*/TM3 were crossed to males with the genotype $Ubx^{1}e^{11}$/TM6B, *Tb*. (The $Ubx^{1}e^{11}$ chromosome had previously been outcrossed for several generations to a wild-type chromosome.) Larvae from this cross were irradiated (1,000 rad X-rays) at 24–48 hours after egg-laying (AEL) or 48–72 hours AEL. Early clones were generated by X-ray irradiating embryos at 4±2 hours AEL with 700 rad. Male offspring without balancer chromosomes were then selected and preserved in 70% ethanol:30% glycerol. Control clones were generated by crossing virgin females with the genotype f^{36a}; f^{+}*87D M*(*3*)*95A*/TM3 to males of the genotype $st^{1}p^{p}e^{11}$. Larvae were irradiated as above and males without the TM3 balancer chromosome were collected. Both experimental and control clones were therefore marked with bristles that were both *forked* and *ebony*. Clones were identified by inspecting the legs of flies for *forked* bristles under a dissecting microscope and all legs from an individual with at least a single clone were then mounted on glass slides in Hoyer's medium (Stern and Sucena, 2000). Images of adult legs were captured with a video camera under darkfield illumination and digitally inverted.

Ubx^{+} overexpression from a heat-shock inducible construct *HSUbx-1a* (Mann and Hogness, 1990) represses trichomes on the posterior T2 and T3 legs from approximately 20–30 hours APF at 25°C (Stern, 1998). We have extended this analysis by performing overexpression studies in flies also carrying an *engrailed* reporter construct (P{*ry*+t7.2=*en-lacZ*(Xho)} en^{Xho25}). White pre-pupae (0±0.5 hours APF) from the cross of HS*Ubx*/TM3 to *en-lacZ*/CyO were aged for 24 hours at 25°C and then heat shocked at 37°C for 1 hour. Pharate adults were dissected from the puparium, fixed in 2.5% glutaraldehyde in Phosphate-buffered saline (PBS: 130mM NaCl; 7mM $Na_2HPO_4.2H_2O$; 3mM $NaH_2PO_4.2H_2O$; pH 7.0) for 5 min, and stained for β-galactosidase activity using standard techniques (Ashburner, 1989). After staining, legs were dissected and mounted in Hoyer's medium (Stern and Sucena, 2000).

The patterns of trichomes and bristles on T1, T2, and T3 femurs possessing *Ubx*$^{-}$ clones were inspected in detail and compared with control clones and wild-type legs.

Pyrosequencing

Whole pupae of *D. melanogaster* (Oregon R) (14 total), *D. simulans* (Tsimbazaza) (14 total) and female hybrids created by mating *D. melanogaster* females and *D. simulans* males (6 collections of 14 pupae each) were collected at 24.5 hours after puparium formation (APF) and flash-frozen in liquid nitrogen. Genomic DNA and total RNA were isolated from these collections and single-stranded cDNA was synthesized twice for each RNA sample (Wittkopp et al., 2004). An amplicon within the *Ubx* homeobox that contains a single-nucleotide difference between *D. melanogaster* and *D. simulans* was amplified from gDNA and cDNA samples (primers: 5′-biotin-ATACACCCGCTACCAGACGCTC-3′ and 5′-TTCTCCGTCTGCGGGTCA-3′; region containing mel/sim difference: 5′-AAGGAGTTCCACACGAATCAT/CTAT-3′) and subsequently Pyrosequenced in the region containing the interspecific difference (Pyrosequencing primer: 5′-AGGAGTTCCACACGAATC-3′). The relative amounts of *D. melanogaster* and *D. simulans Ubx* mRNA represented in *D. melanogaster*/*D. simulans* hybrid cDNA were then determined by comparing the average percent *melanogaster* allele in PCR product amplified from hybrid cDNA with product amplified from hybrid gDNA, with any deviation from 50% in the latter representing allele-specific PCR amplification bias (Wittkopp et al., 2004).

Ubx mutants and genetic crosses

Stocks carrying mutations in the *Ubx* locus were provided by the Bloomington, Umea and Madrid Stock Centers, Michael Akam, Ed Lewis, and Welcome Bender. A full list of the alleles used is available in Supplementary Table 1. All regulatory mutations were crossed to at least one deficiency covering the entire *Ubx* locus (*Df(3)P2*, *Df(3)P9*, or *Df(3)Ubx*^{109}). Legs were dissected and mounted in Hoyer's medium and trichome and bristle patterns were scored under dark-field and bright-field illumination, respectively (Stern and Sucena, 2000). The distribution of trichomes on the posterior T2 and T3 femur was sketched for all genotypes, and we paid particular attention to similarities between T2 and T3 of individual genotypes and to genotypes that removed most or all of the naked cuticle on the posterior T2. Bristles that distinguish the T2 and T3 legs can be found at many positions along the leg (Hannah-Alava, 1958). The presence of ectopic bristles on the posterior T2 and T3 femur were noted and for some genotypes the number of rows of bristles and total number of bristles on the entire femur were counted. Casanova *et al.* (Casanova et al., 1985) and Peifer and Bender (Peifer and Bender, 1986) have previously reported that the *abx* and some of the *bx* mutations in *trans* to a deficiency for *Ubx* generate the *ppx* transformation with low penetrance. We therefore examine the T2 femurs of *abx* mutations for *ppx* transformations.

To mark the compartment boundary in *pbx* hemizygote flies, we generated flies heterozygous for *en-lacZ* on the second chromosome and a *pbx* mutation on the third and crossed these flies either to *Df(3)Ubx*^{109}/TM6B,*Tb* or to *Df(3)P2*/TM6B,*Tb*. Non-*Tubby* pupae from this cross were collected at approximately 48–72 hours APF, fixed and stained for β-galactosidase activity as detailed above. The legs from flies simultaneously displaying a *pbx* haltere phenotype and expressing *en-lacZ* were mounted in Hoyer's medium for examination.

Generation and analysis of new deficiencies

We generated new deficiencies at the *Ubx* locus targeted to the third exon of *Ubx* by P-element-induced male recombination (Preston et al., 1996) using the *rosy*$^{+}$-containing P-element insertion plac(−61) located within the third intron of *Ubx* (McCall et al., 1994). *ry sbd*^{1} *3ry*^{138} *e*^{11}/TM6B males were mated to *Δ2–3 cv v*/FM7c virgins and the resulting *Δ2–3 cv v; ry sbd*^{1} *3ry*^{138} *e*^{11}/+ males were mated to *sbd*^{2} *e*^{ll} virgins. Male progeny were then screened for recombinants (absence of *sbd*$^{-}$ or *e*$^{-}$ phenotypes). Deficiencies among recombinants were initially identified by failure to PCR-amplify DNA just 5′ and 3′ of plac(−61). Excision of the P element in some recombinants was determined by loss of *ry*$^{+}$ eye phenotype when tested in a *ry*$^{-}$ background and by PCR assays. Breakpoints were determined by inverse PCR when it was determined that the P-element had been retained and by Southern analysis when it had been excised.

Enhancer constructs

Enhancer constructs were derived from a previously cloned library of genomic fragments (Bender et al., 1983). The genomic fragments 3109, 3128, 3130, and 3142 were subcloned into pBluescript SKII+ (Stratagene) and then subcloned again into the Gal4 P-element *w*+ vector pPTGAL with an *hsp70* minimal promoter (Sharma et al., 2002). DNA preparations of these constructs were then injected into *w*^{118} embryos at the Duke Model System Genomics Group (http://www.biology.duke.edu/model-system/). Transformants (3 lines of 3109, 1 line of 3128, 2 lines of 3130 and 2 lines of 3142), were crossed to flies carrying *UAS-lacZ*$^{4\text{-}1.2}$ and the resulting progeny examined by anti-β-galactosidase (Capel) staining for embryos and by X-GAL staining for 3rd instar imaginal discs and pupal legs (22–26 hours APF). An internal PCR product (3118int; primers: 5′-GCAATGTAAGCCCTGTTCGTATCTC -3′ and 5′-CCTAAGTAATGGACGCAACTTCAGG -3′) from genomic fragment 3118 was cloned into the TOPO-TA vector pCR-II (Invitrogen) and then subcloned into the nuclear enhanced GFP P-element *w*+ vector pH-Stinger with *hsp70* minimal promoter and flanking gypsy insulating

elements (Barolo et al., 2000). Likewise, an ~3.2 kb 5′ of the first exon (5′ Ubx) was PCR-amplified from genomic OreR DNA (primers: 5′-AGCGGCCGCGAGGGCGTTGAGATAGGCCCCTTCA -3′ (NotI site added at 5′ end) and 5′-AAGATCTCGCGCCTGTTATCCAATCCGTTGC -3′) and cloned into pH-Stinger. The embryos, 3rd instar imaginal discs, and pupal legs of transformants (1 line each for 3118int and 5′ Ubx) were examined by fluorescence microscopy.

RESULTS

Ubx expression is required for specification, repression and migration of different bristles

We first performed clonal analysis to further define *Ubx* requirements in leg patterning. Our analysis of *Ubx*$^{-}$ clones agrees with the findings of Kerridge and Morata (1982) that on the T3 leg, *Ubx*$^{+}$ activity cell-autonomously represses bristles on the posterior femur (Fig. 2a). We interpreted these bristles as representing an ectopic eighth bristle row, located laterally on the posterior femur, although the bristles were never found in a neat row in clones, as they are elsewhere on the leg. The fine bristles normally located proximal-ventrally on T3p (arrow in Fig. 1d) were lost in posterior *Ubx*$^{-}$ clones (Fig. 2a), indicating that *Ubx*$^{+}$ activity is required to specify these bristles.

In posterior *Ubx*$^{-}$ clones, the small bristles normally found dorsally (arrowhead in Fig. 1d) were apparently transformed into more robust bristles (Fig. 2a). In contrast, in anterior *Ubx*$^{-}$ clones these small bristles remain on T3 (arrows in Fig. 2b) indicating that *Ubx*$^{+}$ is required to instruct these bristles to be small. In addition, as will be made clear in subsequent sections, the boundary between naked cuticle and trichomes in the *Ubx*$^{-}$ clones in Figures 2a and 2b represents the dorsal anterior-posterior boundary. Thus, the small dorsal-proximal bristles are found in the anterior compartment in legs with an anterior *Ubx*$^{-}$ clone (Fig. 2b), but not in legs with a posterior *Ubx*$^{-}$ clone (Fig. 2a). In Fig. 2b, it can be seen that these small bristles are not *forked*$^{-}$, indicating that they originated in the posterior compartment. This was also observed with control clones (data not shown). These results indicate that *Ubx*$^{+}$ expression in the posterior is required for these bristles to migrate from the posterior to the anterior compartment.

Finally, we found that *Ubx*$^{-}$ clones derepressed a bristle row on the posterior T2 femur (Fig. 2c), as they did on T3, leading to a total of eight bristle rows on the entire femur. In contrast, Kerridge and Morata (1982) reported that the bristle pattern on the posterior T2 femur was unaltered in *Ubx*$^{-}$ clones generated in the larval period (they found that blastoderm clones generated a *ppx* transformation). This observation led them to suggest that loss of *Ubx*$^{+}$ in the posterior T3 femur generated a homeotic transformation towards T2. Our results suggest, instead, that loss of *Ubx*$^{+}$ has the same effect in both legs: gain of an eighth bristle row.

Ubx expression is required to repress most trichomes in the posterior femur and a subset of anterior trichomes

In wild-type legs, the proximal region of the posterior femur of T2 (Fig. 1b) and most of the posterior femur of T3 (Fig. 1d) secrete smooth cuticle. In addition, as will be discussed in more detail, the proximal dorsal anterior T3 femur is also naked (Fig. 1d). Previous studies indicated that *Ubx*$^{+}$ represses trichome differentiation on the posterior femur of both T2 and T3 (Kerridge and Morata, 1982;Stern, 1998). Our analysis extends these results. First, large clones in the posterior T2 and T3 derepress trichomes over most, but not all of the posterior femur (Fig. 2a,c). On both legs, clones fail to differentiate trichomes in a small patch on the proximal, ventral region of the posterior femur (arrows in Fig. 2a,c).

In addition, *Ubx*$^{+}$ represses trichomes on the dorsal anterior proximal femur of T3 (arrowhead in Fig. 2b), a region that is normally naked (arrowhead in Fig. 1d). Large *Minute*$^{+}$ *Ubx*$^{-}$ clones

on T3p leave a large region of the dorsal T3 femur naked (Fig. 2a). This result was found in all legs carrying large *Minute*$^{+}$ *Ubx*$^{-}$ clones on T3p induced between 24–48 AEL (N=9) and between 48–72 AEL (N=7). In contrast, small *Ubx*$^{-}$ clones induced between 24–48 AEL using the FLP-FRT system (Xu and Rubin, 1993) could be found in the dorsal region and differentiated trichomes (data not shown). This naked dorsal region in large clones is interpreted as the naked cuticle of the anterior compartment that has shifted laterally, and perhaps expanded, because the posterior compartment has shrunk.

The difference between the response of trichome-forming cells in the anterior and posterior compartments to *Ubx* expression that was reported earlier (Stern, 1998) can now be extended to the differential response of subsets of cells within each compartment. In the posterior compartment of the T2 and T3 femur, all cells except a small patch of proximal ventral cells are capable of responding to high levels of *Ubx*$^{+}$ 20–30H APF by repressing trichomes (Stern, 1998); the proximal ventral cells repress trichomes independently of autonomous *Ubx*$^{+}$ activity. In the T2 and T3 anterior femur, only cells in the proximal dorsal region are capable of responding to high levels of *Ubx*$^{+}$ by repressing trichomes (also see Fig. 5).

Global *cis*-regulation of *Ubx* expression has not evolved between *D. melanogaster* and *D. simulans*

To test the hypothesis that the reduction in the size of the naked patch due to *Ubx* resulted from a decrease in the global (that is, non-tissue specific) *cis*-regulation of *Ubx* in *D. melanogaster*, we assayed the relative amounts of *D. melanogaster* and *D. simulans Ubx* mRNA in whole pupae of *D. melanogaster/D. simulans* hybrids by Pyrosequencing (Wittkopp et al., 2004) at the time when trichomes are repressed on T2p. We found that the percent *melanogaster* allele in PCR product amplified from hybrid cDNA was 48.9% (SD = 6.2%) and from hybrid gDNA was 47.6% (SD = 0.3%), which is not significantly different (two-tailed t-test, assuming unequal variances, $t=-0.512$, $df = 5$, $p = 0.63$). The variability observed among replicate measures of *Ubx* expression was larger than the variability observed for other genes using the same technique (Landry et al., 2005;Wittkopp et al., 2004;Wittkopp et al., 2006) and is likely due to the rarity of *Ubx* transcripts versus the relative abundance of previously surveyed mRNA. We attempted to measure the relative levels of mRNA in hybrid pupal legs, but the signal to noise ratio was not sufficiently high to allow robust inference of allelic ratios (data not shown).

Existing aberrations of the *Ubx* locus do not uncover a T2p leg enhancer

Regulatory mutations in *Ubx* cause morphological transformations similar to those characterized by clonal analysis with the *Ubx* null allele (Casanova et al., 1985;Kerridge and Morata, 1982;Peifer and Bender, 1986). We used this observation to attempt to map the regulatory regions of *Ubx* that control *Ubx*$^{+}$ function in different regions of the legs. We examined all existing regulatory mutations of *Ubx* and new deficiencies that we generated by P-element-induced male recombination (Supp. Table 1). Fig. 3 illustrates the primary informative alleles. The results of this analysis are organized into three sections discussing first the regions 5′ of the *Ubx* transcript, the introns and then the regions 3′ of the *Ubx* transcript.

The* postbithorax *region is required for* Ubx *expression in T3p: *postbithorax* (*pbx*) mutations cause transformations of T3p to T2p. The two known *pbx* mutations are deletions that overlap by approximately 5kb in the 5′ upstream regulatory region of *Ubx* (Fig. 3). As Lawrence (1979) reported, *pbx* homozygotes and hemizygotes displayed complete loss of the transverse rows on the T3 basitarsus and this is consistent with the loss of most *Ubx* expression in the posterior compartment of T3 leg imaginal discs (data not shown). The effects of *pbx* mutations on the T3p femur are more complicated.

In *pbx* hemizygotes and homozygotes, an apparently ectopic row of bristles appears on the lateral posterior femur of T3 together with a sharp column of trichomes (Fig. 4b). These legs carry seven bristle rows, which is equal to the wild-type number of rows. In contrast, large Ubx^{-} clones lead to the derepression of an eighth bristle row (see above). Therefore, *pbx* alleles retain the ability to repress an eighth, lateral row of bristles on the posterior femur. The apparently ectopic row of bristles in *pbx* flies lies in the dorsal-most position of the posterior femur (see below). The bristles in this row probably represent the small bristles in the normal dorsal row that have been transformed to a more robust shape by loss of Ubx^{+} function. Finally, *pbx* mutations lead to the loss of the small bristles normally found on proximal T3p (cf. Figs. 1d with 4b). *pbx* T3p femurs still express low levels of *Ubx* at 24 hours APF, similar to the pattern observed in T2p (data not shown), suggesting that high levels of *Ubx* expression are required to specify these small proximal bristles, whereas low levels are sufficient to repress the eighth bristle row.

Four pieces of evidence indicate that the dorsal boundary between trichomes and naked cuticle in *pbx* mutants and $Minute^{+}$ Ubx^{-} clones reflects the anterior-posterior compartment boundary. First, wild type legs carrying *en-lacZ* demonstrate that cells in the dorsal anterior compartment of the proximal T3 femur differentiate naked cuticle (Fig. 5a). Second, the distribution of Ubx protein in the developing T3 legs of *pbx* hemizygotes reveals only one sharp boundary of *Ubx* expression that is coincident with the anterior-posterior compartment boundary, as revealed by marking the anterior compartment with anti-*Ci* staining, in both third-instar larvae and in 24 hour APF pupae (data not shown). Third, flies hemizygous for *pbx* and simultaneously carrying *en-lacZ* show a sharp boundary of *en-lacZ* staining coincident with the dorsal boundary of trichomes and naked cuticle (Fig. 5b). Fourth, ectopic expression of *Ubx* in T2 leads to repression of trichomes in the dorsal anterior region of T2 (Fig. 5c,d), suggesting that this dorsal anterior region is normally competent in both T2 and T3 to repress trichomes in response to high levels of *Ubx* expression. Together these observations support the hypothesis that in wild type flies, the naked cuticle on the proximal dorsal third femur extends into the anterior compartment, as shown in Fig. 1.

The effect of all *bithoraxoid* (*bxd)* mutations, large inversions that break between the *pbx* deletions and the first exon of *Ubx*, are identical to the effects observed with the *pbx* deletions (data not shown). This includes the allele bxd^{HM}, which breaks close to the 5′ end of the *Ubx* transcript. Therefore, the enhancer(s) controlling expression in T2p are not found upstream of the bxd^{HM} breakpoint.

The anterobithorax region is required for Ubx expression in T3a and Ubx function during early development in T2p and T3p—We examined all existing mutations that disrupt the large third intron of *Ubx*. All effects on the femurs resemble the effects of the anterobithorax (abx) mutations (Fig. 4a), which implies that the region defined by the small deficiency abx^{2} is required for *Ubx* expression in the anterior of the third femur. Patterns of Ubx protein expression in the imaginal discs of late-third instar larvae are consistent with this conclusion (data not shown).

abx mutations tested as homozygotes or in *trans* to deficiencies for the *Ubx* locus had no effect on T3p and derepressed proximal dorsal T3a trichomes (arrowhead in Fig. 4a), consistent with the effects of Ubx^{-} clones in T3a (arrowhead in Fig. 2b). With rare exceptions (discussed below), *abx* mutations had no detectable effect on T2p trichome patterning (data not shown). Thus *abx* is not required for the *Ubx* expression that represses T2p trichomes during pupal development. Simultaneous removal of both the *abx* and *pbx* enhancers transformed T3p into the likeness of T2p, with derepression of distal trichomes, but maintenance of a naked proximal patch on both T2p and T3p (Fig. 4d). This result suggests that the regulatory element or elements that drive *Ubx* expression in the proximal patch of pupal legs are normally active in

both T2p and T3p, but that their activity in T3p is normally hidden below the high levels of *Ubx* expression in T3p driven by the *pbx* enhancer.

Alleles of *abx* also generated a low frequency of posterior T2 femurs transformed towards a posterior T1 pattern, the *postprothorax* (*ppx*) transformation, similar to the penetrance observed by others (Casanova et al., 1985;Peifer and Bender, 1986). Rarely do *ppx* transformations completely transform the leg into a T1-like morphology. In cases of complete transformation, however, the transformation towards T1 is compelling, since the pattern of bristles resembles T1 and the legs show complete loss of naked cuticle in the proximal posterior of T2 (data not shown). This pattern of bristles and trichomes is indistinguishable from that obtained with Ubx^{-} clones generated at 4±2 hours AEL (data not shown). In contrast, late $Minute^{+}$ Ubx^{-} clones show a small region of naked cuticle ventrally and a bristle pattern unlike T1 (Fig. 2c). Most *ppx*-transformed legs caused by *abx* contain small regions displaying a T1 transformation that resemble clonal patches. Combined with the observation that only Ubx^{-} clones generated early in embryogenesis can cause the *ppx* transformation, these results suggest that the partial transformations result either from loss of Ubx^{+} function in a variable number of cells in the blastoderm, leading to derepression of *Scr* in these cells (Little et al., 1990;Struhl, 1982), or from derepression of *Scr* in only some Ubx^{-} cells. The latter model is consistent with the observation that not all blastoderm clones show *ppx* transformation and that some clones show partial transformation (see Fig. 6 of Kerridge and Morata, 1982). We have also confirmed Little et al.'s (1990) report that ectopic *Scr* is observed at low frequency in patches in *abx* hemizygote T2 discs (not shown).

These results indicate that the *abx* enhancer region is required early in development to repress *Scr* in the second posterior femur and is required late for expression of *Ubx* in the anterior third femur. We found no evidence that the *abx* region is required for expression of *Ubx* in the posterior second femur after embryogenesis.

DNA 3′ of the Ubx transcript does not contain a T2p enhancer, but the Cbx^{3} inversion represses T2p function in cis—We examined two alleles containing inversions with breaks near the 3′ end of the *Ubx* transcript: Cbx^{Twt} and Cbx^{3}. These alleles were originally identified because they display a dominant transformation of wing tissue towards a haltere phenotype (Bender et al., 1983). This gain of function phenotype is due to ectopic expression of *Ubx* anterior to its normal expression domain in wing tissue (Gonzalez-Gaitan et al., 1990;White and Akam, 1985). Cbx^{Twt} breaks closer to the *Ubx* third exon than does Cbx^{3}. We did not detect any effect of Cbx^{Twt} on patterning of bristles or trichomes on the second (Fig. 4e) or third femur. Therefore, there is unlikely to be a regulatory region downstream of the *Ubx* transcript that is required for patterning bristles and trichomes on the femurs.

We were therefore surprised to find that the Cbx^{3} allele when homozygous or hemizygous displays a strong loss of naked cuticle on the proximal posterior second femur (Fig. 6b). This allele did not cause appearance of an ectopic row of bristles, suggesting that this allele is not leading to a strong loss of *Ubx* function during larval development in the posterior second femur. In addition, since the Cbx^{Twt} allele does not show this loss of naked cuticle (Fig. 4e), and because we have already excluded most of the region upstream of the *Ubx* transcript, we infer that the Cbx^{3} allele causes this loss of *Ubx* function through dominant suppression of an enhancer found within the region encoding the *Ubx* transcript. This model is supported by the fact that while we observe this loss of function in Cbx^{3} homozygotes and when the Cbx^{3} allele is placed in *trans* to a deficiency for the entire locus (Fig. 6b), we do not observe this loss of function phenotype when the Cbx^{3} allele is placed in *trans* to *Ubx* null alleles caused by point mutations (Fig. 6a). One model to explain this result is that the Cbx^{3} allele causes dominant repression of the T2p enhancer region mainly or entirely on the same chromosome (i.e. in

cis). The recovery of *Ubx* function observed when Cbx^3 is placed in *trans* to null point mutations would result from transvection, or the activation of the *Ubx* promoter on the Cbx^3 chromosome by the T2p enhancer on the chromosome carrying the *Ubx* null allele (Lewis, 1954).

This hypothesis suggests an additional way to search for the T2p enhancer. If Cbx^3 represses the T2p enhancer only in *cis*, then we would expect to observe loss of naked cuticle when Cbx^3 is placed in *trans* to chromosomes carrying a deficiency including the T2p enhancer. In contrast, we would expect to observe a wild-type pattern of naked cuticle when Cbx^3 is placed in *trans* to a deficiency that does not include the T2p enhancer. We have, in fact, observed loss of the T2p naked cuticle when Cbx^3 is placed in *trans* to independent deficiencies that remove the first two introns and part of the third intron, but not when placed in *trans* to deficiencies that do not remove this region (Fig. 6c-g). These results support the hypothesis that the T2p enhancer is located within a region defined by the left-hand break of $Df(3)Ubx^{C1}$ and the right-hand break of $Df(3)Ubx^{50\text{-}1}$ (Fig. 6), a region including mainly the first two introns of *Ubx*.

New deficiencies generated by P-element-induced male recombination fail to uncover an enhancer active in T2p

In order to generate additional deficiencies that might reveal the T2p enhancer, we used P-element-induced male recombination (Preston and Engels, 1996;Preston et al., 1996), using the *rosy*-containing P-element insertion plac(−61) located at the 3′ end of the large candidacy region, within the third intron of *Ubx*. We screened ~6,000 progeny and isolated 63 recombinants of which at least 45 were unique (0.75%). Of these 45, only 5 retained the P-element. At least 77% of our recombinants were associated with precise excisions of the P-element. This result is at odds with a previous study that reported that most male recombination events at cytological position 50C retained the P-element at its original site (Preston and Engels, 1996).

Of the five lines retaining the P-element, two were duplications and three were deficiencies in directions consistent with the Hybrid P-element Insertion model (Preston et al., 1996). Unfortunately, all three deficiencies were in the 3′ direction. Two (132B1 amd 82B1) were *Ubx* null with left-hand breakpoints extending beyond the *Ubx* last exon (Fig. 6). One (33B2) was a small deficiency of ~235bp (Fig. 3). Of the 3 lines that underwent imprecise excision of the P-element, one is a 200bp deficiency that gives a *bx* phenotype (B.1) and two are 5′ deficiencies of ~5–7kb (60B2 and 147A1) (Fig. 3). None of the non-Ubx^- deficiencies have any detectable effect on the T2p trichome pattern either as homozygotes or when heterozygous with a deficiency removing the entire *Ubx* locus.

Enhancer constructs containing most of the remaining candidate regions of *Ubx* fail to drive expression in the pupal legs

None of the alleles discussed above definitively revealed a region controlling trichome patterning on T2p. These results rule out large regions 5′, 3′ and within the large last intron as containing *cis*-regulatory elements required for expression in T2p. This leaves two candidate regions.

The first is a small region between the left-hand break of $Df(3)Ubx^{HC71\text{-}1/HC166D}$ and the right-hand break of Cbx^{Twt}, which includes ~2.8kb of the third intron 5′ of the last exon (Fig 3). We have not explored this 3′ region because none of our functional assays provide any support for the hypothesis that this region contains the leg enhancer. For example, none of the *bx* insertions, which presumably disrupt *abx* activity by introducing insulators between *abx* and the *Ubx* promoter (Peifer and Bender, 1986), disrupt the T2p trichome pattern. This suggests that the T2p enhancer is unlikely to be 3′ of the *bx* insertions.

The second region contains DNA between the bxd^{HM} break and the right-hand break of *Df(3)60B2* or *Df(3)147A1*, which contains ~3.2kb 5′ of the first exon, the first two introns and a portion of the third intron. We focused on this region because the transvection tests with the Cbx^3 allele suggested that the T2p enhancer falls in this region.

We generated six enhancer constructs spanning 31kb, covering the entire large candidacy region except for the first exon (Fig. 3). As reported previously, the construct covering ~3.3kb 5′ of the transcription start site, drives expression in the visceral mesoderm in parasegment 7, part of the wild-type *Ubx* pattern, as well as ectopic expression in the gastric caecae in parasegment 4 (Bienz et al., 1988;Hursh et al., 1993;Irvine et al., 1991;Müller et al., 1989). Other than this pattern, we did not observe any expression patterns resembling wild-type expression during the embryonic, larval or pupal stages for any of the constructs.

DISCUSSION

One major challenge facing the field of evolutionary developmental biology is the identification of the individual nucleotide changes responsible for developmental and morphological evolution. This goal has rarely been achieved. This is largely because most evolutionary changes that have been identified are evolved expression patterns and we currently have a poor understanding of enhancer structure and evolution. We do not yet have reliable methods of predicting the location of enhancer regions and their identification typically requires brute-force empirical methods.

The particular evolutionary change we have studied, a change in the distribution of trichomes caused by an apparently slight shift in the quantity or distribution of Ubx protein, presents several challenges. First, one could explain our previous results with two competing hypotheses. The evolutionary change could have resulted from evolution of an enhancer that drives expression specifically in the pupal legs. Alternatively, the same results could be obtained through a change in the global (that is, non-tissue specific) *cis*-regulation of *Ubx* expression levels at the time when trichomes are repressed in T2p. In this case, the observed morphological change would have resulted from the fact that the extent of trichome repression on T2p is more sensitive to altered *Ubx* levels than other morphological features. We therefore tested whether the global levels of *Ubx* expression driven by the *Ubx* promoter have evolved between *D. melanogaster* and *D. simulans*. We used a method that measures relative expression levels between species due to changes in *cis* while controlling for differences in *trans* (Wittkopp et al., 2004). The relative levels of *D. melanogaster* and *D. simulans Ubx* mRNA were measured in *D. melanogaster*/*D. simulans* hybrid whole pupae. This is effectively a test for any global *cis*-regulatory difference that manifests as a difference in mRNA levels, including differences in the basal or other non-tissue specific promoters of *Ubx*, in portions of *Ubx* affecting transcript stability, or in the *cis*-targets of translational regulation mechanisms that result in mRNA degradation. We found no significant differences in the level of *Ubx* mRNA from the two species in hybrids. We conclude, therefore, that there is no evidence for evolved changes in the global *cis*-regulation of *Ubx* between *D. melanogaster* and *D. simulans* and that the most likely location of the evolutionarily relevant *cis*-regulatory change is in a leg-specific enhancer.

Our second challenge was thus to identify an enhancer driving *Ubx* expression in the pupal legs. We therefore embarked on a multi-pronged analysis to identify the regulatory regions controlling *Ubx* expression in T2p. We first performed a detailed analysis of the requirements for Ubx protein in the legs. This analysis confirmed that *Ubx* is required for repressing trichomes on the posterior second and third femurs. In addition, we discovered that *Ubx* is required to repress trichomes in a small region on the dorsal proximal anterior T3 femur and that a small region on the ventral posterior T2 does not require *Ubx* expression for repression

of trichomes. Finally, we found that *Ubx* is required to repress an eighth row of bristles on the posterior second and third femurs and is required for the *presence* of the fine proximal bristles on T3p and to instruct the morphology and migration of the thin proximo-dorsal bristles on T3. When levels of *Ubx* expression are reduced in T3p by mutations of the *pbx* enhancer, some Ubx^{+} function remains that resembles the activity present in T2p. This weak expression, which can be observed by staining with anti-*Ubx* antibody (data not shown), accounts for the repression of trichomes in the proximal posterior femurs and the repression of the eighth bristle row on the posterior femur. This analysis is summarized in a model shown in Fig. 7 illustrating the requirements for expression driven by the *abx* and *pbx* regulatory regions and by unidentified enhancers required for a weak gradient of expression late in development on the posterior femurs of T2 and T3. The model emphasizes that both spatial and temporal differences in *Ubx* expression distinguish the function of *Ubx* in T2 and T3 (Stern, 1998).

We then attempted to identify the T2p regulatory region by an analysis of all existing alleles of the *Ubx* locus. This analysis confirmed that the *pbx* region is required for *Ubx* expression in the posterior third femur and that the *abx* region is required for expression in the anterior third femur and for expression in the blastoderm cells that will give rise to the posterior second femur. We generated several additional deficiencies in the large intron that also did not have any effect on leg patterning. We found no regulatory mutations that altered trichome patterning on T2p with the exception of Cbx^{3}. This allele is an inversion with a breakpoint 3′ of the last exon of *Ubx*. We do not believe this allele removes a T2p enhancer because a different inversion allele with a breakpoint closer to the last *Ubx* exon (Cbx^{Twt}) does not alter trichome patterning. Instead, we infer that the Cbx^{3} allele causes repression of a T2p enhancer found elsewhere in the *Ubx* locus and that this repression is most effective on the enhancer in *cis*. One observation in support of this conclusion is that null alleles caused by small lesions placed in *trans* to Cbx^{3} drive *Ubx* expression in the correct T2p pattern from the Cbx^{3} allele. This is an example of transvection, or activation of a promoter by a *cis*-regulatory region in *trans*. This inference provided a potential method for narrowing down the region containing the T2p enhancer. We placed the Cbx^{3} allele in *trans* to a series of deficiencies that removed different parts of the *Ubx* locus. When the Cbx^{3} allele was placed in *trans* to alleles that removed the first two introns, the legs showed complete or nearly complete loss of naked cuticle. In contrast, when the Cbx^{3} allele was placed in *trans* to deficiencies that removed mainly the third intron, the naked cuticle looked wild type. This experiment suggested that the T2p enhancer is located between the limits defined by the left-hand breakpoint of $Df(3)Ubx^{C1}$ and the right-hand breakpoint of $Df(3)Ubx^{50\text{-}1}$.

We then assayed almost the entire region defined by these breakpoints using enhancer constructs. We found that none of these constructs possessed enhancers that, on their own, are capable of driving expression of a reporter gene in the posterior epidermis of the second femur. There are several possible explanations for our failure to find the T2p enhancer. One trivial explanation is that the enhancer lies in one of the several small regions we have not yet surveyed, including the first and last exons and a 2.8kb region 5′ of the latter. There is little possibility that enhancer elements required for T2p expression exist upstream or downstream of the *Ubx* transcript or in the portion of the third exon that we were able to test with deficiencies (Fig. 3). Another possibility is that the enhancer for T2p does exist in the area of the first two introns, but is complex such that the transcription factor binding sites that drive this expression are scattered across a region larger than any of our individual enhancer constructs (Klingler et al., 1996). A final possibility is that the enhancer for T2p is dependent for its function on its presence within the *Ubx* locus and cannot be identified by removal from this context.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Michael Akam, Marion Rozowski, Henrique Teotonio and several anonymous referees for helpful comments on earlier drafts of this manuscript. Genomic clones were kindly provided by Michael Akam. Fly stocks were kindly provided by Ed Lewis, Michael Akam, Antonio Garcia-Bellido, Welcome Bender, and the Bloomington and Umeå stock centers. We thank Rita Pasini for *Drosophila* injections of construct 3118int, Rob White for providing the FP3.38 antibody, and Robert Holmgren for the anti-*Cubitus interruptus* antibody. We thank Andrew Clark for use of Pyrosequencing equipment and supplies. This research was supported by a NRSA Fellowship to GKD (GM69102), a Damon Runyon Cancer Research Postdoctoral Fellowship to PJW (DRG # 1795-03) and a Junior Research Fellowship from Churchill College, Cambridge, a David Phillips Research Fellowship from the Biotechnology and Biological Sciences Research Council of the U.K., a grant from The National Institutes of Health (GM063622) and a David & Lucile Packard Foundation Fellowship to DLS.

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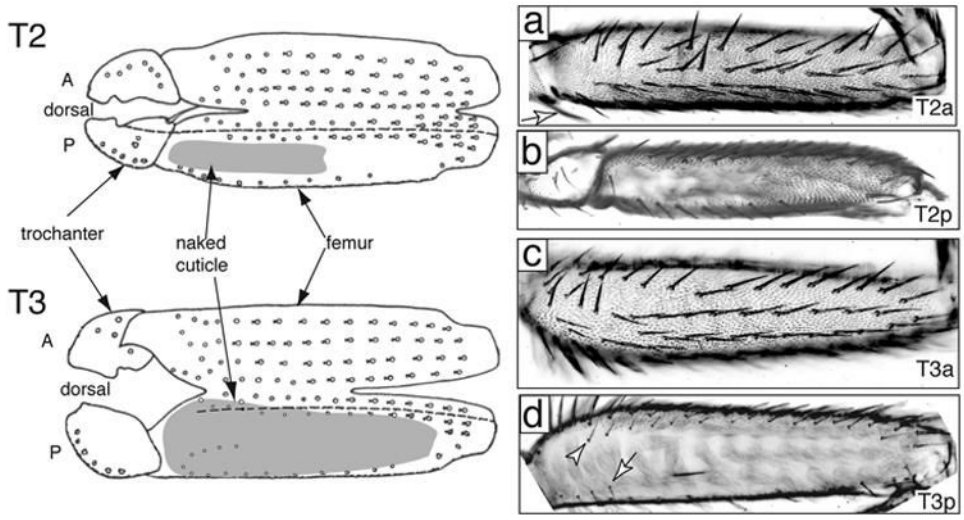

Fig 1.

Wildtype mesothoracic (T2) and metathoracic (T3) femurs. A diagrammatic representation of the legs is shown to the left, with the legs split open along the ventral boundary of the anterior/posterior compartment (modified from Steiner 1976). Anterior and posterior compartments are labelled with A and P and are separated by a dashed line. Regions of naked cuticle are shaded gray. The photographs are oriented in the same way as the diagram: in (a) and (c) ventral is up and in (b) and (d) dorsal is up. (A) Anterior T2 femur has five rows of bristles and is completely covered in trichomes. (B) Posterior T2 has one row of bristles dorsally and one ventral row that is incomplete distally. A proximal lateral patch of naked cuticle varies in size between different strains. The remainder of the posterior surface is covered with trichomes. (C) Anterior T3 has five rows of bristles and is covered in trichomes over all but a dorsal proximal region (see D). (D) Posterior T3 has one row of bristles ventrally, several fine bristles in the proximal region (arrow), and a dorsal row of small bristles. Most of the surface is naked, and a small distal region normally produces trichomes. The dorsal anterior-posterior compartment boundary lies approximately along the dorsal bristle row (arrowhead) and does not reflect an obvious morphological discontinuity. The dorsal boundary between trichomes and naked cuticle extends into the proximal anterior dorsal region.

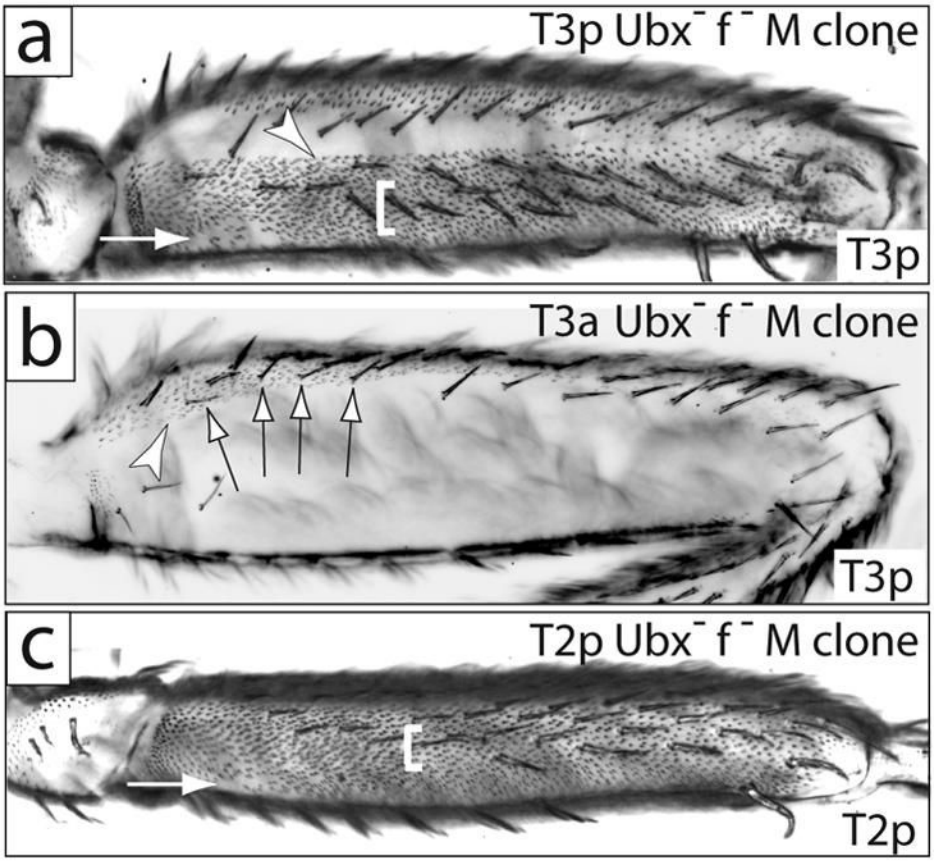

Fig 2.

Ubx^{+} represses most of the trichomes and one lateral bristle row on posterior T2 and T3. (a) Posterior of a T3 femur carrying a *Minute*$^{+}$ *Ubx*$^{-}$ clone that fills the posterior compartment. A small patch of naked cuticle is found proximal-ventrally (arrow). Trichomes differentiate throughout the rest of the posterior compartment. Naked cuticle in the anterior compartment (dorsally) is unaffected. The fine bristles normally found in the proximal lateral region are repressed, and the dorsal bristles are apparently transformed into more robust bristles. Finally, an ectopic, eighth row of bristles differentiates laterally (white bracket). All *Minute*$^{+}$ *Ubx*$^{-}$ clones found on the posterior T3 exhibited a sharp dorsal boundary between derepressed trichomes and naked cuticle (arrowhead). (b) A view of the posterior and dorsal anterior of a femur with a *Minute*$^{+}$ *Ubx*$^{-}$ clone that fills the anterior compartment. Trichomes differentiate in the normally naked cuticle region of the anterior dorsal femur (arrowhead). The small bristles in the dorsal posterior compartment are unaffected by the clone and are *forked*$^{+}$, indicating that they originated in the posterior compartment (arrows). (c) T2 posterior femur carrying a *Minute*$^{+}$ *Ubx*$^{-}$ clone that fills the posterior compartment. Trichomes differentiate throughout most of the posterior femur except for a small patch of cuticle proximal-ventrally (arrow). An ectopic, eighth row of bristles is indicated with a white bracket. Clones in (a) and (c) are marked with *forked* bristles both dorsal and ventral to the small ventral naked patches.

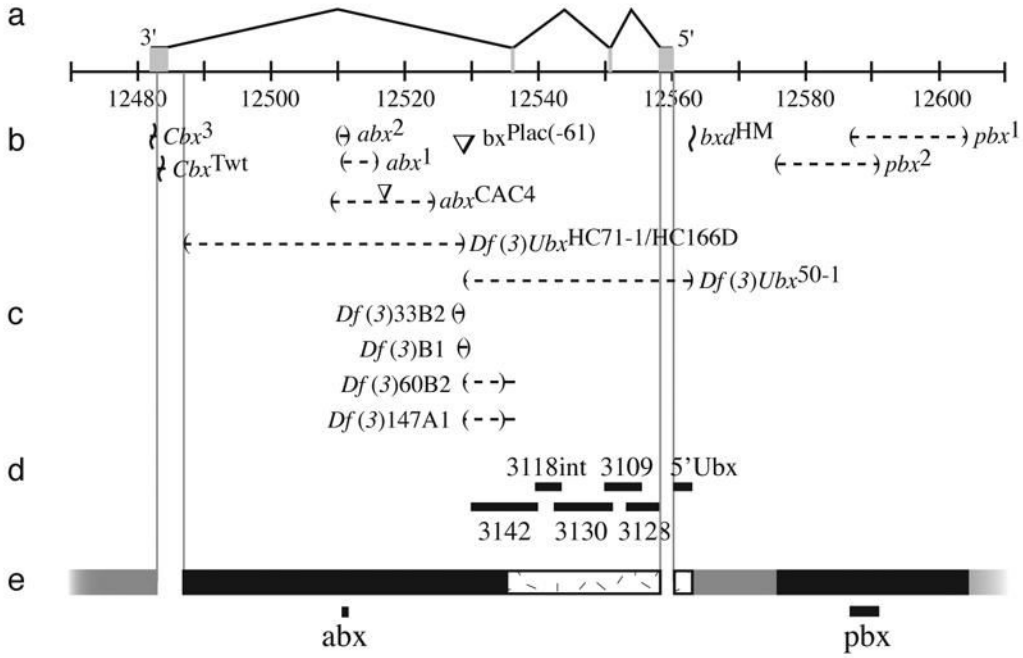

Fig 3.

Map of the *Ubx* locus. (a) The four exons are illustrated above a scale showing genomic positions in kilobases (*Drosophila melanogaster* Genome Release 4.3). The centromere is to the left. (b) The most informative alleles used in this study are shown beside symbols indicating the type of lesion. Deletions are indicated by parentheses separated by a dashed line, insertions by inverted triangles (not to scale), inversion breakpoints by horizontal lines bisected by a wavy line. Uncertainty in the location of breakpoints is indicated by the range of the solid horizontal lines. (c) Four new deficiencies generated in this study by P-element induced male recombination using the $bx^{Plac(-61)}$ insertion are shown. (d) The positions of the six enhancer constructs are indicated with horizontal bars. (e) Regions scanned with deficiencies are indicated by black bars. Regions scanned by inversion are indicated by grey bars. Regions scanned with enhancer constructs are indicated with stippled boxes. The two minimal regions identified by this scan, the previously identified *abx* and *pbx* regions, are indicated with solid lines at the bottom of the figure. Regions of the *Ubx* gene not scanned by any technique are bounded by light lines extending from the molecular map vertically to the bottom of the figure.

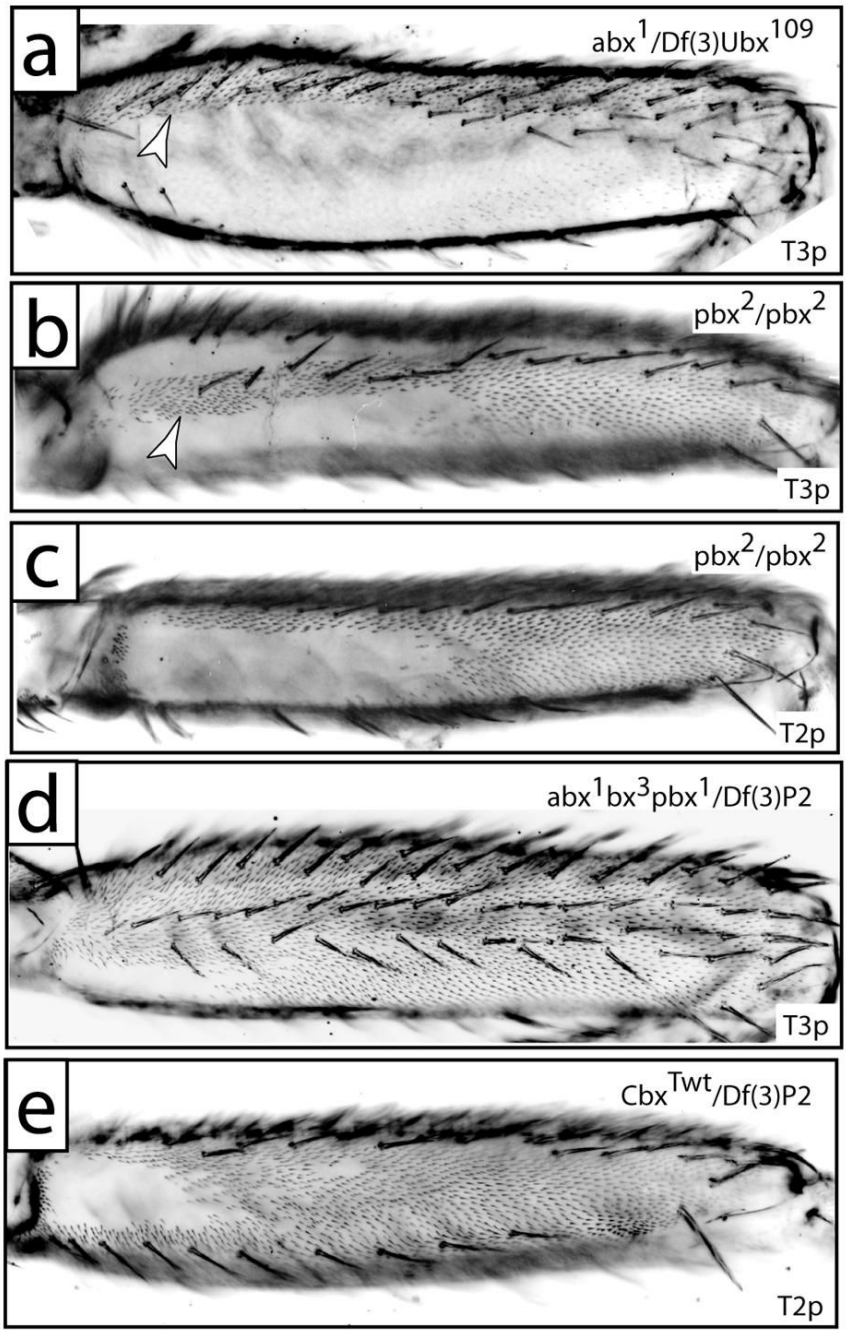

Fig 4.

The posterior femurs of *Ubx* regulatory mutants. (a) T3p from a fly carrying $abx^{1}/Df(3)Ubx^{109}$ displays ectopic trichomes in the dorsal anterior compartment (arrowhead, compare with Fig. 2c). (b) T3p of a pbx^{2} homozygote displays a stripe of ectopic trichomes proximally (arrowhead) and distally. Naked cuticle is observed dorsally and ventrally to the ectopic proximal trichomes. (c) The distribution of naked cuticle on T2p of a pbx^{2} homozygote is approximately equal to the ventral naked cuticle on T3p (b). (d) T3p of a fly carrying $abx^{1}bx^{3}pbx^{1}/Df(3)P2$ displays a trichome pattern that is approximately a composite of the *abx* and *pbx* patterns, with a naked patch of cuticle only on the posterior proximal surface. (e) T2p from a fly carrying $Cbx^{Twt}/Df(3)P2$ displays a wild type distribution of trichomes.

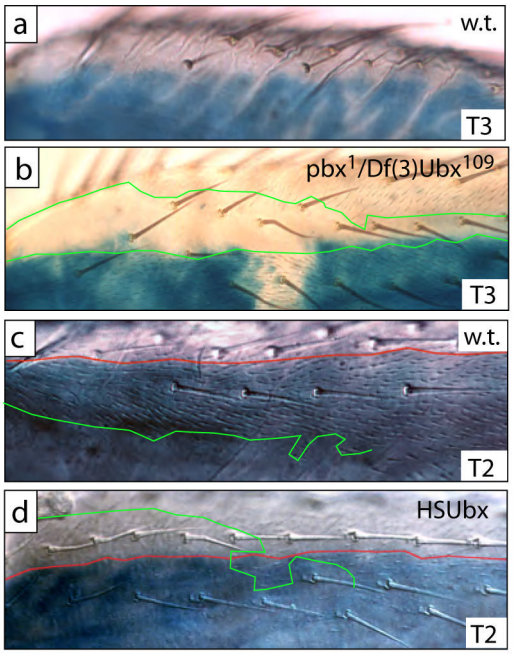

Fig 5.

The boundary of naked cuticle and trichomes does not obey the compartment boundary in T2 or T3, and the proximal dorsal anterior femur of both T2 and T3 is competent to repress trichomes when high levels of *Ubx* are expressed in these cells. (a) In proximal dorsal regions of wild-type T3, naked cuticle is found in both posterior and anterior compartments. Blue *en-lacZ* staining marks the posterior compartment. (b) In a pbx^1 hemizygote naked cuticle is still found in the anterior compartment, but cells in the posterior compartment differentiate trichomes. In this preparation, *en-lacZ* staining failed in a small patch of cells, revealing the faint trichomes. In most specimens this boundary of *en-lacZ* staining is complete and approximately straight, and the ectopic trichomes are only found within the region of *en-lacZ* staining. The boundaries of naked cuticle and trichomes are marked with green lines. (c) In dorsal proximal regions of wild-type T2 legs trichomes differentiate in both the anterior and posterior compartment. The boundary of *en-lacZ* staining is marked with a red line, and the boundary of trichomes is marked with a green line. (d) When Ubx is expressed ectopically at high levels at 24H APF, cells in this dorsal region now differentiate naked cuticle in both the posterior and anterior compartments. The boundary of *en-lacZ* staining is marked with a red line, and the border of naked cuticle is marked with a green line.

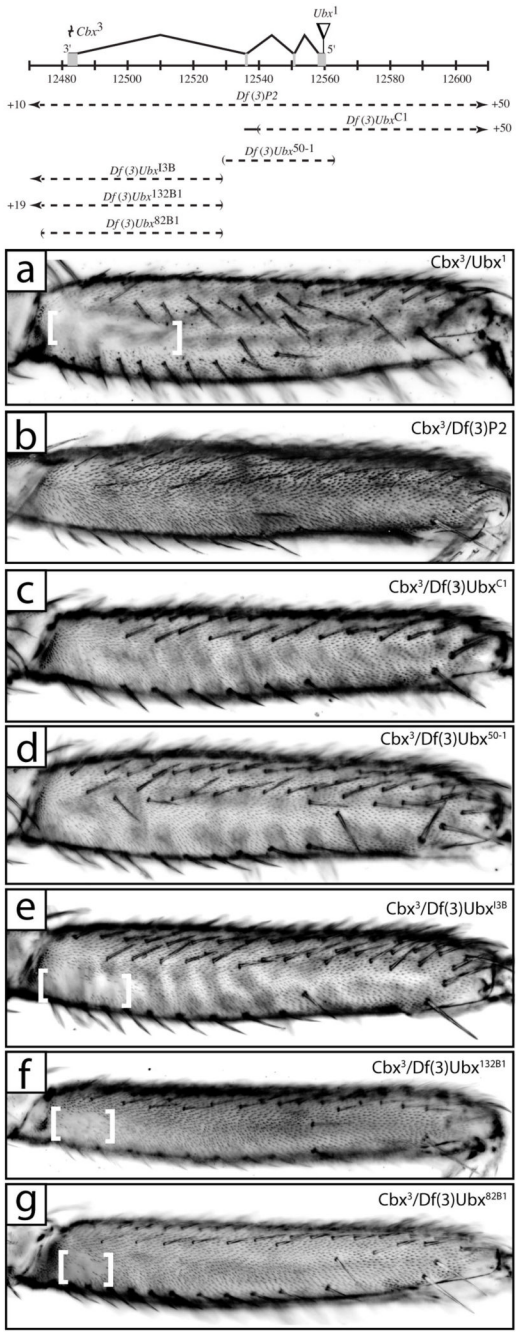

Fig 6.

Cbx^3 causes ectopic T2p and T3a trichomes in a transvection dependent manner. A map of the *Ubx* locus, as in Fig. 3, is shown at the top illustrating *Cbx*^{3} and *Ubx*^{1} lesions above and positions of deficiencies used in transvection mapping below. (a) T2p from a fly with the genotype *Cbx*^{3}/*Ubx*^{1} displays a normal sized patch of naked cuticle. (b) T2p from *Cbx*^{3}/*Df*(*3*)*P2* flies exhibits only a small patch of proximal-ventral naked cuticle similar to that observed in *Ubx* clones (Fig. 2a). (c,d) T2p from flies with the genotype *Cbx*^{3}/*Df*(*3*)Ubx^{C1} (c) and *Cbx*^{3}/*Df*(*3*)Ubx$^{50\text{-}1}$ (d). (e,f,g) T2p from flies with the genotype *Cbx*^{3}/*Df*(*3*)Ubx^{I3B} (e), *Cbx*^{3}/*Df*(*3*)Ubx^{132B1} (f), and *Cbx*^{3}/*Df*(*3*)Ubx^{82B1} (g) show a patch of proximal naked cuticle (between white brackets) indicative of trichome repression due to *Ubx* expression.

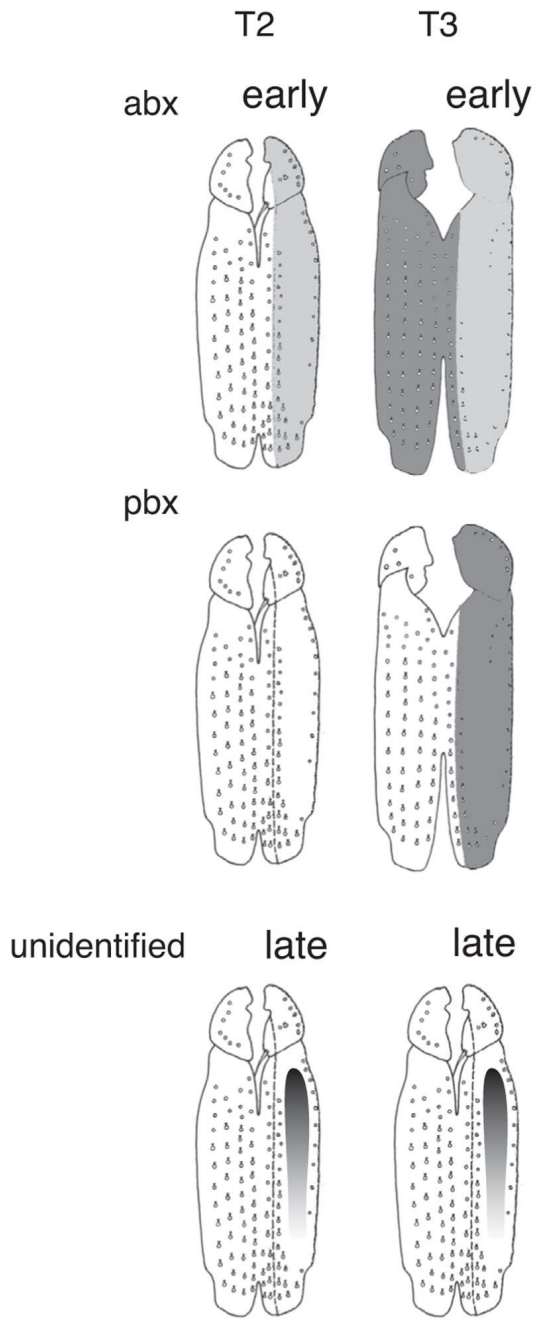

Fig 7.

Domains of *Ubx* regulatory function in the femurs of *Drosophila melanogaster*. Weak expression is illustrated as light gray shading, strong expression as dark gray shading. The *abx* region contains elements promoting early embryonic expression in T2p and T3p and later high levels of expression in T3a. The *pbx* region contains elements required for T3p throughout development. Unidentified regions contain elements driving a proximal-distal expression gradient in T2p and T3p femurs.