Polycomb-dependent Ultrabithorax Hox gene silencing induced by high Ultrabithorax levels in Drosophila

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The Ultrabithorax (Ubx) gene of Drosophila specifies the third thoracic and first abdominal segments. Ubx expression is controlled by several mechanisms, including negative regulation by its own product. We show here that if Ubx expression levels are inappropriately elevated, overriding the auto-regulatory control, a permanent repression of Ubx is established. This continuous repression becomes independent of the presence of exogenous Ubx and leads to the paradoxical result that an excess of Ubx results in a phenotype of Ubx loss. The mechanism of permanent repression depends on Polycomb-group genes. Absence of endogenous Ubx transcription when Ubx levels are highly elevated probably activates Polycomb complexes on a Polycomb response element located in the Ubx major intron. This, in turn, brings about permanent repression of Ubx transcription. Similar results are obtained with the gene engrailed, showing that this mechanism of permanent repression may be a general one for genes with negative auto-regulation when levels of expression are transitorily elevated.

KEY WORDS: Hox, Ultrabithorax, Polycomb, Autoregulation, engrailed

INTRODUCTION

The Hox genes specify the anteroposterior (A/P) axis of bilaterians (Lewis, 1978; Duboule, 2007). They are expressed in defined domains along this axis and their mis-expression frequently causes gross alterations in the body plan. Therefore, Hox gene expression must be tightly regulated throughout development (reviewed by Carroll et al., 2001).

In Drosophila, the expression domains of Hox genes are set in the early embryo by the activity of gap genes (White and Lehmann, 1986; Harding and Levine, 1988; Irish et al., 1988; Casares and Sánchez-Herrero, 1996). After this initial regulation, Hox genes expression domains are maintained by two groups of genes: the Polycomb (Pc) group, which code for proteins that maintain the repression of Hox genes (and other genes); and the trithorax (trx) group, coding for proteins that maintain Hox transcription by preventing Pc silencing (reviewed by Schwartz and Pirrotta, 2007). Pc-group complexes bind to DNA in specific regions called Polycomb response elements (PREs) (reviewed by Müller and Kassis, 2006; Ringrose and Paro, 2007). Hox expression is also regulated by the Hox genes themselves: those expressed more posteriorly along the A/P axis downregulate the expression of those transcribed more anteriorly (Hafen et al., 1984; Struhl and White, 1985). Finally, some Hox genes control their own expression. For example, Deformed maintains its own transcription in cells of the epidermis and central nervous system (Kuziora and McGinnis, 1988; Lou et al., 1995). The opposite effect, negative regulation by its own product, has been described for the Ultrabithorax (Ubx) gene (Irvine et al., 1993).

Ubx expression in the embryonic epidermis extends from parasegment (PS) 5 to PS12. In the larval thorax, Ubx is expressed in imaginal discs of the third thoracic segment (haltere disc and third leg disc) and in the posterior compartment of the second leg disc (Beachy et al., 1985; White and Wilcox, 1985). In this region, Ubx mutations transform the third leg into the second one, and the haltere and metanotum (proximal part of the dorsal metathorax) into wing and mesonotum (corresponding region of the mesothorax), respectively (Lewis, 1963).

Ubx regulates negatively its own expression in the embryonic epidermis and imaginal discs (Irvine et al., 1993; Casares et al., 1997). Thus, increasing the amount of Ubx protein reduces Ubx transcription, and the opposite effect is seen with mutations that reduce its expression (Irvine et al., 1993). Changes in Ubx levels also modify the adult phenotype, as increasing Ubx dose reduces haltere size (Smolik-Utlaut, 1990; Irvine et al., 1993); conversely, heat-shock-induced Ubx expression occasionally brings about a very slight transformation of haltere into wing (Irvine et al., 1993). This transformation is particularly intriguing, as adding more Ubx seems to reduce Ubx activity.

We have studied this surprising result and have found that the increased expression of Ubx produces a strong Ubx mutant phenotype, but only if the high Ubx protein levels are transitorily present in the imaginal disc cells. The transient high Ubx expression causes a Pc-group-dependent permanent inactivation of Ubx, probably owing to the repression of Ubx endogenous transcription. A similar effect is also observed in engrailed (en), a gene required to specify the development of posterior compartments. The mechanism we have uncovered, therefore, seems to be at work in different genes showing negative auto-regulation in Drosophila development.

MATERIALS AND METHODS

Genetics

The Gal4/UAS system (Brand and Perrimon, 1993) was used with the following drivers: MS372-Gal4 (a gift from F. Jiménez), Ubx-Gal4SS2 (A. Sánchez and E.S.-H.), C-765-Gal4 (Guillén et al., 1995), sd-Gal4 (M.C. and G. Morata, unpublished), Ubx-Gal4M4, Ubx-Gal4M5, dpp-Gal4, en-Gal4, ptc-Gal4, ap-Gal4, hh-Gal4 (FlyBase; http://flybase.bio.indiana.edu) and with the following UAS constructs: UAS-Ubx (Castelli-Gair et al., 1994; Michelson, 1994), UAS-Ubx-HA (Ronshaugen et al., 2002), UAS-dsRNA-Ubx (Monier et al., 2005), UAS-ubd-Δ (Michelson, 1994), UAS-Abd-B (m) (Castelli-Gair et al., 1994), UAS-en (Guillén et al., 1995), UAS-UbxU10 (Merabet et al., 2007), UAS-UbxAf (from Artemia

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franciscana) (Ronshaugen et al., 2002) and UAS-Pcl-RNAi (Vienna Drosophila RNAi Center). The Pce1 and trf2 mutations, the Df(1)09 deletion, which eliminates Ubx, and the UAS-GFP reporter construct are described in FlyBase. P-lacZ lines inserted in the Ubx (Ubxlac1) (Casares et al., 1997), abd-A (HC7J41), Abd-B (HC7J99) (Bender and Hudson, 2000) and en (ryxho25) (Hama et al., 1990) were used as reporters of expression of the corresponding genes.

Clonal analysis
Clones expressing Ubx were induced in larvae of the following genotype: y w hs-flp; tub-Gal80s/UAS-Ubx-HA; Ubx+/+ /UAS-flt; y-Gal4. The tub-flt-GFP, y-Gal4 construct has been previously described (Zecca and Struhl, 2002). The Gal80s/Gal4 system (McGuire et al., 2003) was used to control the time of expression of the exogenous Ubx. Clones were marked by the absence of GFP expression and induced according to the following protocol: larvae at 48–72 hours of development, raised at 25°C, were heat-shocked at 37°C for 5 minutes to induce recombination between the FRT sequences, transferred to 29°C for 2 days (to allow Gal4 activity) and then transferred to 17°C to inactivate the Gal4 protein (McGuire et al., 2003). After several days at 17°C, the discs were fixed and scored for clones. In this and similar experiments with several temperature changes, the time of pupation is delayed.

Clones eliminating Pce function in a background in which Ubx permanent repression was established were induced according to this procedure: 24-48 hour larvae of the genotype ds-Gal4 hs-flp; UAS-Ubx tub-Gal80s; Ubx-GFP FRT2A hs-CD2 rP PcXT109 FRT2A, grown at 25°C for 24 hours, heat-shocked for 1 hour at 37°C, grown for 1 day at 29°C and transferred to 17°C for 2 days before fixation. The hs-CD2 rP PcXT109 chromosome is described by Beuchle et al. (Beuchle et al., 2001).

Immunostaining
Imaginal discs were stained according to standard procedures. The antibodies used were mouse and rabbit anti-β-galactosidase (Cappel), mouse Mab4D9 anti-En (Patel et al., 1989), rat anti-haemagglutinin (Roche) and mouse anti-Ubx (White and Wilcox, 1984). Secondary antibodies are coupled to Red-X, Texas Red, FITC and Cy5 fluorochromes (Jackson ImmunoResearch).

In situ hybridization
Haltere discs were hybridized with a Ubx cDNA probe according to standard protocols (Wolff, 2000).

Adult cuticle analysis
Flies were kept in a mixture of ethanol: glycerol (3:1), cooked in 10% KOH at 60°C for 10 minutes, dissected, washed with water, dehydrated with ethanol and mounted in Euparal for inspection under a compound microscope.

X-gal staining and inverse PCR
X-gal staining was carried out as previously described (Wolff, 2000). The P-Gal4 line Ubx-Gal4552 was localized by inverse PCR (http://www.fruitfly.org/about/methods/inverse.pcr.html).

RESULTS
The induction of high Ubx levels in the haltere disc results in absence of the Ubx protein
Ubx represses its own expression in the embryonic epidermis, and in the haltere and third leg imaginal discs (Irvine et al., 1993). This is easily observed when expressing Ubx with the Gal4/UAS system (Brand and Perrimon, 1993) in haltere discs of larvae carrying a Ubx-lacZ reporter insertion, Ubx1lacZ (Casares et al., 1997). In these discs, there is strong reduction of β-galactosidase expression, indicating repression of the endogenous Ubx gene by the exogenous Ubx protein (Fig. 1B, compare with the lacZ expression in Ubx1lacZ discs in Fig. 1A). Flies with increased Ubx expression present, in some GAL4/UAS combinations, a partial transformation of haltere into wing (Fig. 1D,E, compare with the wild type in Fig. 1C).

Fig. 1. Inducing high levels of Ubx eliminates Ubx protein in the haltere disc. Discs are oriented with the anterior compartment towards the left and the ventral compartment upwards. (A) Haltere imaginal disc of a Ubx552/TM6B stock, showing lacZ expression in the disc. (B) In a C765-Gal4/UAS-Ubx Ubx1lacZ+1 haltere disc, lacZ expression is almost abolished and the size of the haltere is reduced. (C) Wild-type haltere. (D,E) Halteres of E132-Gal4/UAS-Ubx (D) and MS372-Gal4/UAS-Ubx (E) flies. Note the increased size, with respect to C, and the appearance of wing bristles and thricomes. (F) In a MS372-Gal4/UAS-Ubx haltere disc, there are patches lacking Ubx protein expression.

weak effect on haltere development has been described after expressing Ubx under heat-shock control (Irvine et al., 1993) but the transformations we observe are much stronger. Other regions of the third thoracic segment (metanotum and third leg) are also transformed into the second thoracic one (see below). By contrast, Ubx expression driven by other Gal4 lines do not transform the halteres into wings but reduce their size. This is similar to what has been previously described (Smolik-Ultlaut, 1990; Irvine et al., 1993), and suggests that there may be some peculiarity in the Gal4 drivers that produce Ubx transformations when expressing Ubx.

These transformations are paradoxical, as providing an excess of Ubx protein results in a phenotype similar to that produced when eliminating the Ubx product. We wondered whether there could be, in addition to the repression of the endogenous Ubx gene, a dominant-negative effect caused by an excess of Ubx protein or whether, by contrast, total Ubx protein levels were reduced. To check this, we stained third instar haltere discs of the MS372-Gal4/UAS-Ubx combination, which produces Ubx transformations (Fig. 1E), with an anti-Ubx antibody. As shown in Fig. 1F, there are patches of cells that lack Ubx expression. Therefore, the Ubx phenotype is due to the absence of the Ubx protein.

High transient expression of exogenous Ubx accounts for the permanent absence of Ubx protein
A question posed by the previous experiments is why only some Gal4 lines elicit Ubx mutant transformations. It could be that these lines are repressed by Ubx. We studied whether this is the case with the MS372-Gal4 line, which drives expression in the haltere disc (Fig. 2A). To distinguish the contribution of endogenous and exogenous Ubx proteins to the Ubx pattern, we monitored the endogenous Ubx gene (Endo-Ubx) with the Ubx1lacZ insertion and the exogenous one (Exo-Ubx) by using an antibody against haemagglutinin (HA) in larvae expressing a Ubx protein tagged with this epitope (Ubx-HA) (Ronshaugen et al., 2002). Several
High Ubx levels permanently repress Ubx

conclusions can be drawn when analyzing UAS-GFP/+; MS372-Gal4/UAS-Ubx Ubx\textsuperscript{act} haltere discs (GFP in green in A) correlates with the absence of Endo-Ubx, detected by the expression of Ubx\textsuperscript{act} (A', grey scale). Merged image in A". The inset indicates a region without GFP, Ubx or Endo-Ubx expression. (B-B') Exo-Ubx and Endo-Ubx expression in UAS-Ubx-HA+/; MS372-Gal4 UAS-GFP/Ubx\textsuperscript{act} haltere discs. GFP is shown in B (in green), the Exo-Ubx protein is detected with an antibody against the haemagglutinin (HA) epitope (B, in red) and the Endo-Ubx with an antibody against the \( \beta \)-galactosidase protein (grey scale in B'). Merged image in B". Note the coincidence of Exo-Ubx and GFP expression but not of Endo-Ubx and the absence of Endo-Ubx in many cells, probably owing to the long stability of the \( \beta \)-galactosidase protein. The inset indicates a region without GFP, Exo-Ubx or Endo-Ubx expression.

(C) Ubx expression in a tub-Gal80\textsuperscript{D}+; dpp-Gal4 UAS-GFP/UAS-Ubx haltere disc transferred from 17 to 29°C and then back to 17°C during the larval period. Ubx is absent in most of the anterior compartment. Discs are oriented with the anterior compartment towards the left and the ventral compartment upwards.

These results are not explained by Ubx needing an exceptionally long time to restore its expression. We used a UAS-daUbxRNA construct (Monier et al., 2005) to prevent Ubx protein synthesis by RNA interference in the ap domain. If the larvae are kept at 29°C, there is no Ubx protein expression in the dorsal compartment (Fig. 3E), but if they undergo our standard protocol, Ubx expression in this compartment is almost completely restored after 4 days at 17°C (Fig. 3F). Such recovery is not observed in ap-Gal4 UAS-GFP/+; UAS-tub-Gal80\textsuperscript{D} larvae that underwent the same temperature changes, not even after 5 or 7 days at 17°C (Fig. 3C-D' and data not shown). We conclude that a mechanism must prevent the restoration of Ubx transcription in the latter case.

In accordance with these results, we observe a different phenotype in halteres of flies expressing Ubx permanently or transiently. In ap-Gal4/UAS-Ubx flies, the halteres are reduced (Fig. 3G), as has been described previously when Ubx levels are increased (Smolik-Ulaut, 1990; Irvine et al., 1993). By contrast, if this increased expression is transient, a transformation of halteres into wing ensues (Fig. 3H). Similar phenotypic effects, under similar experimental regimes, are obtained when we use a scalloped-Gal4 (sd-Gal4) line, which also drives expression in the haltere disc (not shown). These experiments explain the contrasting effects obtained with different Gal4 lines when expressing Ubx. Those that reduce haltere size most probably maintain a fixed domain of expression throughout development, whereas those that show transformations of halteres to wing, like dpp-Gal4 or MS372-Gal4, vary their expression domains with time (Weigmann and Cohen, 1999) (data not shown).
The repression of *Ubx* is maintained cell-autonomously

We wanted to know whether the permanent repression of *Ubx* by its own product is a cell autonomous effect. To this aim, we induced clones expressing transitorily the *Ubx* product and studied exogenous, endogenous and total *Ubx* expression in these clones after several temperature changes (see Materials and methods). We observe in these clones that Endo-*Ubx* expression is continuously repressed in all the cells that previously expressed Exo-*Ubx* (even several days after the exogenous product is no longer present), but not outside it (Fig. 31-L). This suggests that the permanent *Ubx* repression is maintained cell autonomously.

Permanent repression of an *Ubx*-Gal4 line inserted close to a Polycomb response element

The distinct effect of lines with permanent or transient *Ubx* expression has one exception: one *Ubx*-Gal4 line (*Ubx-Gal4SS.2*) produced very strong transformations of haltere to wing (Fig. 4A), metanotum into mesonotum (Fig. 4B) and third leg into second leg (Fig. 4C), even though it drives constant expression in the anterior haltere disc (Fig. 4D). By contrast, when we expressed *Ubx* under the control of two other *Ubx*-Gal4 lines, *Ubx-Gal4M1* and *Ubx-Gal4M3*, the only effect we saw was a reduction of haltere size (not shown). When we co-expressed GFP and *Ubx* with the *Ubx-Gal4SS.2* line, we observed coincident absence of both proteins in large areas of this disc (Fig. 4E-G), indicating there is repression of both *Ubx* and the Gal4 driver. By contrast, in most UAS-GFP/+; *Ubx-Gal4M1*/+ and *Ubx-Gal4M3*/+ UAS-*Ubx* haltere discs, grown for 2 days at 29°C during the larval period, there was strong GFP and *Ubx* expression in the pouch (not shown).

We guessed that the different effect of the *Ubx-Gal4SS.2* line may depend on its location within the *Ubx* gene. *Ubx-Gal4M1* and *Ubx-Gal4M3* have been mapped upstream and close to the *Ubx* transcription start site (de Navas et al., 2006). By contrast, we have located the *Ubx-Gal4SS.2* insertion to position 274.277 (coordinates according to Martin et al., 1985), very close to the Polycomb response element (PRE) of the *bithorax* (*bx*) region of *Ubx* (Orlando et al., 1998; Ringrose et al., 2003; Papp and Müller, 2006; Beisel et al., 2007) (Fig. 4H). We suspect that the particular position of the *Ubx-Gal4SS.2* insertion may account for its different morphological effect when expressing *Ubx*.

The permanent repression of *Ubx* depends on the Pc-group and trx-group genes

The continuous repression of *Ubx* and the location of the *Ubx-Gal4SS.2* insertion (with its particular properties) close to the *bx* PRE suggest that Pc-group genes may be part of the mechanism used for *Ubx* permanent repression. To verify this, we first
examined whether the partial transformation of halteres into wings observed in Ubx-Gal4SS.2 UAS-Ubx flies was modified in a Polycomb or trithorax heterozygous mutant background. In Ubx-Gal4SS.2 UAS-Ubx/Pc3 flies there is a significant reduction in the penetration (and expressivity) of the haltere to wing transformation when compared with the controls. An opposite effect is observed in Ubx-Gal4SS.2 UAS-Ubx/trx2 flies (Fig. 5A).

A first step in the mechanism leading to Ubx lasting repression is suppression of Ubx transcription. We reasoned that, as the abdominal-A (abd-A) and Abdominal-B (Abd-B) Hox genes downregulate Ubx embryonic expression (Struhl and White, 1985), they may also cause permanent repression of Ubx in haltere discs. In MS372-Gal4/UAS-abd-A haltere discs, there are large areas of Ubx repression that resemble those observed in the same discs of MS372-Gal4/UAS-Ubx larvae (Fig. 6A, compare with Fig. 1F), and Ubx-Gal4SS.2/UAS-abd-A adults show a strong transformation of halteres into wings (Fig. 6B). As Abd-A can also make normal halteres (Casares et al., 1996; de Navas et al., 2006), these results strongly suggest that Abd-A can repress Ubx permanently in haltere discs.

A different result is obtained with Abd-B, as in MS372-Gal4/UAS-Abd-B adults there is no or weak transformation of halteres into wings (Fig. 6C). To study in detail whether Abd-B can permanently repress Ubx, we first studied Ubx expression in ap-Gal4 UAS-GFP/UAS-Abd-B(m); tub-Gal80ts/+ haltere discs of larvae grown at 29°C for 3 or more days. In the dorsal compartment of such discs, Ubx signal disappears or is strongly reduced (Fig. 6D-G). By contrast, if larvae of this genotype are grown according to our standard protocol, and the discs fixed 4 days after the last transfer to 17°C, only a small proportion of haltere discs show patches of cells lacking Ubx protein (Fig. 6H). Consistently, only a few flies in this experiment show transformations of halteres into wings. Our conclusion is that Abd-A and Abd-B can repress Ubx transcription in haltere discs, but that only Abd-A can consistently induce permanent Ubx repression.

Ubx and Abd-A proteins share common protein motifs, like the Hexapeptide (HX) and the Abd-A ones, which the Abd-B protein lacks (Chan and Mann, 1993; Bürglin, 1994; Mann and Chan, 1996). To investigate whether any of these domains accounts for the differences we detected between Ubx and Abd-B, we expressed Ubx proteins lacking either the Hexapeptide (UbxHX) or the Ubd-A (UbxUbdA) domains (Merabet et al., 2007), and analyzed whether there is permanent repression of Ubx. Many Ubx-Gal4SS.2 UAS-UbxHX flies show transformations of haltere into wing and, occasionally, of metanotum into mesonotum (Fig. 6I). Consistently, in ap-Gal4 UAS-GFP/UAS-UbxHX; tub-Gal80ts/+ larvae that went through our standard protocol, we see permanent repression of Ubx signal in the dorsal compartment of the haltere disc (Fig. 6J). By contrast, in Ubx-Gal4SS.2/UAS-UbxUbdA flies, the halteres are not transformed into wings (Fig. 6K) and there is no permanent
repression of Ubx in ap-Gal4 UAS-GFP/UAS-Ubx\textsuperscript{EabdA}; tub-Gal80\textsuperscript{TM6B}+/+ haltere discs that underwent the standard treatment (Fig. 6L). After 5 days at 29°C, we see suppression of the Ubx\textsuperscript{lac1} reporter in most cells of the haltere disc (Fig. 6M) and if the larvae are then transferred to 17°C they give rise to adults with wild-type halteres (not shown). These results suggest that the UbdA domain may not be essential for Ubx repression, but for permanent Ubx silencing. However, the Ubx\textsuperscript{EabdA} protein downregulates, but does not suppress, Ubx\textsuperscript{lac1} expression after 3 days at 29°C (not shown), indicating that in our standard protocol the absence of silencing may be due to lack of complete Ubx repression. We have also observed that expressing the Ubx protein from the crustacean Artemia franciscana, with a different C-terminal domain from that of the Drosophila Ubx protein (Galant et al., 2002; Ronshaugen et al., 2002), also leads to permanent repression of Ubx (in Ubx-Gal4\textsuperscript{EabdA}; tub-Gal80\textsuperscript{TM6B}+/+ UAS-Ubx-Af flies there is transformation of halteres into wings). Out of the domains tested, the UbdA may be the only motif in the Ubx protein required for Ubx permanent repression.

**The engramed gene is also permanently repressed by high levels of its protein**

We have observed similar results to those of Ubx with the engramed gene, which is required to determine the posterior identity of Drosophila segments and is also regulated negatively by its own product in the imaginal discs (Guillén et al., 1995; Simmonds et al., 1995; Tabata et al., 1995). Increased expression of the en gene driven by different Gal4 lines causes transformations of posterior-to-anterior compartments in the notum and the wing resembling those observed in weak en mutants (Guillén et al., 1995; Simmonds et al., 1995; Tabata et al., 1995; Casares et al., 1997).
is probably a compensation mechanism to cope with protein level fluctuations during development, which could interfere with the correct expression of target genes that are highly sensitive to Ubx concentration (Tour et al., 2005). High levels of Ubx in the haltere disc, for example, reduce haltere size (Smolik-Utlaut, 1990). This negative regulation demands a precise control of protein levels, which is of high importance to fly development because, as we have seen, if such fine-tuning does not take place, the two genes are permanently inactivated.

This continuous repression is dependent on the Pc-group of genes. Several publications have demonstrated that transcription through a PRE in regulatory domains of the abd-A or Abd-B genes

Fig. 6. Effect of the Abd-A and Abd-B proteins on Ubx permanent repression in the haltere disc. (A) Haltere disc of a MS372-Gal4/UAS-abd-A larva. Ubx is repressed in a large area of the disc. (B) Ubx-Gal4SS-2/UAS-abd-A adults, showing a strong transformation of the haltere into wing. (C) In UAS-Abd-B/+; MS372-Gal4/+ flies, there is barely an increase in haltere size and only the occasional appearance of some bristles or a small amount of wing tissue. (D-G) An ap-Gal4 UAS-GFP/UAS-Abd-B; tub-Gal800/+ haltere disc, grown for 3 days at 29°C. GFP (green, D) and Abd-B (red, E) are expressed in the dorsal compartment, whereas Ubx (greyscale, F) is repressed. Merged image in G. (H) In ap-Gal4 UAS-GFP/UAS-Abd-B; tub-Gal800/+ haltere discs from larvae that were grown at 17°C after 3 days at 29°C, the repression of Ubx is only occasionally maintained. (I) In most UAS-UbxSS-2/+; UbxGal4SS-2/+ adults, there are transformations of haltere into wing (arrowhead). (K) By contrast, in UbxGal4SS-2/+ UAS-UbxAadult flies, the haltieres are abnormal but they are not transformed into wings. These transformations correlate with Ubx expression in discs that went through the standard treatment. (J-L) In ap-Gal4 UAS-GFP+/+; tub-Gal800/+UAS-UbxSS-2 adult discs (I), there is repression of Ubx in the dorsal compartment but such repression is never observed in ap-Gal4 UAS-GFP+/+; tub-Gal800/+UAS-UbxAadult haltere discs (L) under the same experimental conditions. (M) In ap-Gal4 UAS-GFP+/UAS-UbxAadult; UbxSS-2/+ haltere discs, grown at 29°C for 5 days, there is strong repression of the lacZ gene in most dorsal cells. d, dorsal compartment. Magnification in I and K is the same, but different from that in B and C. Discs are oriented with the anterior compartment towards the left and the ventral compartment upwards.

Fig. 7. Permanent repression of the engrailed gene induced by high Engrailed levels. (A-C) Posterior to anterior transformations in the mesonotum (A) and wing (B) of en-Gal4/UAS-en flies. See the duplicated notum (arrow in A) and the presence of anterior bristles in the posterior wing (C, detail of the square in B). A and P indicate anterior and posterior, respectively. (D) En expression in a wild-type wing disc. (E) En expression in an en-Gal4/UAS-en wing disc is confined to small groups of cells and the posterior compartment is enlarged. (F,G) GFP (F) and En (G) expression in an en-Gal4 UAS-GFP++; UAS-en/+ wing disc is coincidently repressed. (H-K) ap-Gal4 UAS-GFP/en-lacZ; UAS-en/tub-Gal800 wing disc from larvae grown for 5 days at 29°C and transferred to 17°C for 3 days. GFP signal (H, green) is still present (though reduced) in the aperuous (dorsal) domain of the disc, and both En (I, in red) and β-galactosidase (J, greyscale) expression disappear in the posterior dorsal compartment (arrows). There is ectopic β-galactosidase signal in the dorsoventral boundary (arrowhead in J). Merged image in K. d and v indicate dorsal and ventral compartments, respectively. Discs are oriented with the anterior compartment towards the left and the ventral compartment upwards.
results in inefficient \( \text{Pc} \) silencing, ectopic expression of the corresponding gene and homeotic transformations (Bender and Fitzgerald, 2002; Hogga and Karch, 2002; Rank et al., 2002; Schmitt et al., 2005). We assume that the phenomenon we have observed (\( \text{Pc} \)-dependent permanent repression of \( \text{Ubx} \)) makes use of a converse mechanism, that is, assembling of \( \text{Pc} \) complexes at the \( \text{PRE} \) following absence of transcription. There is a \( \text{PRE} \) located in the large \( \text{Ubx} \) intron (Chiang et al., 1995; Orlando et al., 1998) and it was suggested that \( \text{Ubx} \) transcription through this \( \text{PRE} \) may contribute to inactivation of \( \text{Pc} \)-complexes (Papp and Müller, 2006). Our results support this view. If \( \text{Ubx} \) transcription is suppressed, as it is when we force high \( \text{Ubx} \) levels, \( \text{Pc} \)-group complexes may become active at this \( \text{PRE} \) and repress the \( \text{Ubx} \) region (Fig. 8). How \( \text{Ubx} \) transcription may inactivate \( \text{Pc} \)-complexes activity is not clear. It has been proposed that transcription through a \( \text{PRE} \) may prevent binding of the complexes to the DNA (Bender and Fitzgerald, 2002; Hogga and Karch, 2002; Rank et al., 2002; Schmitt et al., 2005). However, recent experiments have demonstrated that \( \text{Pc} \)-group proteins are bound to the \( b\alpha \) \( \text{PRE} \) in haltere discs, where \( \text{Ubx} \) is transcribed (Papp and Müller, 2006). Nevertheless, binding of some of these proteins is reduced in the haltere disc, when compared with the wing disc, suggesting that \( \text{Ubx} \) transcription may reduce this binding (Papp and Müller, 2006). Whether affecting \( \text{Pc} \)-group proteins binding or activation, our results favor the view that \( \text{Ubx} \) transcription is a requisite to prevent \( \text{Ubx} \) silencing and that absence of transcription leads to permanent repression.

The results obtained with the \( \text{Ubx-Gal4}^{\text{SS.2}} \) line, inserted close to the \( b\alpha \) \( \text{PRE} \), may be relevant to this hypothesis. When this line directs expression of \( \text{Ubx} \), there is repression of both \( \text{Ubx} \) and the \( \text{Gal4} \) driver in the anterior haltere imaginal disc. We assume that, following repression of \( \text{Ubx} \) transcription, the \( \text{Pc} \)-group complexes at the \( \text{PRE} \) ‘close’ the chromatin in nearby DNA (reviewed by Müller and Kassis, 2006), thus repressing the \( \text{Ubx-Gal4}^{\text{SS.2}} \) driver (Fig. 8). This contrasts with what happens with other \( \text{Ubx-Gal4} \) lines, located far from the \( \text{PRE} \) (de Navas et al., 2006) and in which the inactivation of the driver occurs more rarely. Our hypothesis is that, in the latter, there is repression of the \( \text{Gal4} \) line by the exogenous \( \text{Ubx} \) product. This repression reduces \( \text{Ubx} \) protein levels, relieves endogenous \( \text{Ubx} \) repression, and the subsequent \( \text{Ubx} \) transcription through the \( \text{PRE} \) prevents \( \text{Pc} \)-mediated silencing. It is possible that \( \text{Ubx} \) does not repress the \( \text{Ubx-Gal4}^{\text{SS.2}} \) line as efficiently as the other \( \text{Ubx-Gal4} \) lines owing to its position close to the \( \text{PRE} \). Alternatively, the \( \text{Pc} \)-group complexes may not completely silence \( \text{Ubx-Gal4}^{\text{SS.2}} \) and \( \text{Ubx-Gal4}^{\text{SS.2}} \) insertions.

Although repression of transcription is a requisite for establishing permanent repression in the \( \text{Ubx} \) gene, it may not be sufficient. We have shown that \( \text{abd}-\alpha \), but not \( \text{Abd}-\beta \), consistently achieve \( \text{Ubx} \) silencing in the haltere disc, although both genes repress \( \text{Ubx} \) transcription. This different behavior of \( \text{Ubx/Abd}-\alpha \) and \( \text{Abd}-\beta \) proteins depends neither on the C-terminal region, which contains a conserved block of glutamines and alanines (Galant et al., 2002; Ronshaugen et al., 2002), nor on the HX motif, but may depend on the presence of the \( \text{UbdA} \) domain. The \( \text{Ubx-UbdA} \) protein can partially transform wings into halteres (not shown) and downregulates wing disc-specific targets (Merabet et al., 2007), but is unable to establish permanent \( \text{Ubx} \) repression under our standard protocol conditions. It is possible that the lack of permanent repression we observe when expressing \( \text{Abd}-\beta \) or \( \text{Ubx-UbdA} \) may be due to these proteins allowing very low levels of \( \text{Ubx} \) transcription, enough to prevent \( \text{Pc} \)-mediated permanent repression. In fact, the \( \text{Ubx-UbdA} \) protein needs to be present for a long time (5 days at 29°C in our experiments) to achieve complete repression of the endogenous \( \text{Ubx} \). Alternatively, the \( \text{UbdA} \) domain may be necessary for the \( \text{Ubx} \) protein to collaborate with the establishment of \( \text{Pc} \) silencing.

If this requirement of the \( \text{Ubx} \) protein to establish \( \text{Pc} \)-dependent permanent repression is true, it may be needed only in structures where \( \text{Ubx} \) transcriptionally active, such as the haltere disc. Obviously, in segments anterior to \( \text{PS5} \), repression of \( \text{Ubx} \) by \( \text{Pc} \)-group proteins cannot depend on \( \text{Ubx} \). In this context, it is relevant to mention a specific case of \( \text{Ubx} \) repression: that occurring in the posterior wing disc. The \( \text{Ubx} \) promoter is ectopically expressed in the posterior compartment of the larval wing disc in mutations (like \( \text{bx} \) or \( \text{abx} \) mutations) that eliminate \( \text{Ubx} \) expression in this compartment (Irvine et al., 1993). This suggests there is \( \text{Ubx} \) early expression in this domain that is subsequently shut off by the \( \text{Ubx} \) protein itself (Irvine et al., 1993) and that the repression is maintained by \( \text{Pc} \)-group genes. We have found there is indeed \( \text{Ubx} \) expression in the posterior compartment of the incipient wing disc (in stage 12 embryos) and that this expression disappears from the wing disc at later embryonic stages (stage 16; see Fig. S2 in the supplementary material). Therefore, in the mature posterior wing disc there is a \( \text{Pc} \)-dependent permanent repression of \( \text{Ubx} \) that was set after a transient \( \text{Ubx} \) protein expression, similar to the mechanism we have shown. Such early expression may confer specific properties to cells that initially express \( \text{Ubx} \). Thus, in mutations that inactivate partially a
High Ubx levels permanently repress Ubx

**References**


Pc-group gene (in Pc/+ heterozygous, for example), ectopic Ubx expression is detected in the posterior compartment of the wing disc much more frequently than in the anterior one (or in other anterior discs) (e.g. Cabrera et al., 1985). This suggests that this compartment is somehow ‘poised’ to activate Ubx when the conditions of repression are reduced. There is probably a state in the chromatin that favors Ubx derepression in such a compartment, and this may be due to the early embryonic expression of Ubx.

The en gene, like Ubx, is negatively autoregulated in imaginal discs (Guillén et al., 1995; Simmonds et al., 1995; Tabata et al., 1995) and is also regulated by Pc-group proteins (Busturia and Morata, 1988; Moazed and O’Farrel, 1992; McKeon et al., 1994). There is a PRE in the en gene located upstream its transcription start site (Kassis et al., 1991; Kassis, 1994) and transcription through this PRE has been reported (Schmitt et al., 2005). We have shown that high levels of En protein permanently repress en expression in the wing disc. Analogous to what we have shown with the Ubx gene, we suppose that En may repress the expression of the transcript(s) running through the PRE (or PREs) and trigger a permanent repression through Pc-group complexes binding to, or being active at, the PRE. Other genes are also negatively regulated by their own products in development, such as Drosophila Distal-less (Gorfinkiel et al., 1997), labial (Chouinard and Kaufman, 1991), Suppressor of hairless (Barolo et al., 2000), brinker (Moser and Campbell, 2005) and trithorax-like (Bermúdez et al., 2007), mouse Six1 (Zhu et al., 2002), and Xenopus goosecoid (Danilov et al., 1998). These examples support the idea that many genes need to maintain stable levels of expression in development, as overriding this control may have deleterious effects. It would be interesting to know whether the mechanism we have described take place also in these genes.

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