Evolutionarily conserved domains required for activation and repression functions of the *Drosophila* Hox protein Ultrabithorax

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Summary

While testing the functions of deletion mutants in the Hox protein Ultrabithorax (Ubx), we found that the embryonic repression function of Ubx on *Distal-less* transcription in limb primordia is highly concentration dependent. The steep sigmoidal relationship between in vivo Ubx concentration and *Distal-less* repression is dependent on the Ubx YPWM motif. This suggests that Ubx cooperatively assembles a multi-protein repression complex on *Distal-less* regulatory DNA with the YPWM motif as a key protein-protein interface in this complex. Our deletion mutants also provide evidence for a transcriptional activation domain in the N-terminal 19 amino acids of Ubx. This proposed activation domain contains a variant of the SSYF motif that is found at the N termini of many Hox proteins, and is conserved in the activation domain of another Hox protein, Sex combs reduced. These results suggest that the N-terminal region containing the SSYF motif has been conserved in many Hox proteins for its role in transcriptional activation.

Key words: Ultrabithorax, *Drosophila*, Hox, Transcriptional activation, Transcriptional repression, Sex combs reduced

Introduction

Hox homeodomain proteins are a family of transcription factors that are instrumental in patterning the anterior-posterior axis in metazoan embryos (Balavoine and Adoutte, 1998; Hughes and Kaufman, 2002; Lewis, 1978; McGinnis and Krumlauf, 1992). One of the best-studied Hox proteins, Ultrabithorax (Ubx), is expressed in a complex pattern in the posterior thorax and anterior abdomen of *Drosophila* (Akam, 1983; Bienz et al., 1988), where it controls a variety of morphological decisions by the application of transcriptional activation or repression activities.

In the visceral mesoderm (VM), Ubx activates the transcription of the *decapentaplegic* (*dpp*) gene in parasegment 7 (Capovilla and Botas, 1998; Manak et al., 1995; Muller et al., 1989; Sun et al., 1995; Tremml and Bienz, 1989), where *dpp* is required for the formation of the second midgut constriction (Immerglück et al., 1990; Reuter et al., 1990). In the epidermis of the embryonic trunk, Ubx activation function is required for the maintenance of the transcription of *teashirt* (*tsh*), a homeotic gene that acts in concert with trunk Hox genes to promote trunk identity (Fasano et al., 1991; McCormick et al., 1995; Roder and Kerridge, 1992). Ubx provides specific segmental identity to parasegment 6, in part by repressing the transcription of another Hox gene, *Antennapedia* (*Antp*) (Carroll et al., 1986; Hafen et al., 1984; Saffman and Krasnow, 1994). In the abdominal ventral epidermis, the Ubx and Abd-A Hox proteins prevent the formation of embryonic limbs by directly repressing the transcription of the *Distal-less* (*Dll*) appendage-promoting gene (Vachon et al., 1992).

Ubx homologs from some evolutionarily distant species can appropriately regulate *Drosophila* Ubx target genes in embryonic assays, suggesting evolutionarily conservation of activation and repression functions in these proteins (Galant and Carroll, 2002; Grenier and Carroll, 2000; Ronshaugen et al., 2002). It is therefore of great interest from an evolutionary point of view to understand which regions in Ubx contribute to its activation and repression functions, and whether they are conserved among other Hox proteins.

Many studies have focused on mapping domains required for Ubx limb repression functions in embryos, which is largely due to the ability of Ubx to transcriptionally repress *Dll* (Vachon et al., 1992). Some of these studies have come to different conclusions. For example, a recent study has provided evidence that the domain encoded in the optional exon, present in Ubx isoforms Ia and Ib, but absent from the isoform IVa, is required for the repression of larval limbs (Keilin's organs) and *Dll* transcription (Gebelein et al., 2002). However, three earlier studies found that Ubx isoform IVa was as effective, or nearly as effective, as the Ib isoform at repressing limbs (Busturia et al., 1990; Mann and Hogness, 1990; Subramaniam et al., 1994).

In order to address such inconsistencies, and learn more about Ubx activation and repression functions, we have performed quantitative assays of Ubx function, and find that the repression activity of Ubx in embryos is highly concentration dependent. Using this knowledge and deletion mutants, we have mapped domains required for the repression and activation functions of Ubx protein. A domain required for transcriptional activation, which includes a variant of the Ser-Ser-Tyr-Phe (SSYF) amino acid motif that is evolutionarily

conserved in many Hox proteins, maps to the N-terminal 19 amino acids. Although the YPWM region upstream of the homeodomain is required for Ubx to repress *Dll* with normal cooperativity, no single deletion abolishes the Ubx repression function. Instead, in combination with other findings (Hittinger et al., 2005), our data suggest that the Ubx protein contains multiple regions that contribute additively to its repression function on embryonic targets.

Materials and methods

Construction of the deletion mutants

The deletions in the UbxIa protein were generated by PCR, by first amplifying two fragments, 5′ and 3′ of the deletion, with 34 bp primers that contained overlapping sequences flanking the deletion. The two fragments were then used as a template for the amplification of the full-length protein containing the desired deletion, using 5′ and 3′ end primers. N-terminal deletions of Ubx and Scr were made with a single primer pairs. All cDNAs were cloned into the pUAST vector (Brand et al., 1994). All primer sequences and further details are available upon request.

Immunostaining and quantitation of the protein expression levels.

Experimental and control embryos were collected and processed simultaneously for immunostaining as previously described (McGinnis et al., 1998), except that Western Blocking Reagent (Roche) was used for blocking. Ubx was detected with FP3.38 antibody (White and Wilcox, 1984); HA-tagged proteins were detected with rat anti-HA antibody (Roche). Embryos were mounted in FluoroGuard Antifade Reagent (BioRad) and unsaturated images of ectodermal staining of early stage 11 embryos were taken using confocal microscope (Leica Microsystems), using identical settings between experimental and control samples. Average levels of pixel intensity were measured in the nascent limb field area in the transgenic embryos and in the corresponding area of the first abdominal segment of the wild-type control, using Leica Confocal software. After subtraction of the background, which was measured in ventrolateral thorax of the same stage wild-type embryos, the ratios between the experimentally induced protein levels and endogenous Ubx protein levels were determined. Scr protein concentration was determined similarly, using rabbit anti-Scr antibody; CrebA protein was detected using rat anti-CrebA antibody (both gifts from D. Andrew).

In situ hybridization and quantitation of the transcription levels

In situ hybridization was performed as described by Kosman et al. (Kosman et al., 2004). The *Dll* antisense probe was made from a 1.4 *Eco*RI cDNA fragment (Cohen et al., 1989), the *AntP1* probe was as described by Bermingham et al. (Bermingham et al., 1990), the *dpp* probe was made from a 3.5 kb cDNA in pNB40 (a gift from E. Bier), the *tsh* probe was produced from BSKSNotI-*tsh* plasmid (Fasano et al., 1991), the *wg* probe was as described by Cohen (Cohen, 1990) and the *fkh* probe was produced from a 1.5 kb pBst-*fkh* plasmid. Quantitation of the transcriptional repression of *Dll* and activation of *dpp* was performed using the histogram function of Adobe Photoshop. The background pixel intensity was measured in the same embryo, in the areas adjacent to the signal, and subtracted from the average signal value.

Curve fitting and analysis

The data points of *Dll* transcriptional repression versus Ubx concentration were processed using GraphPad Prism 4 Software as follows: Ubx concentration values were transformed to logarithmic values, a non-linear regression analysis option was chosen and a sigmoidal dose-response (variable slope,

$Y=Top/(1+10^{(LogEC50-X)^{HillSlope}})$ curve was fitted to the data. The goodness of the fit of the resulting curves, measured as the coefficient of determination (R^2), was 0.97 for wild-type Ubx and 0.96 for UbxΔYPWM.

Sequence alignments

Sequence alignments and processing were performed using ClustalW and Boxshade 3.21 programs available at the Swiss node of EMBnet (http://www.ch.embnet.org).

Results

Ubx limb repression function is highly concentration dependent

Although previous studies have suggested that Ubx function is sensitive to protein concentration (Irvine et al., 1993; Mann and Hogness, 1990; Smolik-Utlaut, 1990), most structure-function assays of Ubx protein function using embryonic ectopic expression have used visual comparisons of unknown accuracy to estimate the amounts of control and experimental protein. To better understand the relationship between Ubx concentration and embryonic limb repression, we generated a series of transgenic lines that contained the UbxIa-coding region (hereafter referred to as Ubx) fused at the 5′ end to a sequence consisting of the UAS GAL4 response element/*hsp70* basal promoter, and at the 3′ end to hemagglutinin (HA) tag codons. When these lines were crossed to either of two different *armadillo*-Gal4 drivers, they produced a range of ectopic Ubx concentrations in the embryonic thorax, as measured by antibody staining for the HA tag (Fig. 1D-F). The expression levels of these ectopic Ubx proteins were measured in the nascent limb primordia of fully germ band-extended embryos (early to mid-stage 11) (Campos-Ortega and Hartenstein, 1985). During this stage, but not afterwards, Ubx is capable of repressing *Dll* transcription and limb development (Castelli-Gair et al., 1994; Gonzalez-Reyes and Morata, 1990). We scored the ability of wild-type Ubx to mediate complete repression of larval thoracic limbs (Keilin's organs), as well as its ability to reduce larval limb size by scoring the number of sensory hairs remaining on rudimentary Keilin's organs.

The relationship between Ubx protein concentration and larval limb elimination is plotted in Fig. 1A. From 0-20% of endogenous protein levels, ectopic Ubx did not eliminate Keilin's organs (Fig. 1A, black curve). However, in the interval where ectopic Ubx increased from 20% to 70% of endogenous Ubx protein levels, there was a switch to a limbless state. The Keilin's organs developing in the presence of low Ubx concentration are not unaffected: even at 20% of the endogenous concentration, Ubx eliminates half of the sensory hairs of these rudimentary limbs (Fig. 1A, red curve). At 50% of the endogenous Ubx level, about 80% of the sensory hairs are eliminated and most Keilin's organs consist of the organ's base with a single sensory hair (Fig. 1A; data not shown).

We next tested whether a similar concentration-dependent relationship existed between Ubx protein concentration and *Dll* transcripts in the embryonic limb fields. In stage 11 embryos, *Dll* is transcriptionally activated in the limb primordia of the three thoracic segments (Fig. 1G). These are the cells that will give rise to the Keilin's organs, and *Dll* is required for the formation of both the base and the sensory hairs of the organ (Cohen et al., 1991). The repression of *Dll* transcription by
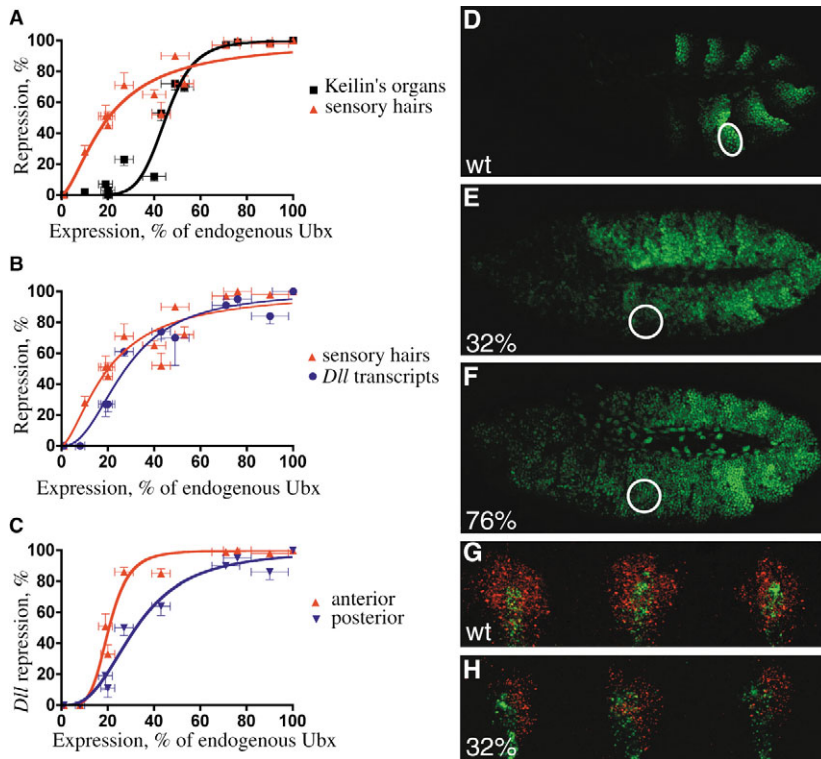
Fig. 1. The repression of larval limbs and *Dll* transcription is highly dependent on Ubx concentration. (A) The repression of Keilin's organs (in black) and the sensory hairs (red) of Keilin's organs as a function of ectopic Ubx concentration in the thorax. Each data point represents a different ectopic expression experiment, within which at least 120 larval limb phenotypes were scored and then averaged. Error bars: standard error of the mean for the limb repression values and 95% confidence intervals for Ubx concentration and *Dll* repression values. (B) The repression of sensory hairs (red) and *Dll* transcriptional repression (blue) plotted against Ubx concentration. (C) *Dll* transcriptional repression as a function of ectopic Ubx levels in the anterior (red) and posterior (blue) compartments of the thoracic segments. (D-F) Ubx protein expression in mid-stage 11 embryos of the following genotypes: (D) wild type, (E) ectopically expressed Ubx at 76% of endogenous levels and (F) ectopically expressed Ubx at 32% of endogenous levels. Ubx protein was detected by staining with FP3.38 anti-Ubx antibody. White ovals indicate the positions where Ubx protein levels were measured. (G,H) Transcripts of *Dll* (red) and *wg* (green) in the limb fields of (G) wild-type embryos, and (H) embryos expressing ectopic Ubx at 32% of endogenous levels. In all figures, anterior is towards the left and dorsal is upwards.

ectopic Ubx is highly concentration dependent, and follows closely the dose-response curve for the repression of sensory hairs (Fig. 1B). The curve that best fits the data points for the Ubx protein concentration-*Dll* transcript repression response has a sigmoidal shape characteristic of cooperative biological regulatory systems in which small changes in concentration trigger an abrupt transition from one state to another (Johnson et al., 1981; Perutz, 1989).

Ubx is a more effective repressor of *Dll* in the anterior compartment of each thoracic segment than in the posterior compartment (Fig. 1C,H). This effect is seen at lower concentrations: at 32% of the Ubx endogenous levels, 85% of *Dll* transcript staining is repressed in the anterior compartment, whereas 57% of *Dll* transcript staining is repressed in the posterior compartment (Fig. 1C,G,H). This is in accord with the compartmental specificities of the DMX *Dll* limb enhancer, which is normally repressed by Ubx protein in the anterior compartment of the first abdominal segment, while the Abd-A protein normally represses the limb enhancer in the rest of the abdomen (Gebelein et al., 2004).

Protein domains required for repression of thoracic limbs

With the above concentration dependence in mind, we tested the larval limb repression functions of eight mutant Ubx proteins (tagged with HA) containing small deletions in regions N-terminal of the homeodomain (Fig. 2A). We placed the borders of our deletions between evolutionarily conserved regions of the Ubx protein sequence (Fig. 2A, see Fig. S1 in the supplementary material). These deletions span over 275 amino acids, covering approximately three-quarters of the Ubx protein. Multiple transgenic lines carrying the mutated forms of UbxIa protein under the control of UAS regulatory sequence
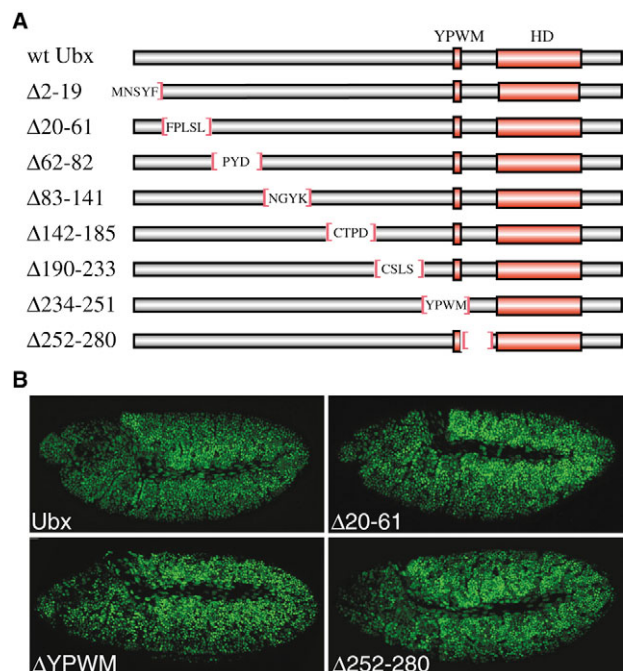

Fig. 2. The Ubx N-terminal region deletions. (A) Diagram of *Drosophila* UbxIa and the eight deletions covering its N-terminal arm. All deletions, except for Δ252-280 (the optional intron region), contained regions highly conserved between Ubx proteins from other arthropod species (see Fig. S1 in the supplementary material). The deletion breakpoints were placed between the conserved regions. (B) Examples of ectopically expressed wild-type Ubx and Ubx deletion mutant proteins (produced at 75-80% of the levels of endogenous Ubx), detected with anti-HA staining in mid-stage 11 embryos.

were generated and crossed to flies carrying *armadillo*-Gal4 drivers. Expression levels of the mutant proteins were compared either directly to the level of the endogenous Ubx in the first abdominal segment (A1) of wild-type embryos, or indirectly, by comparison with a line which ectopically expresses HA-tagged wild-type Ubx at an average of 76% of endogenous levels, and provides 100% limb repression (Fig. 2B). All of the deletion mutants produced proteins that were almost exclusively localized in nuclei, with the exception of UbxΔ2-19, which was slightly defective in this regard. It showed a ratio of nucleus to cytoplasmic protein staining of 3 to 1, so the expression values we report for this mutant have had cytoplasmic levels subtracted.

For some Ubx deletion mutant constructs, we did not obtain transgenic lines that produced the mutant protein at levels identical to endogenous Ubx levels. In these cases, graphical plots of concentration versus limb repression, prepared using a non-linear regression analysis function in the Prism 4 (GraphPad Software) program (Fig. 3B), were used to estimate the repression strength of the mutants at the concentration of the endogenous Ubx. The limb repression strength of the various deletion mutants when expressed at endogenous Ubx concentration levels is shown in Fig. 3A.

The Ubx deletion mutant with the most severe defect in limb repression lacks the YPWM motif and a few adjacent amino acids (UbxΔ234-251). When produced at the levels of

Fig. 3. The YPWM and the 20-61 regions are quantitatively required for Ubx limb repression function. (A) The limb repression values of Ubx deletion mutants when expressed at the level of endogenous Ubx. (B) The limb repression activity of wild-type Ubx, UbxΔ20-61 and UbxΔYPWM deletion mutants as a function of protein concentration. The UbxΔYPWM protein exhibits a flatter concentration-dependence curve of repression activity than wild-type Ubx.

endogenous Ubx, the UbxΔYPWM mutant repressed only 65% of larval limbs (Fig. 3A,B). Even when expressed at 170% of the endogenous concentration, this mutant protein did not completely repress limbs (83% repression, Fig. 3B). The concentration dependence of the UbxΔYPWM-induced limb repression was also notably less steep than is observed for wild-type Ubx (Fig. 3B).

The UbxΔ20-61 deletion mutant also showed a decrease in limb repression function. The 20-61 region contains an YRXFPLXL motif, conserved in all known arthropod Ubx proteins (see Fig. S1 in the supplementary material). At 100% of endogenous Ubx protein levels, this deletion mutant represses 88% of larval limbs (Fig. 3A,B). However, at half of the normal concentration of Ubx protein, UbxΔ20-61 represses only 11% of limbs, sixfold less than the equivalent concentration of wild-type Ubx protein (Fig. 3B). In contrast to UbxΔYPWM, the UbxΔ20-61 mutant still exhibits a steep increase in limb repression ability over a small concentration range, but this range is shifted to higher concentrations than is observed for wild type Ubx (Fig. 3B). A double deletion mutant, lacking both the 20-61 region and the YPWM motif showed an additive defect in limb repression capacity, repressing about 50% of larval limbs (Fig. 3A).

The five other N-terminal deletion mutants were potent repressors of larval limbs when expressed at endogenous Ubx levels (Fig. 3A). They also showed steep concentration dependence curves, although at lower concentrations none repressed limbs quite as effectively as wild type Ubx (data not shown). Although previous research had suggested an important role in limb repression for the alternatively spliced linker region absent in Ubx IVa (Gebelein et al., 2002), our data for UbxΔ252-280 agree with earlier results suggesting that this region is not essential for limb repression (Busturia et al., 1990; Mann and Hogness, 1990; Subramaniam et al., 1994).

The importance of the C-terminal region of Ubx, not covered in our deletion series, was quantitatively assayed by Ronshaugen et al. (Ronshaugen, 2002). In that study, a Ubx mutant without the conserved C-terminal QA motif was expressed at ~80% of the levels of wild-type Ubx, and was found to be 20% less effective at limb repression than wild-type Ubx. We did not pursue a more detailed quantitative analysis of the C-terminal region using ectopic expression assays, as other studies (Hittinger et al., 2005) used allelic replacement to generate a Ubx C-terminal deletion mutant, and found that limb repression activity of the mutant protein was only slightly reduced in embryos.

UbxΔYPWM mutant is an ineffective repressor of *Dll* and *Antp*

We next tested the function of the most defective Ubx deletion mutant, UbxΔYPWM, on two known repression targets of Ubx protein, *Dll* and the *Antp* P1 promoter (Bermingham et al., 1990; Vachon et al., 1992). Wild-type Ubx and UbxΔYPWM mutant proteins were expressed at similar levels (wild-type Ubx 32±5%, UbxΔYPWM 40±4%), and assayed for their ability to repress *Dll* and *Antp* P1 transcripts. Under these conditions, ectopic wild-type Ubx represses ~85% of *Dll* transcript levels in the anterior compartment of the limb field (Fig. 4C). The UbxΔYPWM deletion mutant is a less effective repressor of *Dll* transcription, repressing 57% of *Dll* transcript
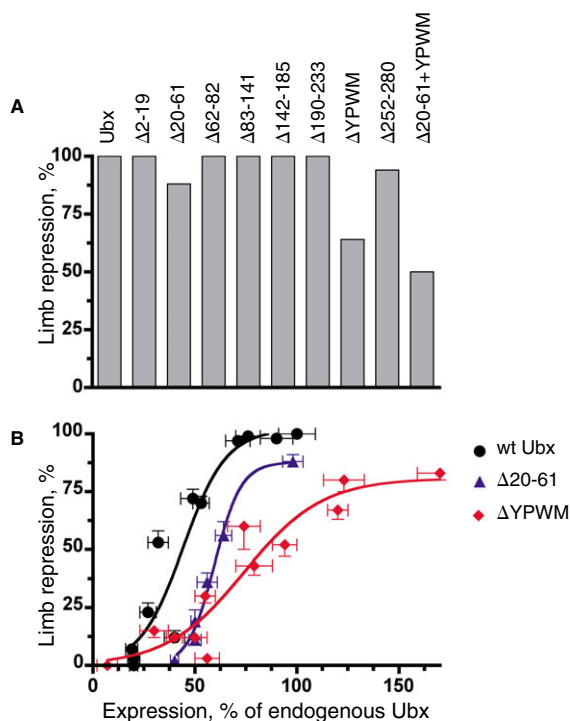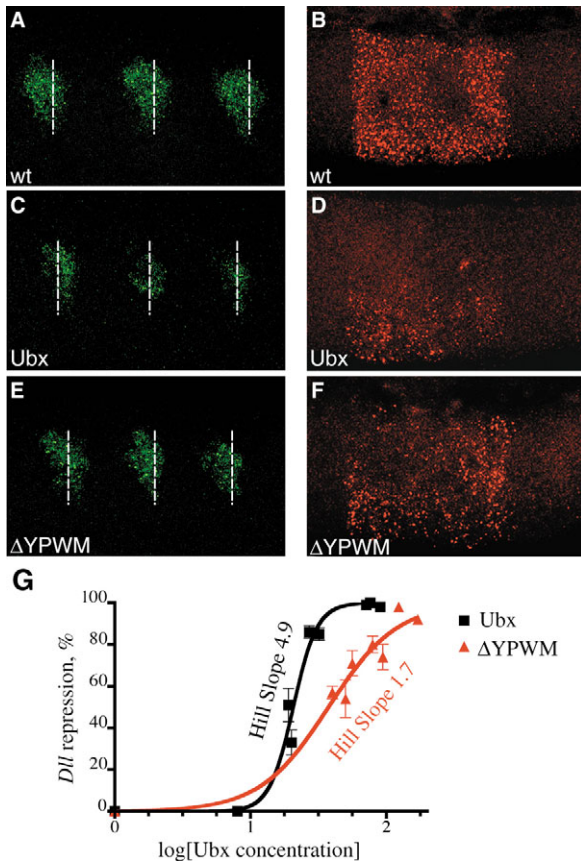
Fig. 4. The UbxΔYPWM protein is a defective transcriptional repressor of *Dll* and *Antp*. (A-F) In situ hybridization of mid-stage 11 embryos, hybridized with *Dll* (green) and *Antp* (red) antisense probes. The broken white lines in A,C,E indicate the posterior boundary of *wg* expression, which was detected in the same embryos (not shown). *Dll* and *Antp* P1 transcripts shown in the thoracic segments of (A,B) a wild type embryo, (C,D) an embryo ectopically expressing wild type Ubx and (E,F) an embryo ectopically expressing UbxΔYPWM protein. (G) A dose-response plot of *Dll* repression as a function of the logarithm of the protein concentration of wild-type Ubx and UbxΔYPWM.

of a 17 amino acid region that includes the YPWM motif results in a Ubx protein with only half to two-thirds of normal repression function on two different downstream target genes.

The Ubx YPWM deletion mutant has decreased repression cooperativity

At wild-type expression levels, the YPWM deletion mutant retains significant limb repression ability, but the curve relating its protein concentration to limb repression is much shallower than for wild-type Ubx. To test whether a similar relationship exists between UbxΔYPWM protein concentration and *Dll* repression, we quantified the repression of *Dll* transcription in the anterior compartments of the thoracic segments of embryos from the transgenic lines expressing a range of ectopic UbxΔYPWM concentrations. Fig. 4G presents these data as a dose-response plot, where *Dll* transcriptional repression is plotted as a function of the log [10] of ectopic protein concentration. For wild-type Ubx, in black, the curve that best fits the data is a steeply rising sigmoid curve. The steepness of the curve can be measured by the Hill slope, which also provides a rough measure of the cooperativity of the repression system. A Hill slope of 1 indicates that the repression system lacks cooperativity, while a Hill slope of more than 1 indicates positive cooperativity. The Hill slope for the wild-type Ubx repression curve is 4.9±2.2 (±two standard errors of the mean). By contrast, the YPWM deletion dose-response curve is much shallower, with a Hill slope of 1.7±0.8. The Hill slopes for wild-type Ubx and UbxΔYPWM curves are statistically significantly different (F test, *P*=0.006), indicating that the repression cooperativity of the YPWM deletion mutant on *Dll* is reduced when compared with wild-type Ubx.

A conserved region required for activation function of Ubx protein

In order to identify the regions required for the transcriptional activation function of Ubx, we assayed the function of the Ubx deletion mutants on two known activation targets of the endogenous Ubx protein, the genes *dpp* and *tsh* (Capovilla and Botas, 1998; McCormick et al., 1995; Roder and Kerridge, 1992; Sun et al., 1995).

Ectopic expression of wild-type Ubx at 100% of endogenous levels induces robust activation of *dpp* transcription in the visceral mesoderm anterior to parasegment 7, as well as in two weaker stripes posterior to parasegment 7 (Capovilla et al., 1994) (Fig. 5B). Although the ectopic expression of the UbxΔYPWM mutant in the visceral mesoderm was at only 60% of endogenous levels, it activated ectopic *dpp* transcription in a pattern and amount indistinguishable from wild-type Ubx (Fig. 5E,F). The UbxΔ20-61 mutant was a poorer *dpp* activator than wild type, inducing no expression posterior to parasegment 7, and 30% lower levels in parasegments 5 and 6 (Fig. 5D,F). This and previous data indicates that UbxΔ20-61 is partially defective in both repression and activation. We conclude that the UbxΔ20-61 mutant has a general defect in gene regulation, perhaps owing to a change in protein structure caused by the deletion.

All but one of the other deletion mutants, including a deletion mutant lacking the conserved C-terminal QA domain (Ronshaugen et al., 2002), produced *dpp* activation levels similar to wild-type Ubx (data not shown). The notable exception to this was UbxΔ2-19, which barely activated *dpp*

levels in the anterior compartment (Fig. 4E). The UbxΔ20-61 protein exhibited a similar defect in *Dll* transcriptional repression (not shown). The other Ubx deletion mutants, including UbxΔ2-19 (which we show later is required for Ubx transactivation function) repressed *Dll* transcription to similar levels as wild-type Ubx, consistent with their strong repression of larval limbs.

The *Antp* P1 promoter is activated in embryonic parasegments 4 and 5 (Bermingham et al., 1990; Martinez-Arias, 1986) (Fig. 4B). Ectopic expression at the indicated levels of wild-type Ubx completely represses *Antp* transcription dorsally and reduces it ventrally (Fig. 4D). Averaged over the entire parasegment 4, this corresponded to repression of 62% of *Antp* transcripts. The UbxΔYPWM mutant was a less effective repressor of *Antp* P1 transcription, partially repressing it dorsally and exerting only slight repression ventrally, resulting in the average repression of 33% of *Antp* transcripts (Fig. 4F). We concluded that the removal

Fig. 5. The N-terminal region of Ubx is required for ectopic activation of *dpp* in visceral mesoderm. (A-E) *dpp* transcripts in the visceral mesoderm of stage 13 embryos. (A) In wild-type embryos, *dpp* transcripts are detected in parasegments 4 and 7 (arrowheads). (B) Ectopic wild-type Ubx activates *dpp* transcripts anterior to and posterior to parasegment 7 (arrows). (C) Ectopic UbxΔ2-19 protein barely activates ectopic *dpp* in parasegments 5 and 6, and represses endogenous *dpp* in parasegments 4 and 7 (arrowheads). (D) Ectopic UbxΔ20-61 protein activates *dpp* transcripts anterior to, but not posterior to parasegment 7. (E) Ectopic UbxΔYPWM protein activates *dpp* transcripts in a pattern and amount indistinguishable to wild-type Ubx. (F) Quantitation of the ectopic Ubx protein levels and *dpp* transcripts in parasegments 5 and 6. Error bars: 95% confidence intervals.
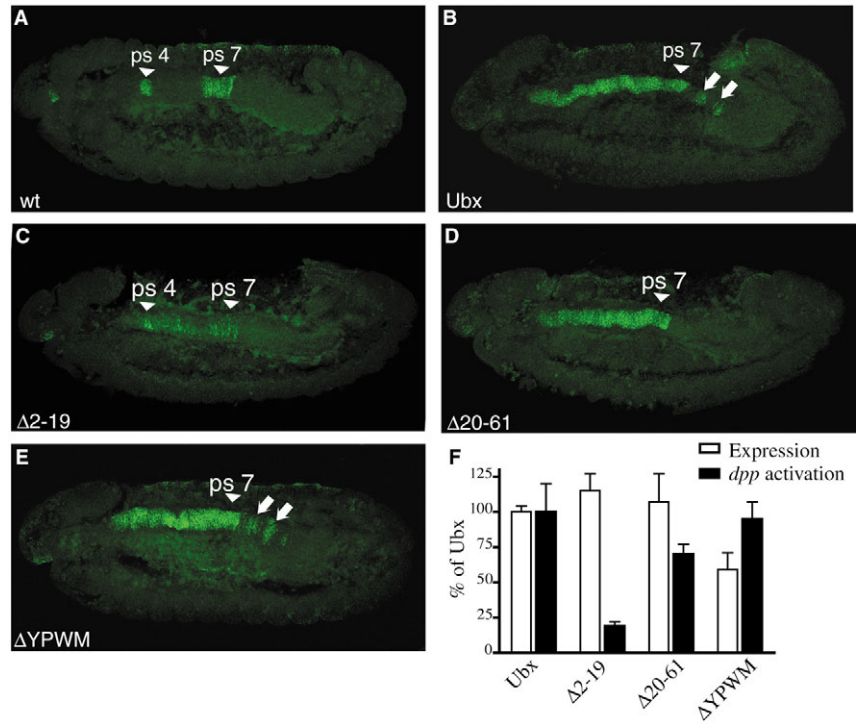
above background levels in parasegments 5 and 6 (Fig. 5C,F). Moreover, the UbxΔ2-19 mutant also repressed transcription of *dpp* in parasegments 4 and 7 to barely detectable levels (compare Fig. 5A with 5C). We concluded that UbxΔ2-19 was a defective activator of *dpp* transcripts, and that the deletion of the Ubx 2-19 region converts it from an activator to a repressor of *dpp*.

To investigate whether the impaired activation function of UbxΔ2-19 was locus-specific, we tested whether this mutant, along with UbxΔ20-61 and UbxΔYPWM controls, could activate *tsh* transcription. Ectopically expressed wild-type Ubx activates *tsh* in the head, including the epidermis of the procephalon, clypeolabrum, mandibular and maxillary segments (McCormick et al., 1995; Roder and Kerridge, 1992)

Fig. 6. The N-terminal region of Ubx protein is required for the activation of *tsh* transcripts in the head epidermis. (A-E) Shown are the head and anterior thorax of late stage 11 embryos, hybridized with a *tsh* antisense probe. (A) In wild-type embryos, *tsh* is transcribed in the epidermis of parasegment 3 (as well as in parasegments 4-13, not shown). (B) Ectopic wild-type Ubx induces *tsh* transcripts in the clypeolabrum (cl), the procephalon (pc), and the mandibular (Md) and maxillary (Mx) segments. (C) Ectopic UbxΔ2-19 activates very low levels of *tsh* transcripts in the pc and cl, and in only a few cells of the Md and Mx segments. (D,E) Ectopic UbxΔ20-61 and UbxΔYPWM proteins activate *tsh* transcripts at similar levels and in similar pattern to wild-type Ubx, but with less uniformity. (F) Quantitation of ectopic protein expression levels and *tsh* transcripts, averaged over the entire head region. Error bars: 95% confidence intervals. (G) Alignment of the N termini of the Ubx proteins from *Drosophila melanogaster* (Dm), *Tribolium castaneum* (Tc), *Porcellio scaber* (Ps) and *Artemia franciscana* (Af). Ten out of the 18 amino acid residues eliminated in the UbxΔ2-19 mutant are identical in the four Ubx homologs.
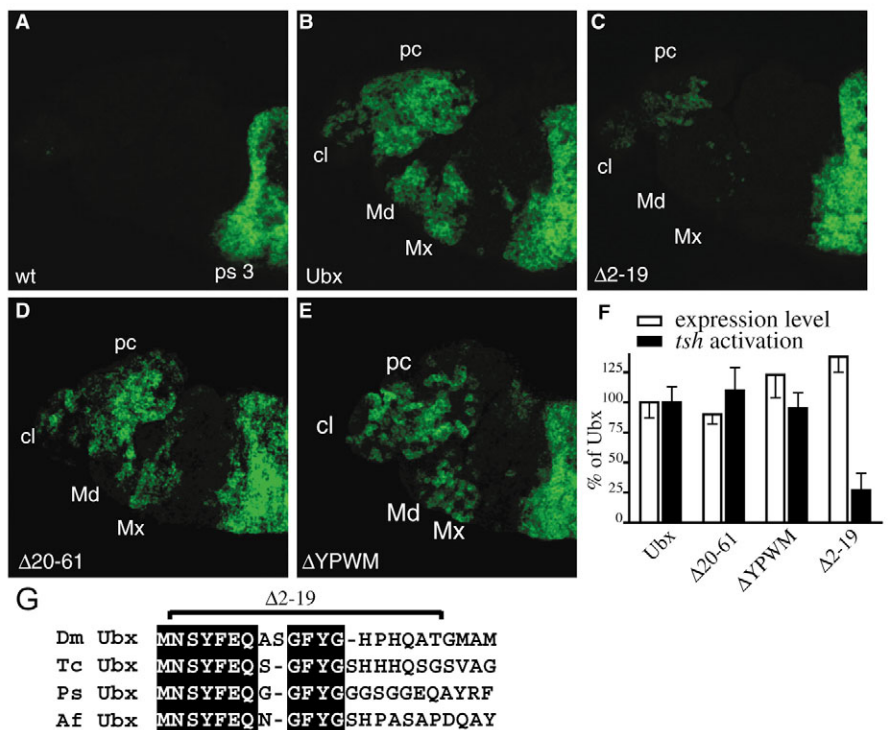
(Fig. 6A,B). Ectopic UbxΔ2-19, although expressed at higher levels than wild-type Ubx, only weakly activated *tsh* in the procephalon, the clypeolabrum and in a few cells of the mandibular and maxillary epidermis (Fig. 6C,F). By comparison, both UbxΔ20-61 and UbxΔYPWM activated *tsh* in similar patterns and at similar levels to wild-type Ubx, albeit in a less uniform fashion (Fig. 6D-F). When averaged over the entire head region, *tsh* activation by UbxΔ2-19 was 27% of the activation produced by wild-type Ubx, even though the UbxΔ2-19 protein was expressed at 138% of wild-type Ubx control levels (Fig. 6F).

Recall that UbxΔ2-19 is a potent repressor of *Dll*. In summary, the evidence indicates that a deletion of amino acids 2-19 results in a Ubx mutant that is specifically disabled in its transcriptional activation function when tested on *dpp*, *tsh* and *Dll*. The amino acid 2-19 region of *Drosophila* Ubx is highly conserved in other arthropod Ubx proteins (Fig. 6G).

The conserved N-terminal region is required for Scr activation function

To test whether the N-terminal region of Hox proteins contains an evolutionarily conserved activation domain, we assayed the function of this region in another Hox protein, Sex combs reduced (Scr). The N terminus of insect Scr proteins also contains an extremely well-conserved region (Fig. 7A) with a significant degree of sequence similarity to the N termini of Ubx and many other Hox proteins (Fig. 7J). To investigate the function of this region, we deleted 17 amino acids, starting with the conserved SSYQFVN sequence (Fig. 7A). Multiple transgenic lines carrying wild-type Scr or its N-terminal deletion mutant (ScrΔSSY) under UAS regulatory element control were generated and crossed to the *armadillo*-Gal4 driver. Expression levels of ectopic wild-type Scr and ScrΔSSY were tested, and lines were selected that ectopically expressed the proteins in the ventral head at levels approximately equal to those of the endogenous Scr protein in ventral parasegment 2 (Fig. 7B).

In wild-type embryos, Scr is required for the formation of salivary glands in ventral parasegment 2 (Andrew et al., 2000; Panzer et al., 1992). It does so by activating a battery of genes, among them genes for the transcription factors Fork head (Fkh) (Panzer et al., 1992) and CrebA (Andrew et al., 1994). Both genes are ectopically activated by ectopic Scr protein, and *fkh* is a direct activation target of Scr (Ryoo and Mann, 1999).

Ectopic wild-type Scr induced robust activation of *fkh* transcription in parasegment 1 (Fig. 7D, arrow). Ectopic *fkh* transcription was also activated in the ventral region of the mandibular segment (Fig. 7D, asterisk) and in the procephalon. Ectopic ScrΔSSY protein was a much weaker activator of ectopic *fkh* transcription, activating it only in a few cells of parasegment 1 and the procephalon (Fig. 7D,E).
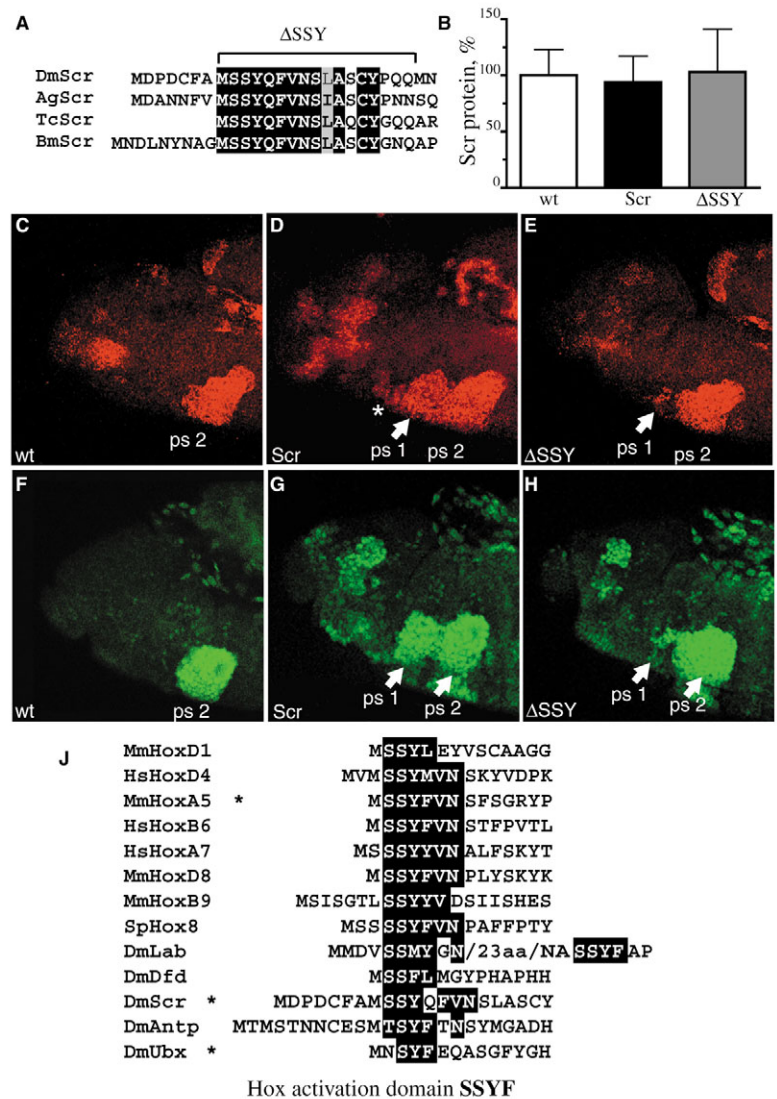
The ScrΔSSY protein was also a defective

Fig. 7. The conserved N-terminal region of Scr is required for the activation of *fkh* and CrebA expression. (A) Alignment of the N-termini of insect Scr proteins [*Drosophila melanogaster* (Dm), *Anopheles gambiae* (Ag), *Tribolium castaneum* (Tc) and *Bombyx mori* (Bm)]. In the region deleted in the ScrΔSSY mutation (bracket), 12 out of 17 amino acid resides are identical. (B) Expression levels of ectopic wild-type Scr (Scr) and the ScrΔSSY mutant (ΔSSY) in ventral parasegment 1 (ps 1), compared with the levels of the endogenous Scr protein (wt) in ventral parasegment 2 (ps 2). Error bars: 95% confidence intervals. (C-E) Anterior regions of mid-stage 11 embryos, hybridized with a *fkh* transcript antisense probe. (C) In wild-type embryos, *fkh* is activated in ventral parasegment 2. (D) Ectopic wild-type Scr activates *fkh* transcripts in ventral parasegment 1, the anterior mandibular segment (asterisk) and in the procephalon. (E) Ectopic ScrΔSSY protein activates *fkh* transcripts in only a few cells of parasegment 1. (F-H) Mid-stage 11 embryos stained with anti-CrebA antibody. (F) In wild-type embryos, CrebA expression is limited to ventral parasegment 2. (G) Ectopic wild-type Scr activates CrebA in parasegment 1 and the procephalon. (H) Ectopic ScrΔSSY protein activates CrebA in only a few cells of parasegment 1. (J) Alignment of the N termini of human (*Homo sapiens*), mouse (*Mus musculus*), sea urchin (*Strongylocentrotus purpuratus*) and fly (*Drosophila melanogaster*) Hox proteins. In all of these proteins, the N terminus conserves an SSYF motif or a subtle variant. Asterisks indicate Hox proteins in which a requirement of the N-terminal region for transcriptional activation in embryos has been demonstrated.

activator of the *CrebA* gene. Ectopic wild-type Scr induced abundant ectopic expression of CrebA protein in parasegment 1 (Fig. 7G, arrow). In addition, patches of CrebA expression were activated in the procephalon and the ventral head area. The ScrΔSSY deletion mutant induced only a small patch of ectopic CrebA expression in the posterior portion of parasegment 1 (Fig. 7H, arrow), and ectopic activation was also reduced in the procephalon and the ventral head (Fig. 7H).

Discussion

An evolutionarily conserved Hox transactivation domain

Our results suggest that many Hox N-terminal regions possess a conserved transcriptional activation domain that includes an evolutionarily conserved SSYF motif (Fig. 7J). This region was required for the *Drosophila* Ubx and Scr proteins to activate four different downstream target genes with differing tissue-specific expression patterns. In Ubx, this domain is not just required for general functional activity, as the deletion of Ubx N-terminal sequences dramatically reduces transcriptional activation function, but has no influence on repression function. In fact, the deletion of the region containing the Ubx variant of the SSYF motif (NSYF) appears to convert it from an activator to a repressor of *dpp* transcription.

The most relevant previous work on Hox N-terminal function in *Drosophila* embryos involved tests of mouse HoxA5 deletion mutants (Zhao et al., 1996). The authors found that multiple regions N-terminal to the homeodomain were required for HoxA5 to activate a *forkhead* promoter-reporter gene. One of the required regions included amino acid residues 2-39, and the authors proposed this region might be required for activation function or co-factor specificity. Similarity of Hox protein N-terminal sequences in *Drosophila* and mammals has been long noted, and is a characteristic of Hox proteins from a wide variety of animal species (Martinez et al., 1997; Schughart et al., 1988; Zhao et al., 1996). In both mammal and *Drosophila* Hox proteins, the core conserved motif in this N-terminal region is a Ser-Ser-Tyr-Phe (SSYF) amino acid sequence (Fig. 7J).

We do not yet know the mechanism through which the Hox SSYF activation domain operates: it may interact with DNA-binding transcription factors dedicated to transcriptional activation or with co-activator protein complexes (Glass et al., 1997). One possible SSYF interactor is the histone acetyltransferase CBP (CREB-binding protein) (Chan and La Thangue, 2001). Mutations in the *Drosophila CBP* gene were found to be dose-sensitive modifiers of *Deformed* and *Ubx* biological function (Florence and McGinnis, 1998). In addition, CBP was found to increase the transactivation activity of human HOXB7 protein in breast cancer cells and to interact with the N-terminal region of HOXB7 in GST pull-down assays, in a manner that required the presence of the first 18 N-terminal amino acids of HOXB7 (Chariot et al., 1999b). In another study, mammalian CBP was shown to interact with the first 141 N-terminal amino acids of human HOXD4 in co-immunoprecipitation assays, and to increase transactivation activity of HOXD4-PBX complexes on a synthetic element containing five HOX/PBX sites in cultured human embryonic kidney cells (Saleh et al., 2000). Another possibility is that the

N terminus interacts with the IκBα protein, which binds to the N-terminal regions of human HOXB7 (Chariot et al., 1999a), a region of HOXB7 that is required for normal function in a murine myelomonocytic cell line (Yaron et al., 2001).

A detailed analysis of Ubx domains required for transactivation function in *Drosophila* cultured S2 cells, which are derived from embryonic hemocytes (Armknecht et al., 2005), was carried out recently by Tan et al. (Tan et al., 2002). In their assays, the N-terminal 67 amino acid residues were not required for Ubx-dependent transcriptional activation. The disparity between our results and those from Tan et al. (Tan et al., 2002) might be explained by the different assay systems (cultured S2 cells versus embryos), the different target elements, and/or the exact size and extent of the deletion mutants that were tested.

Cooperativity in Ubx transcriptional repression function

Our results indicate that at least for its limb and *Dll* repression functions, Ubx contributes to a cooperative on/off switch over a small concentration range. When *Dll* repression is plotted as a function of Ubx concentration, the best-fit curve has a Hill slope of 4.9±2.2. These results suggest a highly cooperative assembly of a multiprotein repression complex containing Ubx on *Dll* regulatory DNA. Although our repression dose-response curves cannot be extrapolated into the number of cooperative protein-protein interactions within a repression complex, they are a surprisingly good fit to the model of Gebelein et al. (Gebelein et al., 2004). In this model, the Ubx-mediated repression of a *Dll* limb enhancer requires at least five clustered DNA sites that cooperatively bind two molecules of Ubx, Extradenticle (Exd) and Homothorax, while the fifth site binds the Sloppy paired 1 protein (Gebelein et al., 2004). The high sensitivity of Ubx phenotypes to concentration may explain why previous experiments using ectopic expression of Ubx have come to different conclusions, and illustrates why the validity of conclusions from ectopic expression studies should be interpreted with caution, unless great care is taken to achieve near-normal physiological levels.

Why is the Ubx repressive effect on *Dll* so concentration sensitive? It is instructive to look at other biological systems with similar concentration-dependent transcriptional switches. For example, the steep concentration dependence of the lambda transcriptional repressor allows prophages in *E. coli* cells to switch, at crucial levels of cellular distress, from one stable state to another, lysogenic to lytic (Johnson et al., 1981). For Ubx, one likely reason for the highly concentration-dependent effects on *Dll* expression and limb development is to ensure that all the cells in a limb field are stably programmed to adopt either the limb state, or body wall fate. At least in extant *Drosophila*, a mosaic appendage that developed from a mixed field of limb and body wall cells would presumably be little benefit to the animal that carried it, and thus selected against during evolution.

Cooperative repression and the Ubx YPWM region

Tests of mutant Hox proteins in *Drosophila* and in mice have demonstrated the importance of the YPWM motif for Hox function in vivo, although both loss- and gain-of-function phenotypes were observed (Chan et al., 1996; Galant et al., 2002; Medina-Martinez and Ramirez-Solis, 2003; Merabet et

al., 2003; Remacle et al., 2004; Zhao et al., 1996). In vitro, the YPWM region has been shown to mediate Hox interactions with the PBC family of homeodomain proteins (Chang et al., 1995; Johnson et al., 1995; Knoepfler and Kamps, 1995; Neuteboom et al., 1995; Passner et al., 1999; Phelan et al., 1995; Piper et al., 1999; Shanmugam et al., 1997). The PBC proteins (Exd protein in *Drosophila*, Pbx proteins in mammals) bind cooperatively with Hox proteins on composite DNA sites, and are important co-factors in the regulation of many Hox target genes (Featherstone, 2003).

Galant et al. (Galant et al., 2002) found that a Ubx protein with a YAAA substitution for YPWM exhibited reduced cooperative binding with Exd on a consensus composite Ubx-Exd DNA-binding site. Reduced affinity between UbxΔYPWM and Exd might compromise the assembly of the entire repression complex proposed by Gebelein et al. (Gebelein et al., 2004), resulting in an inefficient transcriptional repression of *Dll* in the anterior segmental compartments.

Our in vivo results are also consistent with models in which the YPWM region contributes in other ways to repression cooperativity. For example, the YPWM region appears to influence Hox activation and repression functions in a manner that is independent of its role in enhancing the affinity of Hox/PBC protein complexes for binding sites (Chan et al., 1996; Merabet et al., 2003). In vitro, Ubx is also known to bind cooperatively to DNA in homomeric complexes (Beachy et al., 1993), and the YPWM motif might be required for the formation of such complexes on *Dll* regulatory sequences.

No single deletion abolishes the Ubx repression function, although some regions are required for robust repression. Hox protein repression function appears to be quite complex. Our embryonic tests of the deletion mutants, and the results of others (Hittinger et al., 2005), suggest that Ubx contains multiple regions that additively contribute to repression. In addition, previous studies (Catron et al., 1995; Li et al., 1999; Zhang et al., 1996) suggest that the homeodomain also contributes directly to transcriptional repression function in a manner that is independent of its DNA-binding function.

The Ubx YPWM region and transcriptional activation

The deletion of the Ubx YPWM region had little detectable effect on the transcriptional activation of the *dpp* and *tsh* genes. As *exd* genetic function is required for normal levels of *dpp* and *tsh* activation in Ubx-expressing cells (Chan et al., 1994; McCormick et al., 1995; Rauskolb and Wieschaus, 1994; Sun et al., 1995), this result is difficult to reconcile with a simple model in which the YPWM motif is required for Exd recruitment to activation target sites in *dpp* and *tsh* enhancers. However, it is consistent with studies that tested the effect of YPWM mutations on the activation abilities of the Labial and Abd-A Hox proteins in embryos (Chan et al., 1996; Merabet et al., 2003). A YPWM to AAAA mutant of Labial was a more potent activator than wild-type Labial protein of a sequence derived from the *Hoxb1* autoregulatory region (Chan et al., 1996), whereas a YPWM-to-AAAA mutant of Abd-A converted this protein from a repressor into an activator of *dpp* transcription (Merabet et al., 2003). In addition, this YPWM mutation had no effect on the activation function of Abd-A on *wingless*. The ability of Labial and Abd-A YPWM mutants to retain their transactivation functions is correlated with their ability to bind Exd in vitro in a YPWM-independent fashion (Chan et al., 1996; Merabet et al., 2003). The YPWM-independent interactions between Hox proteins and Exd can be mediated by Hox homeodomains and the C-terminal regions (Li et al., 1999; Chan et al., 1996).

As the Ubx-responsive elements from *dpp* and *tsh* loci possess a mixture of Ubx monomer and Ubx-Exd heterodimer-binding sites (Sun et al., 1995; McCormick et al., 1995), possible reasons for the ability of the Ubx YMPM deletion mutant to activate these downstream target genes are: (1) Hox activation of target genes often involves a mixture of Exd-dependent and Exd-independent functions (Pearson et al., 2005); (2) removal of the YPWM motif does not completely abolish Exd-Ubx binding interactions (Galant et al., 2002); and (3) the YPWM apparently serves other functions besides binding Exd in the context of developing embryos (Chan et al., 1996; Merabet et al., 2003).

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/132/23/5271/DC1

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