

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

Ella Tour¹, Chris Todd Hittinger² and William McGinnis^{1,*}

¹Section in Cell and Developmental Biology, Division of Biology, University of California, San Diego, La Jolla, CA 92093, USA

²Howard Hughes Medical Institute, Laboratory of Genetics, University of Wisconsin-Madison, Madison, WI 53706, USA

*Author for correspondence (e-mail: mcginnis@biomail.ucsd.edu)

Accepted 28 September 2005

Development 132, 5271-5281

Published by The Company of Biologists 2005

doi:10.1242/dev.02138

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 evolutionary 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 *EcoRI* 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,

$Y = \text{Top} / (1 + 10^{(\text{LogEC}_{50} - X) \cdot \text{HillSlope}})$ 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 Ubx Δ YPWM.

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

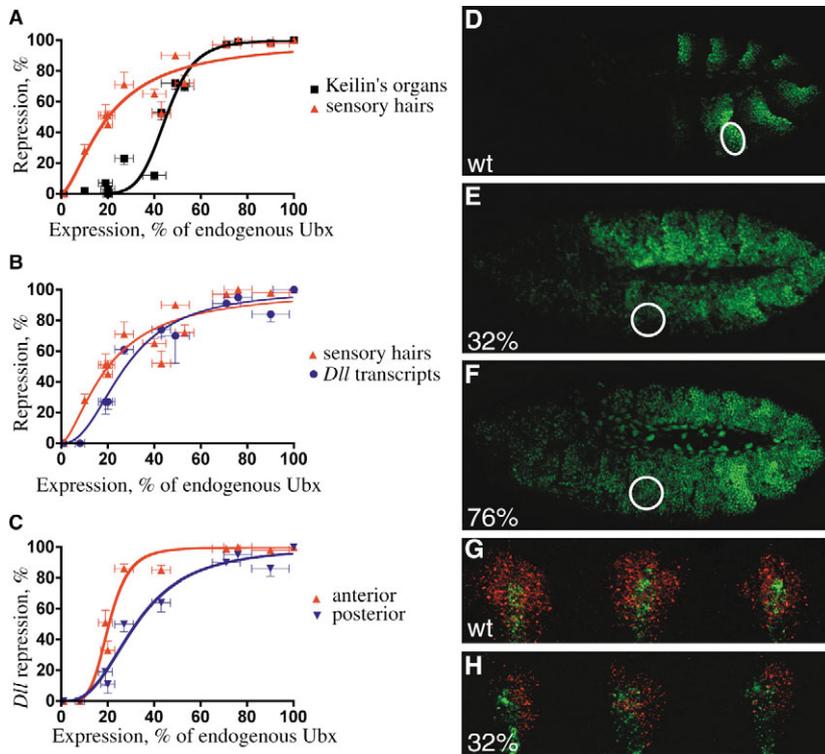


Fig. 1. The repression of larval limbs and *Dll* transcription is highly dependent on Ubx concentration. (A) The repression of Keilin's organs (in black) and the sensory hairs (red) of Keilin's organs as a function of ectopic Ubx concentration in the thorax. Each data point represents a different ectopic 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

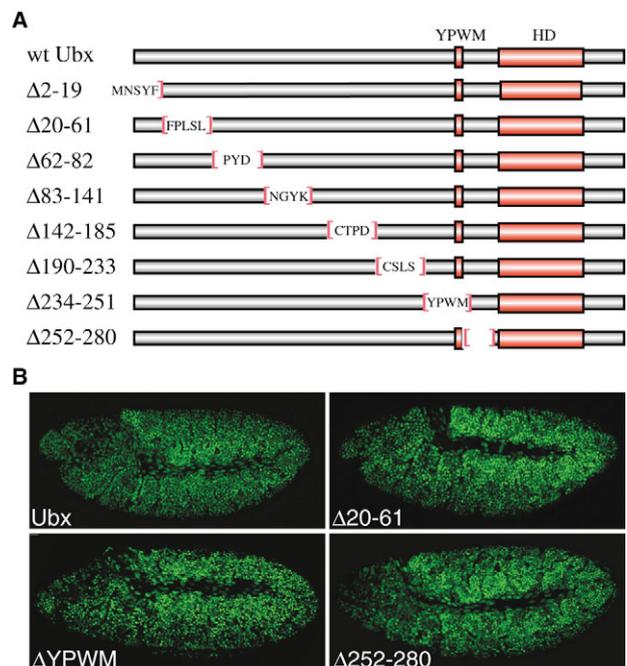


Fig. 2. The Ubx N-terminal region deletions. (A) Diagram of *Drosophila* UbxIa and the eight deletions covering its N-terminal arm. All deletions, except for Δ252-280 (the optional intron region), contained regions highly conserved between Ubx proteins from other arthropod species (see Fig. S1 in the 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 Ubx Δ 2-19, 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 (Ubx Δ 234-251). When produced at the levels of

endogenous Ubx, the Ubx Δ YPWM 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 Ubx Δ YPWM-induced limb repression was also notably less steep than is observed for wild-type Ubx (Fig. 3B).

The Ubx Δ 20-61 deletion mutant also showed a decrease in limb repression function. The 20-61 region contains an YRXPFLXL 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, Ubx Δ 20-61 represses only 11% of limbs, sixfold less than the equivalent concentration of wild-type Ubx protein (Fig. 3B). In contrast to Ubx Δ YPWM, the Ubx Δ 20-61 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 Ubx Δ 252-280 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.

Ubx Δ YPWM mutant is an ineffective repressor of *Dll* and *Antp*

We next tested the function of the most defective Ubx deletion mutant, Ubx Δ YPWM, 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 Ubx Δ YPWM mutant proteins were expressed at similar levels (wild-type Ubx 32 \pm 5%, Ubx Δ YPWM 40 \pm 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 Ubx Δ YPWM deletion mutant is a less effective repressor of *Dll* transcription, repressing 57% of *Dll* transcript

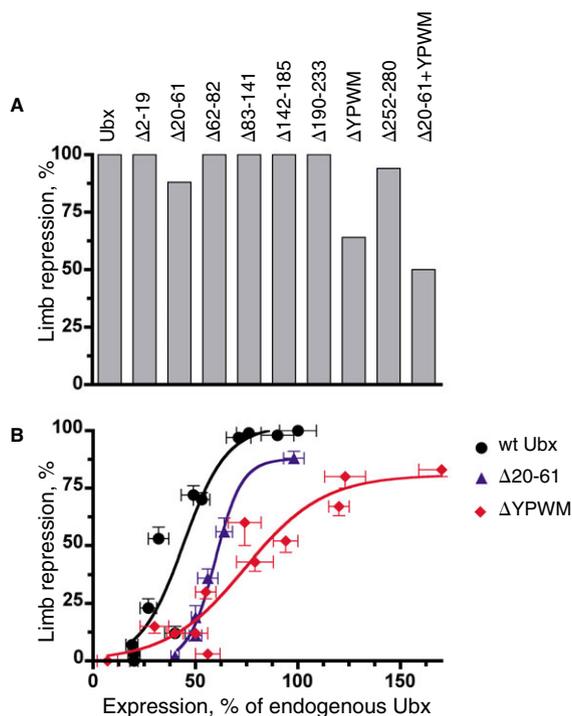


Fig. 3. The YPWM and the 20-61 regions are quantitatively required for Ubx limb repression function. (A) The limb repression values of Ubx deletion mutants when expressed at the level of endogenous Ubx. (B) The limb repression activity of wild-type Ubx, Ubx Δ 20-61 and Ubx Δ YPWM deletion mutants as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

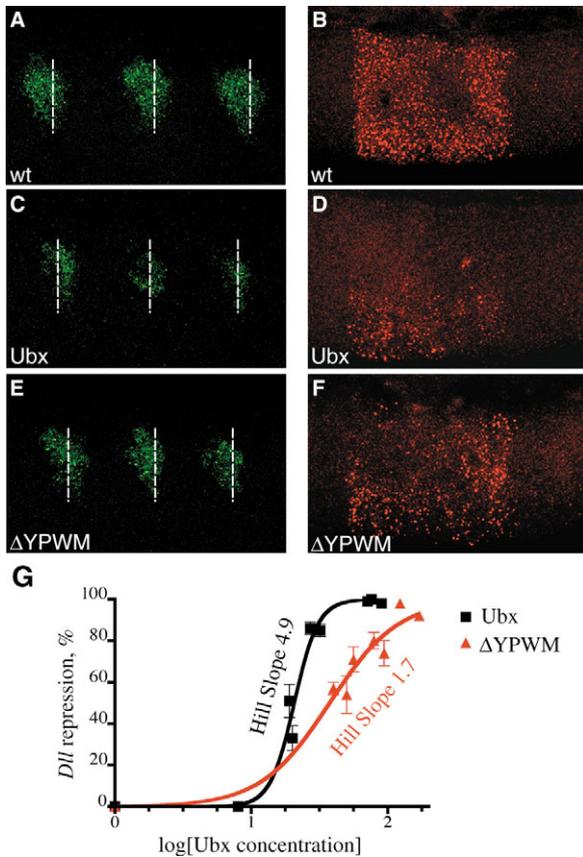


Fig. 4. The Ubx Δ YPWM protein is a defective transcriptional repressor of *Dll* and *Antp*. (A-F) In situ hybridization of mid-stage 11 embryos, hybridized with *Dll* (green) and *Antp* (red) antisense probes. The broken white lines in A,C,E indicate the posterior boundary of *wg* expression, which 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 Ubx Δ YPWM protein. (G) A dose-response plot of *Dll* repression as a function of the logarithm of the protein concentration of wild-type Ubx and Ubx Δ YPWM.

levels in the anterior compartment (Fig. 4E). The Ubx Δ 20-61 protein exhibited a similar defect in *Dll* transcriptional repression (not shown). The other Ubx deletion mutants, including Ubx Δ 2-19 (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 (Birmingham 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 Ubx Δ YPWM 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

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 Ubx Δ YPWM 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 Ubx Δ YPWM 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 (\pm 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 Ubx Δ YPWM 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.

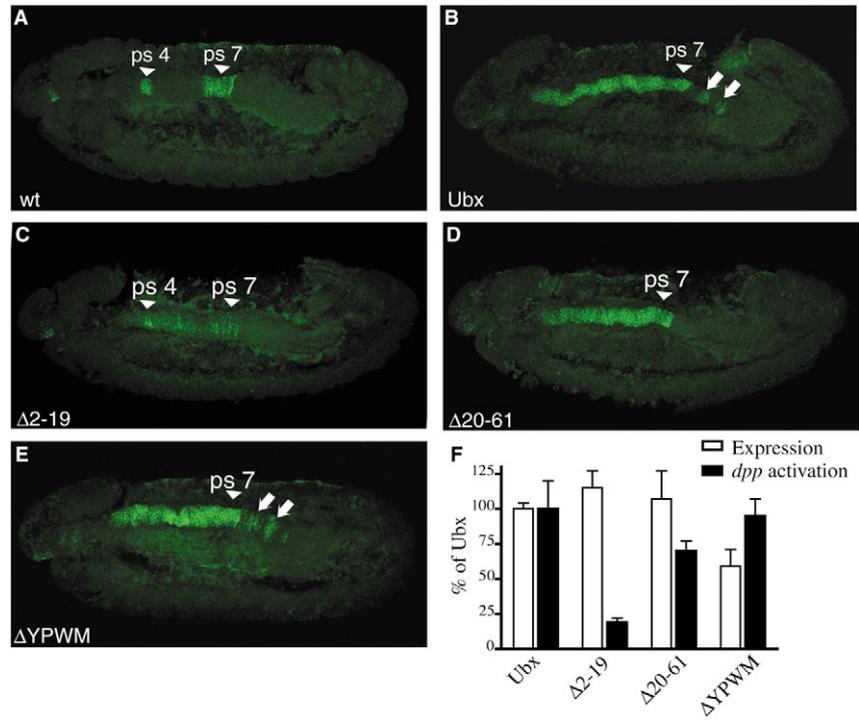
A conserved region required for activation function of Ubx protein

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 Ubx Δ YPWM 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 Ubx Δ 20-61 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 Ubx Δ 20-61 is partially defective in both repression and activation. We conclude that the Ubx Δ 20-61 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 Ubx Δ 2-19, which barely activated *dpp*

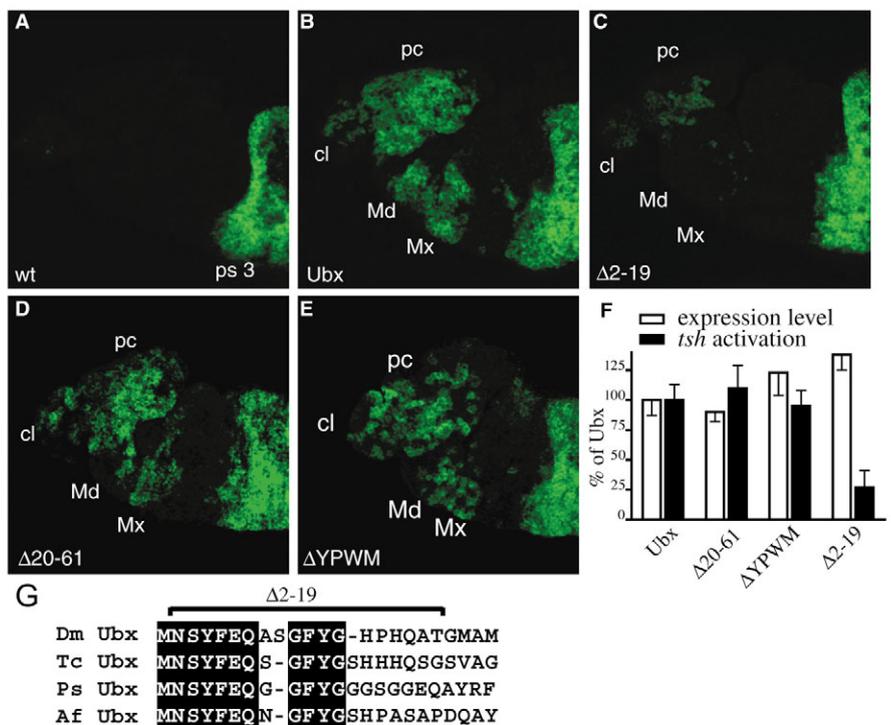
Fig. 5. The N-terminal region of Ubx is required for ectopic activation of *dpp* in visceral mesoderm. (A-E) *dpp* transcripts in the visceral mesoderm of stage 13 embryos. (A) In wild-type embryos, *dpp* transcripts are detected in parasegments 4 and 7 (arrowheads). (B) Ectopic wild-type Ubx activates *dpp* transcripts anterior to and posterior to parasegment 7 (arrows). (C) Ectopic Ubx Δ 2-19 protein barely activates ectopic *dpp* in parasegments 5 and 6, and represses endogenous *dpp* in parasegments 4 and 7 (arrowheads). (D) Ectopic Ubx Δ 20-61 protein activates *dpp* transcripts anterior to, but not posterior to parasegment 7. (E) Ectopic Ubx Δ YPWM 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 Ubx Δ 2-19 mutant also repressed transcription of *dpp* in parasegments 4 and 7 to barely detectable levels (compare Fig. 5A with 5C). We concluded that Ubx Δ 2-19 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 Ubx Δ 2-19 was locus-specific, we tested whether this mutant, along with Ubx Δ 20-61 and Ubx Δ YPWM 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 Ubx Δ 2-19 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 Ubx Δ 20-61 and Ubx Δ YPWM 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 Ubx Δ 2-19 mutant are identical in the four Ubx homologs.



(Fig. 6A,B). Ectopic Ubx Δ 2-19, 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 Ubx Δ 20-61 and Ubx Δ YPWM 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 Ubx Δ 2-19 was 27% of the activation produced by wild-type Ubx, even though the Ubx Δ 2-19 protein was expressed at 138% of wild-type Ubx control levels (Fig. 6F).

Recall that Ubx Δ 2-19 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 (Scr Δ SSY) under UAS regulatory element control were generated and crossed to the *armadillo*-Gal4 driver. Expression levels of ectopic wild-type Scr and Scr Δ SSY 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 Scr Δ SSY 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 Scr Δ SSY protein was also a defective

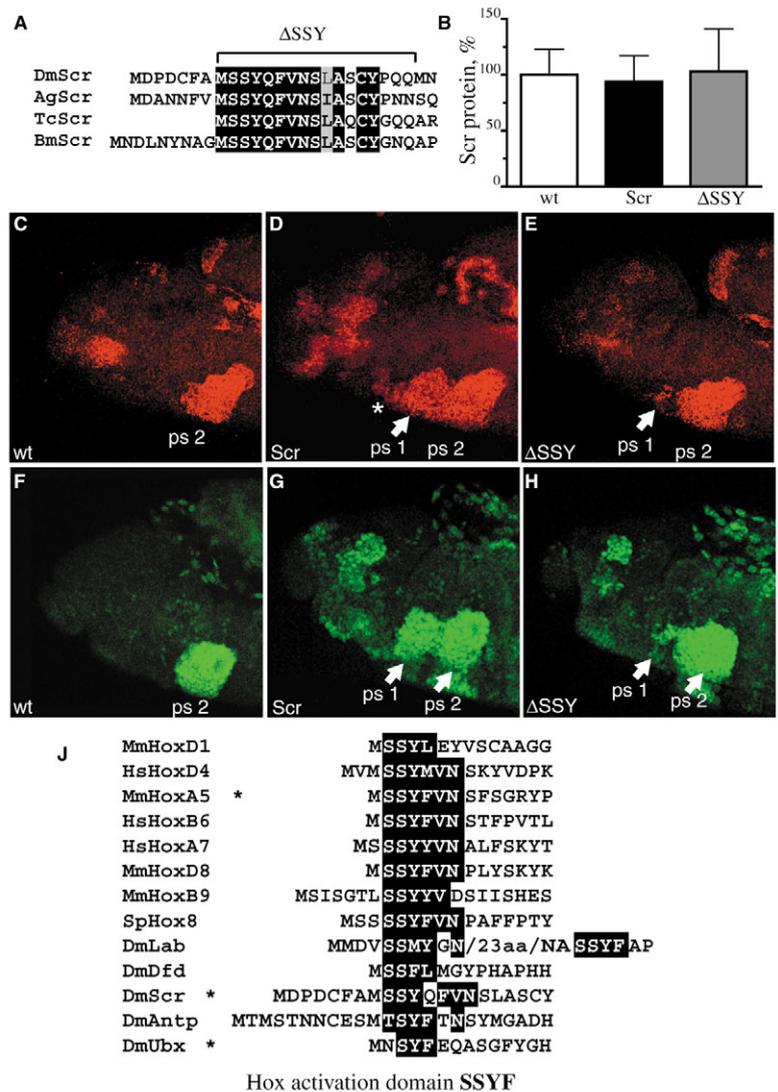


Fig. 7. The conserved N-terminal region of Scr is required for the activation of *fkh* and CrebA expression. (A) Alignment of the N-termini of insect Scr proteins [*Drosophila melanogaster* (Dm), *Anopheles gambiae* (Ag), *Tribolium castaneum* (Tc) and *Bombyx mori* (Bm)]. In the region deleted in the Scr Δ SSY mutation (bracket), 12 out of 17 amino acid residues are identical. (B) Expression levels of ectopic wild-type Scr (Scr) and the Scr Δ SSY mutant (Δ SSY) 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 Scr Δ SSY 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 Scr Δ SSY 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 Scr Δ SSYF 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 I κ B α 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 (Armknrecht 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 Ubx Δ YPWM 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 YPWM 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).

We thank Dave Kosman for invaluable help with in situ hybridization and confocal imaging, Michael Hannon for suggesting Ubx concentration curves and the members of the McGinnis laboratory for critically reading the manuscript. We are grateful to Rob White for anti-Ubx antibody, Brian Gebelein for helpful discussion and reagents, Debbie Andrew for anti-CrebA and anti-Scr antibodies, Thom Kaufman for a *Scr* cDNA clone, Kathy Vaccaro for UAS-*Scr* injections, and Sean Carroll for mentoring and supporting the research of C.T.H. C.T.H. is a Howard Hughes Medical Institute predoctoral fellow. This work was supported by NIH Grant HD28315 to W.M.

Supplementary material

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

References

- Akam, M. E. (1983). The location of *Ultrabithorax* transcripts in *Drosophila* tissue sections. *EMBO J.* **2**, 2075-2084.
- Andrew, D. J., Horner, M. A., Pettitt, M. G., Smolik, S. M. and Scott, M. P. (1994). Setting limits on homeotic gene function: restraint of Sex combs reduced activity by teashirt and other homeotic genes. *EMBO J.* **13**, 1132-1144.
- Andrew, D. J., Henderson, K. D. and Sessaiah, P. (2000). Salivary gland development in *Drosophila melanogaster*. *Mech. Dev.* **92**, 5-17.
- Armknacht, S., Boutros, M., Kiger, A. A., Nybakken, K., Mathey-Prevot, B. and Perrimon, N. (2005). High-throughput RNA interference screens in *Drosophila* tissue culture cells. *Methods Enzymol.* **392**, 55-73.
- Balavoine, G. and Adoutte, A. (1998). One or three Cambrian radiations? *Science* **280**, 397-398.
- Beachy, P. A., Varkey, J., Young, K. E., von Kessler, D. P., Sun, B. I. and Ekker, S. C. (1993). Cooperative binding of an Ultrabithorax homeodomain protein to nearby and distant sites. *Mol. Cell. Biol.* **13**, 6941-6856.
- Bermingham, J. R., Martinez-Arias, A., Pettitt, M. G. and Scott, M. P. (1990). Different patterns of transcription from the two *Antennapedia* promoters during *Drosophila* embryogenesis. *Development* **109**, 553-566.
- Bienz, M., Saari, G., Tremml, G., Muller, J., Züst, B. and Lawrence, P. A. (1988). Differential regulation of Ultrabithorax in two germ layers of *Drosophila*. *Cell* **53**, 567-576.
- Brand, A. H., Manoukian, A. S. and Perrimon, N. (1994). Ectopic expression in *Drosophila*. In *Methods in Cell Biology* (ed. L. S. B. Goldstein and E. Fyrberg), pp. 635-654. New York: Academic Press.
- Busturia, A., Vernos, I., Macias, A., Casanova, J. and Morata, G. (1990). Different forms of Ultrabithorax proteins generated by alternative splicing are functionally equivalent. *EMBO J.* **9**, 3551-3555.
- Campos-Ortega, J. A. and Hartenstein, V. (1985). *The Embryonic Development of Drosophila melanogaster*. Berlin: Springer-Verlag.
- Capovilla, M. and Botas, J. (1998). Functional dominance among Hox genes: repression dominates activation in the regulation of *Dpp*. *Development* **125**, 4949-4957.

- Capovilla, M., Brandt, M. and Botas, J. (1994). Direct regulation of *decapentaplegic* by Ultrabithorax and its role in *Drosophila* midgut morphogenesis. *Cell* **76**, 461-475.
- Carroll, S. B., Layman, R. A., McCutcheon, M. A., Riley, P. D. and Scott, M. P. (1986). The localization and regulation of Antennapedia protein expression in *Drosophila* embryos. *Cell* **47**, 113-122.
- Castelli-Gair, J., Greig, S., Micklem, G. and Akam, M. (1994). Dissecting the temporal requirements for homeotic gene function. *Development* **120**, 1983-1995.
- Catron, K. M., Zhang, H., Marshall, S. C., Inostroza, J. A., Wilson, J. M. and Abate, C. (1995). Transcriptional repression by Msx-1 does not require homeodomain DNA-binding sites. *Mol. Cell. Biol.* **15**, 861-871.
- Chan, H. M. and La Thangue, N. B. (2001). p300/CBP proteins: HATs for transcriptional bridges and scaffolds. *J. Cell Sci.* **114**, 2363-2373.
- Chan, S. K., Jaffe, L., Capovilla, M., Botas, J. and Mann, R. S. (1994). The DNA binding specificity of Ultrabithorax is modulated by cooperative interactions with extradenticle, another homeoprotein. *Cell* **78**, 603-615.
- Chan, S. K., Popperl, H., Krumlauf, R. and Mann, R. S. (1996). An extradenticle-induced conformational change in a HOX protein overcomes an inhibitory function of the conserved hexapeptide motif. *EMBO J.* **15**, 2476-2487.
- Chang, C. P., Shen, W. F., Rozenfeld, S., Lawrence, H. J., Largman, C. and Cleary, M. L. (1995). Pbx proteins display hexapeptide-dependent cooperative DNA binding with a subset of Hox proteins. *Genes Dev.* **9**, 663-674.
- Chariot, A., Princen, F., Gielen, J., Merville, M. P., Franzoso, G., Brown, K., Siebenlist, U. and Bours, V. (1999a). IkappaB-alpha enhances transactivation by the HOXB7 homeodomain-containing protein. *J. Biol. Chem.* **274**, 5318-5325.
- Chariot, A., van Lint, C., Chapelier, M., Gielen, J., Merville, M. P. and Bours, V. (1999b). CBP and histone deacetylase inhibition enhance the transactivation potential of the HOXB7 homeodomain-containing protein. *Oncogene* **18**, 4007-4014.
- Cohen, B., Wimmer, E. A. and Cohen, S. M. (1991). Early development of leg and wing primordia in the *Drosophila* embryo. *Mech. Dev.* **33**, 229-240.
- Cohen, S. M. (1990). Specification of limb development in the *Drosophila* embryo by positional cues from segmentation genes. *Nature* **343**, 173-177.
- Cohen, S. M., Brönnner, G., Küttner, F., Jürgens, G. and Jäckle, H. (1989). *Distal-less* encodes a homeodomain protein required for limb development in *Drosophila*. *Nature* **338**, 432-434.
- Fasano, L., Roder, R., Core, N., Alexandre, E., Vola, C., Jacq, B. and Kerridge, S. (1991). The gene *teashirt* is required for the development of *Drosophila* embryonic trunk segments and encodes a protein with widely spaced zinc finger motifs. *Cell* **64**, 63-79.
- Featherstone, M. (2003). Hox proteins and their co-factors in transcriptional regulation. In *Murine Homeobox Gene Control of Embryonic Patterning and Organogenesis*, Vol. 13 (ed. Lufkin, T.), pp. 1-42. Amsterdam: Elsevier.
- Florence, B. and McGinnis, W. (1998). A genetic screen of the *Drosophila* X chromosome for mutations that modify Deformed function. *Genetics* **150**, 1497-1511.
- Galant, R. and Carroll, S. B. (2002). Evolution of a transcriptional repression domain in an insect Hox protein. *Nature* **415**, 910-913.
- Galant, R., Walsh, C. M. and Carroll, S. B. (2002). Hox repression of a target gene: extradenticle-independent, additive action through multiple monomer binding sites. *Development* **129**, 3115-3126.
- Gebelein, B., Culi, J., Ryoo, H. D., Zhang, W. and Mann, R. S. (2002). Specificity of *Distalless* repression and limb primordia development by abdominal Hox proteins. *Dev. Cell* **3**, 487-498.
- Gebelein, B., McKay, D. J. and Mann, R. S. (2004). Direct integration of Hox and segmentation gene inputs during *Drosophila* development. *Nature* **431**, 653-659.
- Glass, C. K., Rose, D. W. and Rosenfeld, M. G. (1997). Nuclear receptor coactivators. *Curr. Opin. Cell Biol.* **9**, 222-232.
- Gonzalez-Reyes, A. and Morata, G. (1990). The developmental effect of overexpressing a Ubx product in *Drosophila* embryos is dependent on its interactions with other homeotic products. *Cell* **61**, 515-522.
- Grenier, J. K. and Carroll, S. B. (2000). Functional evolution of the Ultrabithorax protein. *Proc. Natl. Acad. Sci. USA* **97**, 704-709.
- Hafen, E., Levine, M. and Gehring, W. J. (1984). Regulation of *Antennapedia* transcript distribution by the bithorax complex in *Drosophila*. *Nature* **307**, 287-289.
- Hittinger, C. T., Stern, D. L. and Carroll, S. B. (2005). Pleiotropic functions of a conserved insect-specific HOX peptide motif. *Development* **132**, 5261-5270.
- Hughes, C. L. and Kaufman, T. C. (2002). Hox genes and the evolution of the arthropod body plan. *Evol. Dev.* **4**, 459-499.
- Immerglück, K., Lawrence, P. A. and Bienz, M. (1990). Induction across germ layers in *Drosophila* mediated by a genetic cascade. *Cell* **62**, 261-268.
- Irvine, K. D., Botas, J., Jha, S., Mann, R. S. and Hogness, D. S. (1993). Negative autoregulation by *Ultrabithorax* controls the level and pattern of its expression. *Development* **117**, 387-399.
- Johnson, A. D., Poteete, A. R., Lauer, G., Sauer, R. T., Ackers, G. K. and Ptashne, M. (1981). lambda Repressor and cro – components of an efficient molecular switch. *Nature* **294**, 217-223.
- Johnson, F. B., Parker, E. and Krasnow, M. A. (1995). Extradenticle protein is a selective cofactor for the *Drosophila* homeotics: Role of the homeodomain and YPWM amino acid motif in the interaction. *Proc. Natl. Acad. Sci. USA* **92**, 739-743.
- Knoepfler, P. and Kamps, M. (1995). The pentapeptide motif of Hox proteins is required for cooperative DNA binding with Pbx1, physically contacts Pbx1, and enhances binding by Pbx1. *Mol. Cell. Biol.* **15**, 5811-5819.
- Kosman, D., Mizutani, C. M., Lemons, D., Cox, W. G., McGinnis, W. and Bier, E. (2004). Multiplex detection of RNA expression in *Drosophila* embryos. *Science* **305**, 846.
- Lewis, E. B. (1978). A gene complex controlling segmentation in *Drosophila*. *Nature* **276**, 565-570.
- Li, X., Murre, C. and McGinnis, W. (1999). Activity regulation of a Hox protein and a role for the homeodomain in inhibiting transcriptional activation. *EMBO J.* **18**, 198-211.
- Manak, J. R., Mathies, L. D. and Scott, M. P. (1995). Regulation of a *decapentaplegic* midgut enhancer by homeotic proteins. *Development* **120**, 3605-3619.
- Mann, R. S. and Hogness, D. S. (1990). Functional dissection of Ultrabithorax protein in *D. melanogaster*. *Cell* **60**, 597-610.
- Martinez, P., Lee, J. C. and Davidson, E. H. (1997). Complete sequence of *SpHox8* and its linkage in the single Hox gene cluster of *Strongylocentrotus purpuratus*. *J. Mol. Evol.* **44**, 371-377.
- Martinez-Arias, A. (1986). The *Antennapedia* gene is required and expressed in parasegments 4 and 5 of the *Drosophila* embryo. *EMBO J.* **5**, 135-141.
- McCormick, A., Core, N., Kerridge, S. and Scott, M. (1995). Homeotic response elements are tightly linked to tissue-specific elements in a transcriptional enhancer of the *teashirt* gene. *Development* **121**, 2799-2812.
- McGinnis, N., Ragnhildstveit, E., Veraksa, A. and McGinnis, W. (1998). A cap 'n' collar protein isoform contains a selective Hox repressor function. *Development* **125**, 4553-4564.
- McGinnis, W. and Krumlauf, R. (1992). Homeobox genes and axial patterning. *Cell* **68**, 283-302.
- Medina-Martínez, O. and Ramírez-Solis, R. (2003). In vivo mutagenesis of the Hoxb8 hexapeptide domain leads to dominant homeotic transformations that mimic the loss-of-function mutations in genes of the Hoxb cluster. *Dev. Biol.* **264**, 77-90.
- Merabet, S., Kambris, Z., Capovilla, M., Berenger, H., Pradel, J. and Graba, Y. (2003). The hexapeptide and linker regions of the Abd-A Hox protein regulate its activating and repressive functions. *Dev. Cell* **4**, 761-768.
- Muller, J., Thuringer, F., Biggin, M., Züst, B. and Bienz, M. (1989). Coordinate action of a proximal homeoprotein binding site and a distal sequence confer the *Ultrabithorax* expression pattern in the visceral mesoderm. *EMBO J.* **8**, 4143-4151.
- Neuteboom, S., Peltenburg, L., van Dijk, M. and Murre, C. (1995). The hexapeptide motif LFPWMR in Hoxb-8 is required for cooperative DNA binding with Pbx1 and Pbx2 proteins. *Proc. Natl. Acad. Sci. USA* **92**, 9166-9170.
- Panzer, S., Weigel, D. and Beckendorf, S. K. (1992). Organogenesis in *Drosophila melanogaster*: embryonic salivary gland determination is controlled by homeotic and dorsoventral patterning genes. *Development* **114**, 49-57.
- Passner, J. M., Ryoo, H. D., Shen, L., Mann, R. S. and Aggarwal, A. K. (1999). Structure of a DNA-bound Ultrabithorax-Extradenticle homeodomain complex. *Nature* **397**, 714-719.
- Pearson, J. C., Lemons, D. and McGinnis, W. (2005). Modulating Hox gene functions during animal body patterning. *Nat. Rev. Genet.* (in press).
- Perutz, M. F. (1989). Mechanisms of cooperativity and allosteric regulation in proteins. *Q. Rev. Biophys.* **22**, 139-237.
- Phelan, M. L., Rambaldi, I. and Featherstone, M. S. (1995). Cooperative interactions between HOX and PBX proteins mediated by a conserved peptide motif. *Mol. Cell. Biol.* **15**, 3989-3997.
- Piper, D. E., Batchelor, A. H., Chang, C. P., Cleary, M. L. and Wolberger, C. (1999). Structure of a HoxB1-Pbx1 heterodimer bound to DNA: role of

- the hexapeptide and a fourth homeodomain helix in complex formation. *Cell* **96**, 587-597.
- Rauskolb, C. and Wieschaus, E.** (1994). Coordinate regulation of downstream genes by *extradenticle* and the homeotic selector proteins. *EMBO J.* **13**, 3561-3569.
- Remacle, S., Abbas, L., De Backer, O., Pacico, N., Gavalas, A., Gofflot, F., Picard, J. J. and Rezsöhazy, R.** (2004). Loss of function but no gain of function caused by amino acid substitutions in the hexapeptide of Hox1 in vivo. *Mol. Cell. Biol.* **24**, 8567-8575.
- Reuter, R., Panganiban, G. E. F., Hoffmann, F. M. and Scott, M. P.** (1990). Homeotic genes regulate the spatial expression of putative growth factors in the visceral mesoderm of *Drosophila* embryos. *Development* **110**, 1031-1040.
- Roder, V. and Kerridge, S.** (1992). The role of the *teashirt* gene in trunk segmented identity in *Drosophila*. *Development* **115**, 1017-1033.
- Ronshaugen, M., McGinnis, N. and McGinnis, W.** (2002). Hox protein mutation and macroevolution of the insect body plan. *Nature* **415**, 914-917.
- Ryoo, H. D. and Mann, R. S.** (1999). The control of trunk Hox specificity and activity by *Extradenticle*. *Genet. Dev.* **13**, 1704-1716.
- Saffman, E. E. and Krasnow, M. A.** (1994). A differential response element for the homeotics at the *Antennapedia* P1 promoter of *Drosophila*. *Proc. Natl. Acad. Sci. USA* **91**, 7420-7424.
- Saleh, M., Rambaldi, I., Yang, X.-J. and Featherstone, M. S.** (2000). Cell signaling switches HOX-PBX complexes from repressors to activators of transcription mediated by histone deacetylases and histone acetyltransferases. *Mol. Cell. Biol.* **20**, 8623-8633.
- Schughart, K., Utset, M. F., Awgulewitsch, A. and Ruddle, F. H.** (1988). Structure and expression of *Hox-2.2*, a murine homeobox-containing gene. *Proc. Natl. Acad. Sci. USA* **85**, 5582-5585.
- Shanmugam, K., Featherstone, M. S. and Saragovi, H. U.** (1997). Residues flanking the HOX YPWM motif contribute to cooperative interactions with PBX. *J. Biol. Chem.* **272**, 19081-19087.
- Smolik-Utlaut, S. M.** (1990). Dosage requirements of *Ultrabithorax* and *bithoraxoid* in the determination of segment identity in *Drosophila melanogaster*. *Genetics* **124**, 357-366.
- Subramaniam, V., Bomze, H. M. and Lopez, A. J.** (1994). Functional differences between *Ultrabithorax* protein isoforms in *Drosophila melanogaster*: evidence from elimination, substitution and ectopic expression of specific isoforms. *Genetics* **136**, 979-991.
- Sun, B., Hursh, D. A., Jackson, D. and Beachy, P. A.** (1995). *Ultrabithorax* protein is necessary but not sufficient for full activation of *decapentaplegic* expression in the visceral mesoderm. *EMBO J.* **14**, 520-535.
- Tan, X. X., Bondos, S., Li, L. and Matthews, K. S.** (2002). Transcription activation by *Ultrabithorax* I β protein requires a predicted alpha-helical region. *Biochemistry* **41**, 2774-2785.
- Tremml, G. and Bienz, M.** (1989). Homeotic gene expression in the visceral mesoderm of *Drosophila* embryos. *EMBO J.* **8**, 2677-2685.
- Vachon, G., Cohen, B., Pfeifle, C., McGuffin, M. E., Botas, J. and Cohen, S. M.** (1992). Homeotic genes of the bithorax complex repress limb development in the abdomen of the *Drosophila* embryo through the target gene *Distal-less*. *Cell* **71**, 437-450.
- White, R. A. H. and Wilcox, M.** (1984). Protein products of the bithorax complex in *Drosophila*. *Cell* **39**, 163-171.
- Yaron, Y., McAdara, J. K., Lynch, M., Hughes, E. and Gasson, J. C.** (2001). Identification of novel functional regions important for the activity of HOXB7 in mammalian cells. *J. Immunol.* **166**, 5058-5067.
- Zhang, H., Catron, K. M. and Abate-Shen, C.** (1996). A role for the Msx-1 homeodomain in transcriptional regulation: Residues in the N-terminal arm mediate TATA binding protein interaction and transcriptional repression. *Proc. Natl. Acad. Sci. USA* **93**, 1764-1769.
- Zhao, J. J., Lazzarini, R. A. and Pick, L.** (1996). Functional dissection of the mouse Hox-a5 gene. *EMBO J.* **15**, 1313-1322.

	$\Delta 2-19$	$\Delta 20-61$
Dm Ubx	MNSYFEQASGFYGHPHQATGMAMGSGGHHDOTASAAAAAYRGFPLSLG-MSPYAN-----	
Jc Ubx	MNSYFEQC-GFYG---AHGVHQG-GGGGDO-----YRGFPLGLT-YAOPHA-----	
Tc Ubx	MNSYFEQS-GFYGSHHHQSGSVAG---HHHEQSAAAAAYRSFPLSLG-MSPYASSQHSHH	
Af Ubx	MNSYFEQN-GFYG---SHPASAP-----DQ-----AYR-FPLGLNPYSATAA-----	
	$\Delta 62-82$	$\Delta 83-141$
Dm Ubx	-HHLQRTTODSPYDASITAA-CNKIYG-DGAG-----AYKQ-DCLNIKADAVNGY---	
Jc Ubx	-LHQPRP-ODSPYDASVAAA-C-KLYAGEQQ-----YAKA-DCSKAGGEQONGYGG-	
Tc Ubx	HHLQARPP-ODSPYDASVAAA-C-KLYSSEGOONSNYSSNSKP--DCSKGNADQ-NGYASV	
Af Ubx	LANIRQTGHEQPYDTANPAAPC-KLY--EHQ-----Y-KLPIDLQSSKEQ--NAYGLP	
	$\Delta 83-141$	$\Delta 142-185$
Dm Ubx	-----KD---IWNVTGGSNGGGGGGGGGGGAGGTGGAGNANGGNAANANGQNNPAGGM	
Jc Ubx	-----KE---A G-----LGAL-----	
Tc Ubx	VAAAAVKD---VWQS---ATSGGGANLTN-----SLTG-----	
Af Ubx	S-----KDTNLAWLQQ-----	
	$\Delta 142-185$	$\Delta 190-233$
Dm Ubx	PVRPSACTPDSRVGG-YLDTSGGSPVSHRGGSAAGNVSVSGGNGNAGGVQSGVGVAGAGT	
Jc Ubx	-VRPAACTPEAR---YSESSS-----PGRAL	
Tc Ubx	PVRPAACTPDSRVGYGSGVGLVGGDPASSPGAAAG-----RTGNSL	
Af Ubx	GQLDPTRTTDLSQGGPRYPESNAMRNVVSQGP-----	
	$\Delta 190-233$	$\Delta 234-259$
Dm Ubx	AWNANCTISGAAQTAAASSLHQA-----SN-HTFYPWMAIAGKIRSDLTQ	
Jc Ubx	PWGNOCALPGAAAS--AQPVOHQP-----TN-HTFYPWMAIAG-----	
Tc Ubx	SWNNPCSINSTSSQ-PVGTQIHQQ-----TN-HTFYPWMAIA-----	
Af Ubx	-WSP-CSLSANNSNASNGSRPQTVPOGQGTPEOQHSPSQPAAAFYPWMAIAG-----	
	$\Delta 252-280$	
Dm Ubx	YGGISTDMGKRYSESLAGSLLPDWLG-TNGLRRRGRQTYTRYQTLELEKEFHTNHYLTRRR	
Jc Ubx	-----ANGLRRRGRQTYTRYQTLELEKEFHTNHYLTRRR	
Tc Ubx	----DSMT-----FCANGLRRRGRQTYTRYQTLELEKEFHTNHYLTRRR	
Af Ubx	-----ANGLRRRGRQTYTRYQTLELEKEFHTNHYLTRRR	
Dm Ubx	RIEMAHALCLTERQIKIWFQNRMKLKKEIQAIKELNEQEKQAQAQKAAAAAAAAAAVQG	
Jc Ubx	RIEMAHALCLTERQIKIWFQNRMKLKKEIQAIKELNEQEKQAQAQKAAAAAAAAAAQ	
Tc Ubx	RIEMAHALCLTERQIKIWFQNRMKLKKEIQAIKELNEQEKQAQAQKAAAAAAVAAQVD	
Af Ubx	RIEMAHSLCLTERQIKIWFQNRMKLKKEIQAIKELNEQDKRITPSKLSNCSSTGDIS	
Dm Ubx	GHLDQ---	
Jc Ubx	GHPFH---	
Tc Ubx	PN-----	
Af Ubx	DDEKDEKL	