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Specificity of *Distalless* Repression and Limb Primordia Development by Abdominal Hox Proteins

Brian Gebelein,[1] Joaquim Culi,[1] Hyung Don Ryoo,[1,3] Wen Zhang,[1] and Richard S. Mann[1,2]
[1]Department of Biochemistry and Molecular Biophysics
Columbia University
701 West 168th Street
HHSC 1104
New York, New York 10032

Summary

In *Drosophila*, differences between segments, such as the presence or absence of appendages, are controlled by Hox transcription factors. The Hox protein Ultrabithorax (Ubx) suppresses limb formation in the abdomen by repressing the leg selector gene *Distalless*, whereas Antennapedia (Antp), a thoracic Hox protein, does not repress *Distalless*. We show that the Hox cofactors Extradenticle and Homothorax selectively enhance Ubx, but not Antp, binding to a *Distalless* regulatory sequence. A C-terminal peptide in Ubx stimulates binding to this site. However, DNA binding is not sufficient for *Distalless* repression. Instead, an additional alternatively spliced domain in Ubx is required for *Distalless* repression but not DNA binding. Thus, the functional specificities of Hox proteins depend on both DNA binding-dependent and -independent mechanisms.

Introduction

The enormous diversity of body plans in the animal kingdom is due, at least in part, to variations in the way Hox transcription factors regulate gene expression. Most animals have one or more clusters of *Hox* genes, and each *Hox* gene controls the development of a specific region of the body plan (McGinnis and Krumlauf, 1992). Altering their patterns of expression, their targets, and their functional domains have all contributed to body plan diversification during animal evolution (Carroll, 1995; Gellon and McGinnis, 1998; Weatherbee and Carroll, 1999; Galant and Carroll, 2002; Ronshaugen et al., 2002). Thus, determining the mechanisms by which Hox proteins regulate gene expression is important for understanding both animal development and evolution.

One important morphological variation in the animal kingdom is the presence or absence of legs in the abdominal segments of arthropods. *Distalless* (*Dll*), an evolutionarily conserved homeobox gene, is a marker for leg primordia throughout the animal kingdom (Panganiban et al., 1997). Some arthropods, such as the fruit fly *Drosophila melanogaster*, have evolved a mechanism to suppress appendage development in the abdomen by repressing *Dll* expression. In *Drosophila*, *Dll* is required for limb development and its expression is directly repressed by the abdominal Hox proteins Ultrabithorax (Ubx) and Abdominal-A (Abd-A) (Vachon et al., 1992). In contrast, *Dll* is not repressed by the thoracic Hox protein Antennapedia (Antp), allowing legs to form in the thorax.

Observations such as these raise a fundamental question: how do Hox proteins, and transcription factors in general, achieve specificity in vivo? As each Hox protein contains a DNA binding homeodomain, one possibility is that Hox proteins selectively bind specific DNA sequences in vivo, and thereby regulate unique sets of target genes (Graba et al., 1997). To achieve a sufficient degree of DNA binding specificity, Hox proteins are thought to bind DNA together with cofactors. In *Drosophila*, the best-characterized Hox cofactor is the homeodomain protein Extradenticle (Exd; Peifer and Wieschaus, 1990; Mann and Chan, 1996). Exd cooperatively binds DNA with Hox proteins, thereby expanding the Hox binding site from ∼6 to ∼10 base pairs (Mann and Chan, 1996). In addition to making a larger binding site, Exd selectively enhances the ability of individual Hox proteins to interact with specific DNA sequences (Chang et al., 1996; Ryoo and Mann, 1999; Ryoo et al., 1999). For example, an enhancer from the *forkhead* (*fkh*) gene that is specifically activated by the Hox protein Sex combs reduced (Scr) in vivo, is weakly bound by both Scr and Antp as monomers (Ryoo and Mann, 1999). Exd selectively stimulates the binding of Scr/Exd dimers over other Hox/Exd dimers to this element (Ryoo and Mann, 1999). These findings, together with similar experiments carried out on other enhancers, suggest that the DNA binding specificity of Hox proteins, and homeodomain proteins in general, is enhanced through interactions with cofactors, and that this specificity plays an important role in how these transcription factors execute their specific functions in vivo (Chan et al., 1997; Florence et al., 1997; Guichet et al., 1997; Yu et al., 1997; Ryoo and Mann, 1999; Ryoo et al., 1999; Henderson and Andrew, 2000; Nasiadka et al., 2000).

In addition to Exd, Homothorax (Hth) is another cofactor required for Hox function (Mann and Affolter, 1998). Hth contains a homeodomain and interacts directly with Exd, suggesting that Hth also contributes to Hox DNA binding specificity. In support of this view, several Hox-activated enhancers from both vertebrates and invertebrates have been identified that contain Hox, Exd, and Hth binding sites (Jacobs et al., 1999; Ryoo et al., 1999; Ferretti et al., 2000). In most cases, the activity of these enhancers requires all three homeodomain binding sites. Thus, the requirement for an Hth binding site in addition to Hox and Exd binding sites provides even greater target gene selectivity for Hox proteins.

Once Hox proteins bind the correct target genes, it is likely that their ability to activate or repress transcription is also regulated. Support for this idea stems in part from experiments showing that, when fused to heterologous transcriptional activation or repression domains, Hox proteins have altered properties in vivo (Li and

[2]Correspondence: rsm10@columbia.edu
[3]Present address: The Rockefeller University, 1230 York Avenue, Box 252, New York, New York 10021.

McGinnis, 1999; Li et al., 1999). Another powerful approach to study Hox specificity has been to examine the in vivo properties of chimeric Hox proteins. For some pair-wise comparisons, most of the specificity maps to the homeodomain (Lin and McGinnis, 1992; Furukubo-Tokunaga et al., 1993; Zeng et al., 1993). However, other chimera studies demonstrate that sequences outside the homeodomain contribute to Hox specificity in vivo (Chan and Mann, 1993; Chauvet et al., 2000; Grenier and Carroll, 2000; Galant and Carroll, 2002; Ronshaugen et al., 2002). Some of these experiments have been interpreted to suggest that non-DNA binding mechanisms, such as the regulated recruitment of a corepressor protein, may contribute to Hox specificity (Galant and Carroll, 2002; Ronshaugen et al., 2002). In no case, however, has there been a chimera study that, in addition to examining functional specificity in vivo, also measured DNA binding to the relevant in vivo binding site with the known Hox cofactors. Thus, it remains unclear what the relative contributions of these two mechanisms—cofactor-mediated DNA binding specificity on the one hand and non-DNA binding-dependent mechanisms on the other—are for determining Hox specificity.

In this study, we address this question by asking why Ubx and Antp have different potentials to repress *Dll*. Consistent with the idea that cofactors play a critical role in Hox specificity, we show that Exd and Hth specifically stimulate Ubx, but not Antp, binding to a Hox/Exd/Hth trimer binding site within a *Dll* regulatory sequence. DNA binding by this trimer, mediated by all three homeodomains, is necessary for full repression. However, we also show that DNA binding is not sufficient for repression. Instead, an alternatively spliced sequence present in a subset of Ubx isoforms is also necessary for *Dll* repression. Finally, we demonstrate that a Ubx/Exd/Hth complex can mediate both transcriptional repression and activation of the same promoter in the same cells. These data underscore the role that cofactors play in Hox target gene selection, and demonstrate that the same Hox/Exd/Hth complex can be used for both gene activation and repression. In addition, they also suggest that Hox proteins use DNA binding-independent mechanisms to activate or repress target genes.

Results

The overall goal of this work is to understand why Antp and Ubx have different abilities to repress *Dll* in vivo. Our approach was to study chimeric Ubx-Antp proteins. To determine whether DNA binding and/or non-DNA binding mechanisms play a role, two measurements were made: the ability of these chimeras to repress *Dll* in vivo and their ability to bind to the relevant Hox binding site in *Dll*. Therefore, we begin by characterizing a Ubx binding site in the *Dll* gene that is critical for *Dll* repression, and show that both Exd and Hth play a role in Ubx binding and repression.

A Hox, Exd, and Hth Repressor Element Is Present within a Dll Enhancer

The Dll304 enhancer is sufficient to recapitulate the early expression pattern of *Dll* in the embryonic leg primordia (Vachon et al., 1992). In addition to activation functions, Dll304 contains two Hox binding sites, Bx1 and Bx2 (Figure 1A), that repress its activity in the abdomen and thereby restrict *Dll* expression to the thorax. Most of the repression activity is conferred by Bx1, a sequence bound by Ubx and Abd-A (Vachon et al., 1992). In agreement with this result, a Distalless minimal element (DME) that lacks the Bx2 site accurately recapitulates the expression of Dll304 in the embryonic thorax (Figures 1C–1F). The DME enhancer also shows no derepression within the abdomen, suggesting that Bx1 is sufficient to fully repress *Dll*.

To better understand how Bx1 represses *Dll*, we searched for the presence of Exd and Hth binding sites near the previously characterized Hox binding site. We identified a consensus Exd site, also found by White et al. (2000), and a near consensus Hth site in close proximity to the Hox site of Bx1 (Figure 1). The Hox/Exd site, however, is unlike other previously characterized Hox/Exd binding sites because it contains an additional base pair in between the Hox and Exd half-sites (Figure 1A). We refer to the Bx1 region containing this Hox/Exd/Hth site as the Distalless repression element (DllR; Figure 1A). To determine whether DllR is required to repress DME expression in the abdomen, we deleted it from the DME enhancer (DME^{act}) and tested its ability to activate a reporter gene in vivo. DME^{act} drives gene expression in all abdominal segments as well as in the thoracic region (Figures 1G and 1H). Because the thoracic expression driven by DME^{act} is similar to that of DME, the DllR region is not required for DME activation but solely functions in the repression of *Dll* in the abdomen.

To determine whether Exd and Hth stimulate Hox binding to DllR, we performed electrophoretic mobility shift assays (EMSAs) with purified Ubx, Exd, and Hth proteins. Unless stated otherwise, all of these experiments were performed with UbxIa, the most widely expressed of several Ubx isoforms (Kornfeld et al., 1989). By themselves, Ubx or an Exd/Hth heterodimer are capable of weakly interacting with DllR (Figure 1B, lanes 2 and 3, respectively). The combination of all three proteins results in a slower migrating band indicating the formation of a Ubx/Exd/Hth/DNA complex (Figure 1B, lanes 4–6). The formation of this protein/DNA complex is highly cooperative when compared to the amount of binding observed with Ubx or Exd/Hth alone. To test the contribution of each binding site, we introduced point mutations within the individual Hox, Exd, and Hth sites (Figure 1A). Mutation of any one of these sites results in a decrease in the formation of the trimeric protein/DNA complex, suggesting that all three are required for optimal binding to DllR (Figure 1B).

To test whether the Hox, Exd, and Hth binding sites are also required for Dll repression in vivo, we created reporter constructs containing the *lacZ* gene under the control of mutant versions of the DME enhancer. Mutation of the Hox site (DME^{Hox}) results in a similar level of derepression of reporter gene expression throughout the abdomen as the complete deletion of DllR (Figure 1I). Mutation of the Exd (DME^{Exd}) and Hth (DME^{Hth}) sites individually also results in derepression, albeit slightly weaker than mutation of the Hox site (Figures 1J and 1K). However, if both the Exd and Hth sites are mutated together, full derepression is observed (Figure 1L). Taken together, these results demonstrate that the efficient formation of a Hox/Exd/Hth trimeric complex on DllR is required for *Dll* repression within the abdomen.

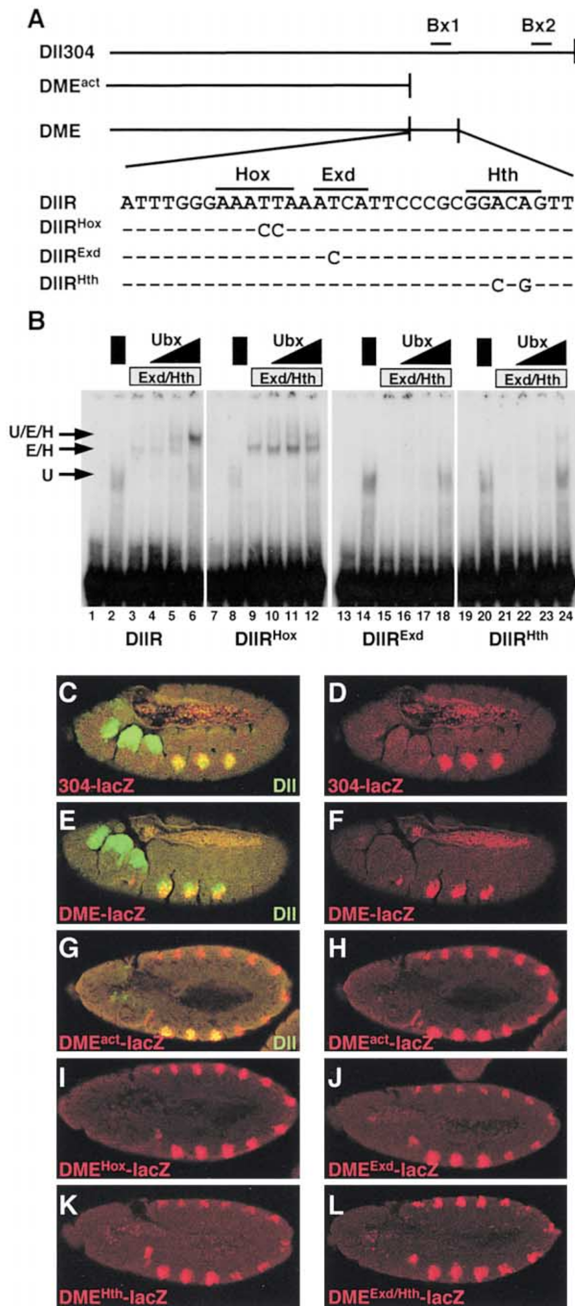

Figure 1. Hox/Exd/Hth Binding Sites Are Required for the Repression of *Dll*

(A) Maps of Dll304, DME, and DME^{act}. The sequence of DllR and the Hox, Exd, and Hth point mutations are indicated.

(B) EMSAs using labeled DllR, $DllR^{Hox}$, $DllR^{Exd}$, and $DllR^{Hth}$ probes and purified Ubx and Exd/Hth proteins as indicated. The arrows highlight the presence of slower migrating complexes that represent Ubx monomers (U), Exd/Hth dimers (E/H), and trimeric protein complexes (U/E/H). Point mutations of the Hox, Exd, or Hth binding sites result in a reduction of the Ubx/Exd/Hth/DNA complex.

(C–L) β-gal expression (red) in wild-type embryos driven by the *Dll304* (C and D), *DME* (E and F), DME^{act} (G and H), DME^{Hox} (I), DME^{Exd} (J), DME^{Hth} (K), and $DME^{Exd/Hth}$ (L) enhancers.

(C, E, and G) Embryos were costained for endogenous *Dll* expression (green). Deletion or point mutations of the Hox, Exd, and Hth binding sites result in derepression of reporter gene expression within the abdomen. The $DME^{Exd/Hth}$ sequence contains the mutations present in both DME^{Exd} and DME^{Hth}.

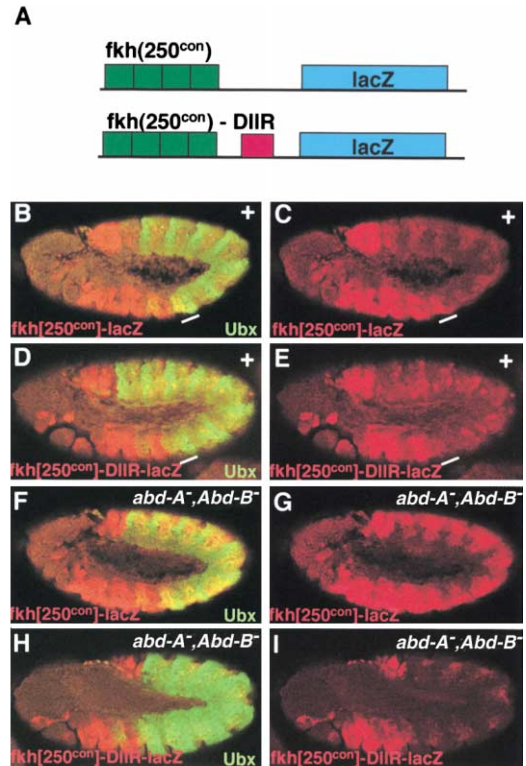

Figure 2. Ubx Can Repress *fkh(250^{con})* Expression through the DllR Element

(A) Maps of *fkh(250^{con})-lacZ* and *fkh(250^{con})-DllR-lacZ*.

(B–I) In wild-type embryos, *fkh(250^{con})-lacZ* (B and C) expression (red) overlaps with Ubx expression (green) in PS 6 (underlined), whereas *fkh(250^{con})-DllR-lacZ* (D and E) is not expressed within this PS. In $abd\text{-}A^{-}$ $Abd\text{-}B^{-}$ embryos (F–I), Ubx is derepressed in the abdomen, resulting in the activation of *fkh(250^{con})-lacZ* expression (F and G). Expression of *fkh(250^{con})-DllR-lacZ* (H and I) in this genetic background, however, is repressed in the abdomen, indicating that Ubx represses gene expression through the DllR site.

Hox/Exd/Hth Complexes Are Used for Both Activation and Repression

The above data support a model in which a Ubx/Exd/Hth complex bound to DllR is necessary for *Dll* repression. We next tested whether a single copy of DllR is sufficient to repress a heterologous enhancer element. An artificial enhancer, called fkh(250^{con}), is activated by Scr, Antp, and Ubx (with Exd and Hth), and thus provides a useful heterologous activator to test for DllR function (Ryoo and Mann, 1999). We created a reporter construct under the control of both fkh(250^{con}) and DllR (Figure 2A). Unlike fkh(250^{con}), which is expressed in parasegments (PS) 2–6, the composite enhancer (fkh250^{con}-DllR) is not expressed in PS 6, where Ubx is expressed (Figures 2B–2E). Ubx-mediated repression of fkh(250^{con})-DllR is more obvious in embryos mutant for *abd-A*, which derepress *Ubx* and, consequently, fkh(250^{con}) throughout the abdomen (Figures 2F and 2G). In this genetic background, fkh(250^{con})-DllR is still only active in PS 2–5 (Figures 2H and 2I). Furthermore, misexpression of Ubx throughout the embryo activates fkh(250^{con}) but represses fkh(250^{con})-DllR (data not shown; Ryoo and Mann, 1999). Taken together, these results indicate that DllR is sufficient to confer Ubx-mediated repression of

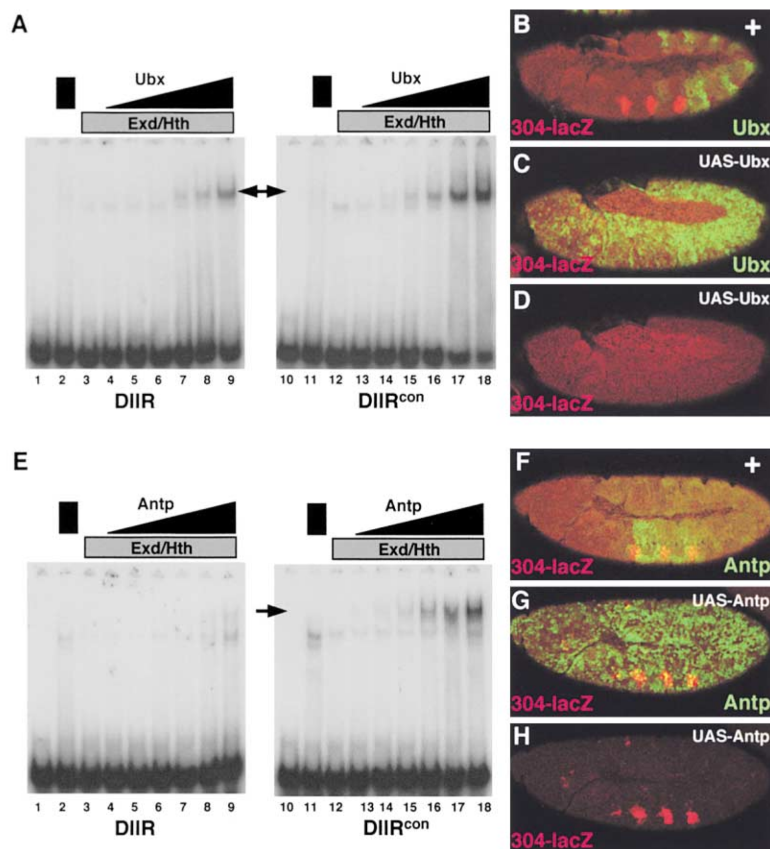

Figure 3. UbxIa, but Not Antp, Binds Cooperatively with Exd and Hth and Represses *Dll*

(A and E) EMSAs using the DllR and DllR^{con} probes and UbxIa, Antp, and Exd/Hth as indicated. The arrows indicate the formation of trimeric protein complexes.

(B–D and F–H) *Dll304-lacZ* expression (red) and Ubx or Antp (green) as indicated. Misexpression of UbxIa results in Dll304 repression (C and D). Wild-type Antp expression overlaps with *Dll304-lacZ* expression (F) and misexpression of Antp does not affect *Dll304* activity (G and H).

a heterologous enhancer. In addition, these results also illustrate that Ubx/Exd/Hth complexes can mediate repression through DllR in the same cells as it mediates activation through fkh(250^{con}) (see Discussion).

Ubx, but Not Antp, Cooperatively Binds DllR with Exd and Hth

DllR is essential for keeping *Dll* off in the abdomen but is not required for *Dll* expression in the thorax. Therefore, Antp, which is expressed in the thorax (Figure 3F), appears to be unable to regulate *Dll* expression through this element. Even if Antp is expressed at high levels, repression of Dll304 or *Dll* is not observed (Figures 3G and 3H). In contrast, ectopic Ubx expression completely abolishes Dll304 activity (Figures 3C and 3D). One potential reason for this difference is that Antp is unable to efficiently form trimeric protein complexes on DllR. To test whether Exd and Hth selectively stimulate the binding of Ubx to DllR, we performed EMSAs using purified proteins. As shown above, Ubx cooperatively binds with Exd and Hth to the DllR element (Figure 3A). In contrast, equimolar amounts of Antp do not readily form a trimeric complex with Exd and Hth on DllR (Figure 3E). As a control for these and all subsequent DNA binding experiments, we tested whether Antp could form complexes with Exd/Hth on a consensus Hox/Exd/Hth binding site, DllR^{con}. DllR^{con} differs from DllR in that the wild-type Hox/Exd binding site in DllR (5′-AAATTAAATCA-3′) was replaced with the sequence 5′-CCATAAATCA-3′ (see Figure 7B for the complete sequence), which is readily bound by many Hox/Exd dimers (Chang et al., 1996). Using the DllR^{con} probe, Antp forms complexes with Exd and Hth nearly as well as Ubx (Figure 3E). We conclude that there are differences between Ubx and Antp that confer specific, Exd- and Hth-dependent binding to DllR. These findings support a model in which Antp is unable to repress *Dll*, at least in part, because it is unable to efficiently bind DllR with Exd and Hth.

Role of the Ubx Linker, Homeodomain, and C Terminus

The homeodomains of Antp and Ubx are very similar, differing by only 6 of 60 amino acids (Figure 4A). Outside the homeodomain the only sequence shared by these proteins is the YPWM motif, which directly contacts Exd's homeodomain in Hox/Exd heterodimers (Passner et al., 1999; Piper et al., 1999). To identify which domains in Ubx are required for it to specifically bind DllR and repress *Dll*, we characterized Ubx-Antp chimeric proteins. These proteins were divided into four parts: an N terminus (start of protein to YPWM), a linker (YPWM to homeodomain), a homeodomain, and a C-terminal tail. Each portion of the protein is represented by either A for Antp or U for Ubx (Figure 4B). These chimeric proteins were tested in EMSAs with the DllR and DllR^{con} probes to determine whether they were capable of efficiently complexing with Exd and Hth. The Ubx-Antp chimeras were also expressed in embryos and assayed for their ability to repress *Dll* and *Dll304-lacZ*. Care was taken to compare transformants that had similar levels of chimera expression, as determined by immunostaining and

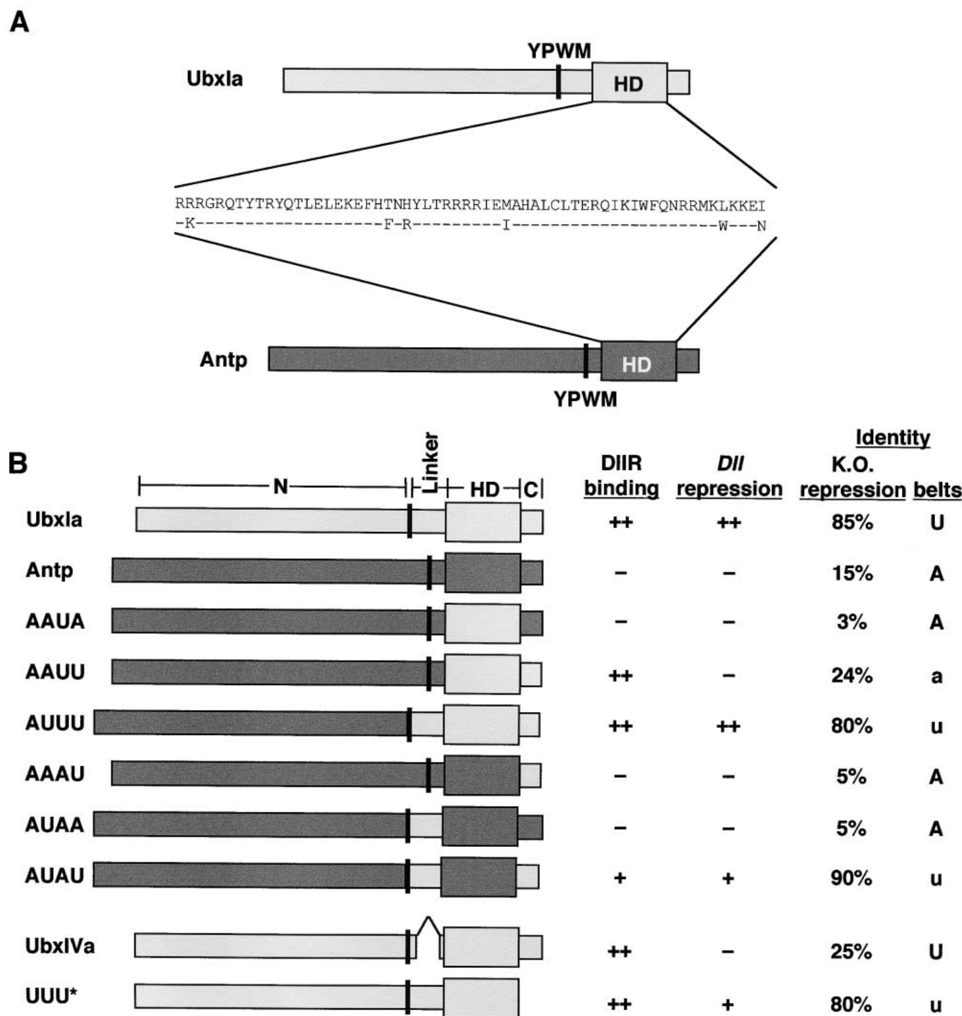

Figure 4. Maps of the Ubxla-Antp Chimeras and Summary of Their Properties

(A) Ubxla (light shade) and Antp (dark shade) contain a YPWM motif (black bar) and a highly conserved homeodomain (HD).

(B) The Ubxla-Antp chimeras are divided into four parts: the N terminus (N, up to the YPWM), linker (YPWM to homeodomain), homeodomain (HD), and C terminus (C). Each portion of the protein is represented by either A for Antp or U for Ubxla. On the right-hand side, the ability of these proteins to bind DllR, repress *Dll*, suppress Keilin's organ formation, and generate Antp (A)-like or Ubx (U)-like cuticle transformations is summarized. Strong phenotypes are indicated with upper case letters (A or U) and weaker phenotypes are indicated with lower case letters (a or u). Examples of cuticle preparations for the different constructs are shown in Supplemental Figure S1.

confocal microscopy (not shown). Finally, embryonic cuticle phenotypes were examined to determine whether the chimeras generate Antp- or Ubx-like phenotypes (Figure 4 and Supplemental Figure S1, available at http://www.developmentalcell.com/cgi/content/full/3/4/487/DC1).

We first tested whether replacing Antp's homeodomain with Ubx's homeodomain is sufficient to change its properties, as measured by these assays. This chimera, AAUA, is unable to efficiently bind DllR in EMSAs (Figure 5A). Consistent with this finding, the misexpression of AAUA in embryos does not repress Dll304 activity (Figure 5A) or endogenous *Dll* levels (data not shown). Further, like Antp, AAUA transforms the T1 denticle belt to a T2 identity and does not suppress the formation of Keilin's organs (Figure 4). The results of these assays indicate that AAUA behaves like Antp.

The finding that AAUA cannot repress *Dll* suggests that Ubx sequences outside the homeodomain are required for this function. Replacing both the homeodomain and C terminus of Antp with the equivalent regions of Ubx results in a protein (AAUU) that binds DllR with similar affinities as Ubx (Figure 5B). Surprisingly, however, AAUU is unable to repress *Dll* expression (Figure 5B) or suppress Keilin's organ formation (Figure 4). The denticle belts from AAUU cuticle preparations are also mostly Antp-like; however, a few A1-like denticles within each belt suggest that AAUU has partial Ubx-like characteristics. These findings suggest that the Ubx C terminus stimulates DNA binding to DllR, but that this DNA binding activity is insufficient for *Dll* repression.

We next tested AUUU to determine whether the linker region of Ubx participates in *Dll* repression. This chimera binds DllR similarly to Ubx and represses *Dll304-lacZ* and *Dll* (Figure 5C and data not shown). Furthermore, the denticle belt transformations were mostly A1-like

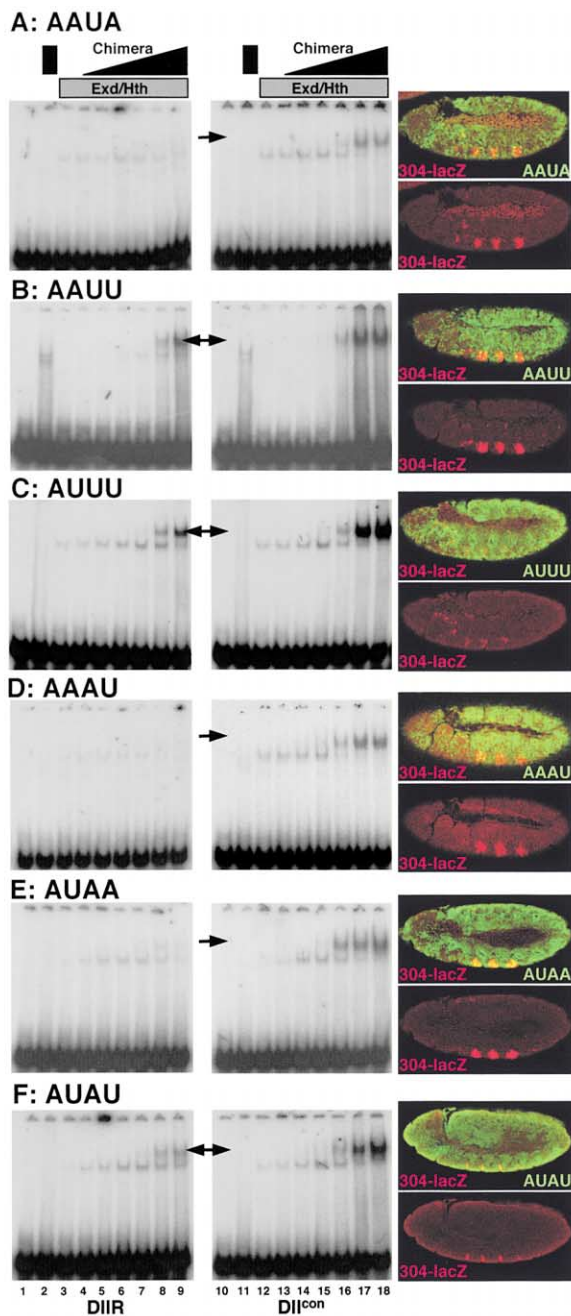

Figure 5. DNA Binding and *Dll* Repression Properties of Ubx-Antp Chimeras

EMSAs were performed using DllR and DllR^{con} probes and the Ubx-Antp chimeras with Exd/Hth as indicated. Trimeric protein complex formation is indicated by arrows. The AAUA (A), AAAU (D), and AUAA (E) proteins only efficiently form a trimeric protein complex with Exd/Hth on DllR^{con}, whereas AAUU (B) and AUUU (C) also bind DllR. The AUAU (F) protein has DNA binding properties on DllR in between those for Ubx and Antp. Embryos misexpressing the chimeras (detected with an anti-Antp antibody; green) were analyzed for *Dll304-lacZ* expression (red). Only the AUUU (C) and AUAU (F) proteins repress *Dll304-lacZ*.

in nature, and AUUU was very efficient at suppressing Keilin's organ formation (Figure 4). Taken together, these results suggest that swapping the linker, homeodomain, and C terminus is sufficient to change the properties of Antp into a Ubx-like protein. The inability of these domains to confer a complete A1 denticle belt transformation suggests that the N-terminal region of Ubx contains additional functions required for this transformation.

AAUU, but not AAUA, efficiently binds DllR, suggesting that either the C terminus of Ubx stimulates or the Antp C terminus inhibits binding to DllR. To distinguish between these possibilities, we tested AAAU and found that this protein is unable to efficiently bind DllR and is also unable to repress *Dll* (Figure 5D). This finding indicates that the Ubx C terminus is not sufficient to convey Ubx-like binding to DllR. Furthermore, it suggests that the Antp C terminus does not inhibit DNA binding, a hypothesis also supported by the finding that a truncated Antp protein completely lacking a C terminus (AAA*) does not bind DllR any better than full-length Antp (data not shown). However, to rule out the possibility that the Antp homeodomain simply has a weaker affinity for DllR, we also tested AAU*, which lacks a C terminus but contains the Antp N terminus and linker and the Ubx homeodomain. Like Antp, AAU* is unable to bind DllR with Exd and Hth, but readily forms trimeric protein complexes on the DllR^{con} probe (data not shown). Therefore, because AAUU, but not AAUA or AAU*, binds DllR, we conclude that the C terminus of Ubx stimulates binding to this site.

Swapping the Linker and C Terminus Is Sufficient to Confer Ubx-like Properties on Antp

Even though both AAUU and AUUU bound DllR well, only AUUU was able to repress *Dll* in vivo, indicating that the Ubx linker is required for this function. The Ubx linker by itself, however, was unable to confer this ability to Antp, because AUAA does not repress *Dll* (Figure 5E). This result is not surprising, because AUAA is also unable to efficiently bind DllR (Figure 5E). Because the Ubx C terminus stimulates binding to DllR, we tested the AUAU chimera in these assays. AUAU forms trimeric complexes with Exd and Hth on DllR, although not as well as Ubx (Figure 5F), suggesting that the Ubx linker, in the presence of the Ubx C terminus, contributes to DNA binding. Moreover, misexpression of AUAU is sufficient to repress *Dll* and *Dll304-lacZ*, (Figure 5F and data not shown), suppress Keilin's organ formation, and, like AUUU, partially transform thoracic segments to an A1 morphology (Figure 4). These results indicate that the Ubx linker plus C terminus is sufficient to convert Antp into a *Dll* repressor.

The Linker Region of UbxIa Is Required for Efficient Repression

All of the above in vitro and in vivo experiments were based on the UbxIa isoform, whose linker region is 41 amino acids in length. Based on these data, we conclude that the UbxIa linker and C terminus are sufficient to confer *Dll* repression activity when present in Antp. But are these domains necessary for Ubx to repress *Dll*? The *Ubx* gene is alternatively spliced and the UbxIVa isoform has a shorter linker than the UbxIa isoform (7 versus 41 amino acids; Kornfeld et al., 1989; Figure 4B). UbxIVa binds to DllR with Exd and Hth as well as UbxIa,

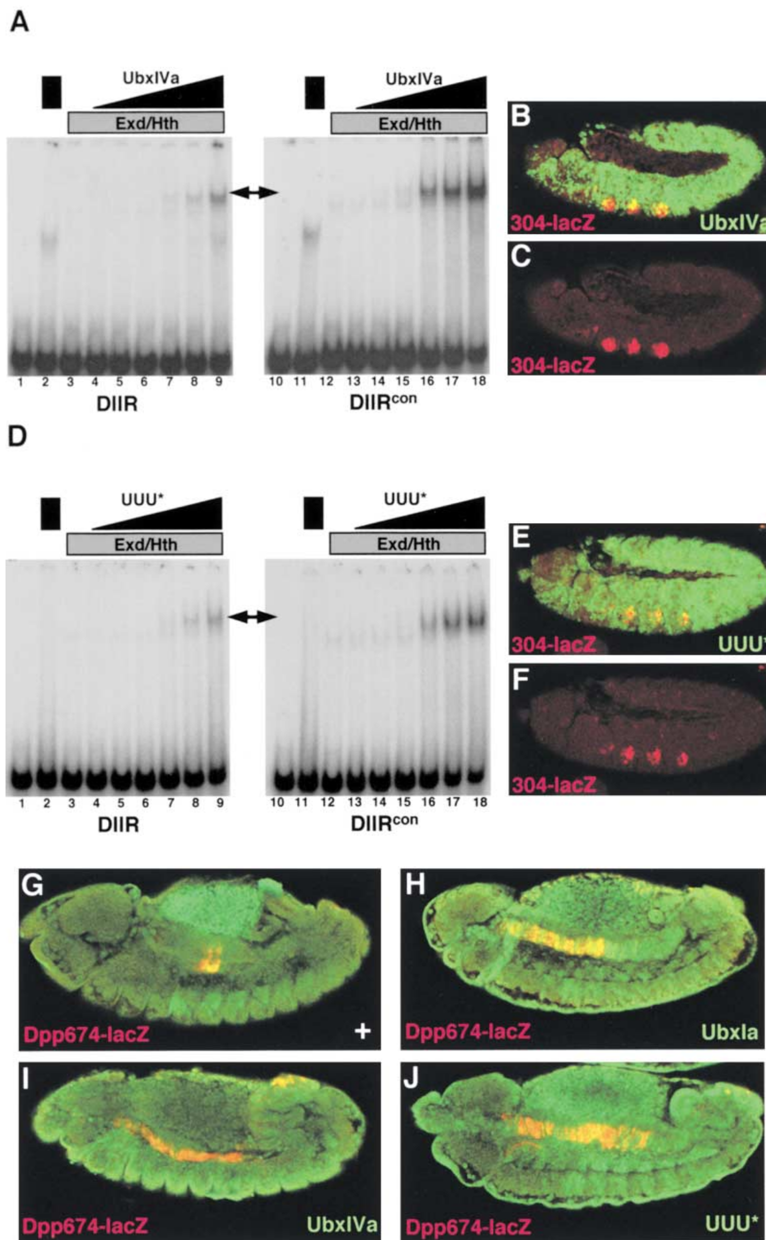

Figure 6. Role of the Linker and C Terminus of Ubx in Gene Activation and Repression

(A and D) EMSAs were performed with UbxIVa and UUU* as indicated. Both of these proteins efficiently bind DllR and DllR^{con} with Exd and Hth (arrows).

(B, C, E, and F) Misexpression of UbxIVa ([B and C]; green) resulted in no significant decrease in *Dll304-lacZ* (red). UUU* ([E and F]; green) partially represses *Dll304-lacZ* (red).

(G–J) Ubx (green) and *Dpp674-lacZ* (red) expression were detected by immunofluorescence. In wild-type embryos (G), Ubx is expressed in PS 7 of the vm. Misexpression of UbxIa (H), UbxIVa (I), and UUU* (J) activates *Dpp674-lacZ* in the anterior vm.

indicating that the linker is not required for binding to this DNA (Figure 6A). However, when expressed at equivalent levels as UbxIa, UbxIVa is unable to repress *Dll* (Figures 6B and 6C) and fails to suppress Keilin's organ formation (Figure 4). However, UbxIVa is able to fully transform thoracic denticle belts to an A1 identity and can also activate *Dpp674-lacZ*, a Ubx target in PS 7 of the visceral mesoderm (vm; Capovilla et al., 1994; Sun et al., 1995; Figures 6G–6I). These findings are consistent with the chimera data described above and demonstrate that the linker of UbxIa is required for efficient *Dll* repression, but not for DllR binding.

To determine the role of the Ubx C terminus in the regulation of *Dll*, we created a truncated form of UbxIa that lacks this domain (UUU*). Somewhat surprisingly, this protein is able to bind DllR as well as UbxIa (Figure 6D). However, UUU* is only partially able to repress *Dll* and *Dll304-lacZ* (Figures 6E and 6F and data not shown). UUU* can also activate Dpp674 in the vm (Figure 6J). These results suggest that while the Ubx C terminus stimulates DNA binding to DllR, it is not absolutely required for this function.

The DllR^{con} Binding Site Does Not Confer Repression

The above results suggest that, in the context of a Hox/Exd/Hth complex bound to DllR, both the Ubx linker and C terminus are necessary for efficient repression. One possible interpretation of these data is that these domains function by directly recruiting factors that repress transcription. To test whether these elements are sufficient to repress transcription, we carried out cell culture transcription assays in *Drosophila* S2 cells. We found

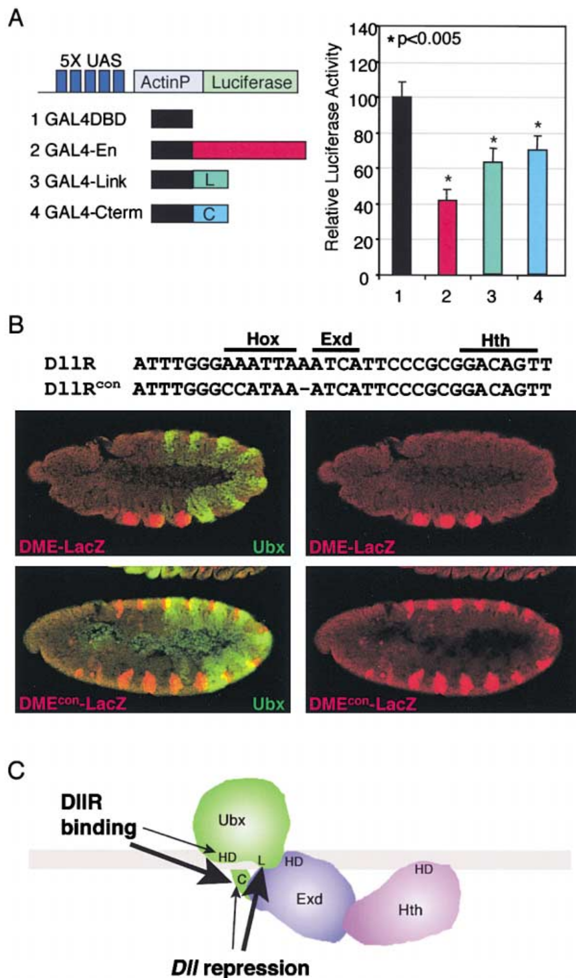

Figure 7. DllR^{con} Does Not Support Repression

(A) Cell culture transcription assays in S2 cells. The diagram on the top left shows the reporter construct and below it the four experimental conditions tested. Gal4DBD contains only the Gal4 DNA binding domain; the other proteins are fusions with either the Ubxla linker (L) or the Ubx C terminus (C).

(B) The sequences of DllR and DllR^{con} are shown. Expression of *DME-lacZ* and *DME^{con}-lacZ* (red) in wild-type embryos is compared with Ubx (green).

(C) The contribution of Ubx domains required for *Dll* repression. The arrows point to domains within Ubxla that make this Hox protein distinct from Antp. The size of the arrow indicates the relative contribution each domain has toward either DllR binding or *Dll* repression. HD, homeodomain; C, C terminus; L, linker.

that, when bound to DNA via a Gal4 DNA binding domain, both the Ubxla linker and C terminus could repress expression of a heterologous promoter by about 30% (Figure 7A). This level of repression was about half that observed for the well-characterized repressor domain from Engrailed, which served as a positive control for repression. These results suggest that both the Ubxla linker and C terminus have some potential to act as repressor domains.

Because Ubxla can both activate and repress transcription depending on the target gene, we reasoned that the repressor activities of the linker and C terminus must depend on their in vivo context. To test this idea, we determined whether a Ubx/Exd/Hth complex was able to repress transcription when bound to DllR^{con}, which has a higher affinity for Ubx/Exd/Hth than DllR (Figure 3). We substituted DllR with DllR^{con} in the DME enhancer to make DME^{con} and tested its ability to activate a reporter gene in vivo. Interestingly, *DME^{con}-lacZ* is expressed in more segments than *DME-lacZ*, including all abdominal segments (Figure 7B). The only difference between DME^{con} and DME is in four positions within the Hox/Exd binding site (Figure 7B). One of these differences is that the Hox/Exd site present in DME contains an additional A between the Hox and Exd half-sites. Partial derepression is also observed when the additional A is either deleted or changed to a T, C, G, or AA (data not shown). These results suggest that a Ubxla/Exd/Hth complex, bound to DllR^{con}, is unable to repress transcription. We conclude that either the conformation of the trimer bound to DME^{con} cannot support repression or that another factor required for repression cannot bind with Ubxla/Exd/Hth to DME^{con}.

Discussion

A general question for all transcription factors is how they achieve specificity in vivo. For the Hox proteins, a large number of studies have implicated sequences both within and outside the homeodomain as being important for their in vivo specificities (Kuziora and McGinnis, 1989; Gibson et al., 1990; Lin and McGinnis, 1992; Chan and Mann, 1993; Furukubo-Tokunaga et al., 1993; Zeng et al., 1993; Chauvet et al., 2000; Grenier and Carroll, 2000; Galant and Carroll, 2002; Ronshaugen et al., 2002). But how do these sequences function? Because DNA binding domains, including homeodomains, can also be protein interaction domains, studies that map the domains necessary for target gene regulation cannot answer this question by themselves. Instead, direct transcriptional targets must be identified and, once binding sites are characterized, DNA binding, in addition to target gene regulation, must be measured. We characterized a Hox/Exd/Hth binding site (DllR) that mediates *Dll* repression in the *Drosophila* abdomen. Our results allow us to discriminate two steps in the repression of *Dll* by Ubx. First, Exd and Hth stimulate Ubx, but not Antp, binding to DllR. In contrast, Ubx/Exd/Hth and Antp/Exd/Hth have similar affinities for a consensus binding site, suggesting that subtle differences in the DNA sequence, in addition to differences between Ubx and Antp, contribute to specificity. We found that a C-terminal peptide in Ubx stimulated this cofactor-dependent binding to DllR (Figure 7C). DNA binding, however, was not sufficient for *Dll* repression. Instead, an additional linker domain included in only a subset of Ubx isoforms was required for repression (Figure 7C). Thus, a second step, the recruitment of additional factors to the Ubx/Exd/Hth complex bound to DllR, is implied by these data. In addition to the Ubxla linker, this step also requires the specific sequences and conformation imposed on the Ubx/Exd/Hth trimer by DllR.

Multiple Domains within Ubx Contribute to Specific Binding to DllR

Although the Ubx C terminus plays an important role in cofactor-dependent binding to DllR, additional domains

contribute to optimal binding (Figures 4B and 7C). In the presence of Exd and Hth, the AAUU chimera, but not AAUA or AAAU, bound DllR, suggesting that both the Ubx homeodomain and C terminus are important for optimal DNA binding to this site. The C terminus is not absolutely required for binding because a Ubx protein that lacks this domain (UUU*) was still able to bind well to DllR. Last, the finding that UUU*, but not AAU*, binds DllR suggests that a domain N terminal to the homeodomain also enhances DllR binding. Based on the crystal structures of Hox/Exd/DNA complexes (Passner et al., 1999; Piper et al., 1999), this difference could be due to the YPWM motif (see below). Taken together, the data suggest that multiple regions of Ubx contribute to binding DllR and that no one domain is sufficient for full binding activity (Figure 7C). This finding may be understood in light of the fact that the entire Ubx coding sequence has been constrained over millions of years of insect evolution to maintain leg (and *Dll*) repression in the abdomen.

How might the Ubx C terminus and YPWM motifs contribute to DNA binding? We suggest that these regions could make additional protein-DNA contacts and/or protein-protein interactions that help stabilize the DllR-bound form of the trimeric complex. In support of this idea, the C termini of other homeodomain proteins also contribute to DNA binding. The Exd C terminus, for example, consists of an α helix that packs against its homeodomain and contributes to DNA binding (Piper et al., 1999). The C terminus of the MATα2 protein from yeast forms an α helix that contacts the MATa1 homeodomain to stabilize heterodimer formation on DNA (Li et al., 1995). Interestingly, the two Hox proteins that repress *Dll* expression, Ubx and Abd-A, share sequence homology in their C termini, and are the only *Drosophila* Hox proteins predicted to form an α helix after their homeodomains.

The Ubx YPWM motif may also help stabilize complex formation on DllR. In the Hox/Exd/DNA crystal structures, this motif, together with flanking amino acids, directly contacts a hydrophobic pocket within the Exd homeodomain (Passner et al., 1999; Piper et al., 1999). These protein-protein contacts are thought to stabilize protein-DNA contacts made by the complex. The amino acids surrounding the YPWM motifs are different in Ubx and Antp and thus could contribute to DNA binding specificity by such an indirect mechanism.

Different Isoforms of Ubx and Transcriptional Regulation

Our finding that UbxIa, but not UbxIVa, is able to repress *Dll* suggests that the linker region in UbxIa is required for repression. In addition, these results suggest that alternative splicing has the potential to modulate Ubx's control of gene expression. In support of this view, the expression of Ubx isoforms is temporally and spatially regulated (Kornfeld et al., 1989; Lopez and Hogness, 1991). In addition, misexpression experiments using UbxIa and UbxIVa have shown that while both perform many of the same functions, only UbxIa efficiently transforms the peripheral nervous system (Mann and Hogness, 1990). Our finding that UbxIa and UbxIVa have different transcriptional regulatory properties provides a possible explanation for their distinct abilities to transform this tissue.

One argument against the idea that the different Ubx isoforms have distinct functions is that flies containing a genetic inversion that prevents the inclusion of the second microexon are, for the most part, normal (Busturia et al., 1990). Although this mutation prevents the expression of UbxIa, it is unclear which other Ubx isoforms are expressed in this mutant because the inversion does not include both microexons. Furthermore, the effect that this mutation has on *Dll* expression has not been examined. A definitive test of the idea that Ubx isoforms have unique functions will require determining whether a *Ubx* allele in which both microexons are eliminated can provide all *Ubx* functions in vivo.

Evolution of Functional Domains among Ubx Orthologs

As in *Drosophila*, *Dll* expression is a marker for leg primordia in many animal phyla (Panganiban et al., 1997). Animals with appendages on their abdominal segments, such as crustaceans and onychophora, coexpress *Ubx* with *Dll*, demonstrating that Ubx is not a repressor of *Dll* in these species (Palopoli and Patel, 1998; Grenier and Carroll, 2000). The ability of Ubx to repress *Dll* probably arose in a subset of arthropods, the hexapods. Consistent with our findings, two recent studies suggest that one relevant difference between Ubx orthologs that repress *Dll* (for example, *Drosophila* Ubx) and Ubx orthologs that do not repress *Dll* (for example, onychophoran Ubx) maps to the C-terminal regions of these Hox proteins (Galant and Carroll, 2002; Ronshaugen et al., 2002). These two groups, however, propose different mechanisms for how these sequences function. Galant and Carroll suggest that the *Drosophila* Ubx C terminus actively represses transcription via a polyalanine motif that is present in the Ubx orthologs from all hexapods (Galant and Carroll, 2002). Ronshaugen et al. suggest that the *Drosophila* Ubx C terminus is only permissive for repression. Instead, they argue that crustaceans, which have abdominal legs, evolved a C-terminal sequence that inhibits *Dll* repression (Ronshaugen et al., 2002). However, neither group analyzed the binding of these proteins to the relevant binding sites in *Dll*, leaving open the possibility that the effects they observe could also be due to effects on DNA binding.

Our data provide additional insights into how repression mechanisms may have evolved in these different species. We found that the *Drosophila* Ubx C terminus contributes to DllR binding but is not sufficient for *Dll* repression in vivo. Thus, the positive role, observed by Galant and Carroll, that the *Drosophila* sequence plays in *Dll* repression, could be due to an effect on DNA binding (Galant and Carroll, 2002). Our experiments also implicate the linker region of UbxIa as important for repression, but not DNA binding. Because some of the onychophora/*Drosophila* and crustacean/*Drosophila* chimeras lack this linker but are able to repress *Dll*, the crustacean and onychophoran Ubx orthologs must have repression domains that are different from the one we identified in *Drosophila* Ubx.

Ronshaugen et al. suggested that the phosphorylation of serine and threonine residues in the crustacean Ubx

C terminus is necessary for it to prevent *Dll* repression (Ronshaugen et al., 2002). This is an intriguing possibility in light of the fact that phosphorylation of a Hox C terminus can inhibit cooperative DNA binding with Exd (Jaffe et al., 1997). Taken together with our data that the C terminus of Ubx enhances DNA binding to DllR, we suggest that the inhibition of *Dll* repression by the crustacean C terminus may be due to a reduced ability to bind DllR with Exd and Hth. This model accounts for why a *Drosophila* UbxIa protein containing the crustacean C terminus is unable to repress *Dll* (Ronshaugen et al., 2002) and for the inability of onychophora Ubx, which also contains a putative phosphorylation site in its C terminus, to repress *Dll*. Taken together, we suggest that the evolution of limb suppression by Hox proteins, and probably many other Hox functions, depended upon the modification of both DNA binding-dependent and -independent mechanisms controlling Hox specificity.

Transcriptional Activation versus Repression by Hox/Exd/Hth Complexes

Although our experiments focused on understanding why Antp is different from Ubx, the results provide some insights into the mechanism of transcriptional repression. The data strongly argue that a DNA-bound Ubx/Exd/Hth complex is necessary, but not sufficient, for repression. First, in addition to repressing *Dll*, Ubx/Exd/Hth activates fkh(250^{con}) (Ryoo and Mann, 1999). When both fkh(250^{con}) and DllR simultaneously regulate the same reporter gene, DllR was able to repress gene expression in the same cells that fkh(250^{con}) normally activates gene expression. This result suggests that the repressor proteins required for DllR activity are not cell type specific and are widely expressed in the embryo. Further, these results suggest that differences between the fkh(250^{con}) and DllR sequences determine whether transcription is activated or repressed. These sequences may recruit additional DNA binding factors that interact with the trimeric complex. These factors, which have not yet been identified, might provide or reveal a latent activation or repression domain within the Hox/Exd/Hth complex. Alternatively, another DNA binding factor may not be needed. Instead, the unique arrangement or spacing of the Hox, Exd, and Hth sites in these two elements may result in distinct conformations of the trimeric complex that recruit different coactivators or corepressors. Such a mechanism has been suggested for the nuclear receptor family of transcription factors and for the POU domain protein Pit-1, where a difference in spacing in a Pit-1 dimer binding site regulates the recruitment of a corepressor (Lefstin and Yamamoto, 1998; Scully et al., 2000). Consistent with such a mechanism, we found that the $DllR^{con}$ binding site, which has one less base pair between the Hox and Exd half-sites than the DllR binding site, fails to repress transcription despite having a higher affinity for Ubx/Exd/Hth complexes. In addition, although we were able to measure repression activity for the UbxIa linker and C terminus in S2 cells, our experiments suggest that their activities are context dependent. The abdominal expression of *DME^con^-lacZ* suggests that the mere presence of these domains is not sufficient for repression. Thus, the data suggest that transcription factor domains have distinct properties when assayed by themselves versus when they are part of a multiprotein complex. Further, we conclude that the unique architecture of the complex assembled on DllR is necessary for efficient repression.

Experimental Procedures

Plasmids

DME (residues 1–713 of Dll304) and DME^{act} (residues 1–680 of Dll304) were cloned into *hs43-lacZ*. Mutation of the Hox, Exd, or Hth sites was carried out by PCR. The *fkh(250^{con})-lacZ* construct has been described (Ryoo and Mann, 1999) and *fkh(250^{con})-DllR-lacZ* was assembled by cloning DllR (residues 633–713 of Dll304) between the fkh(250^{con}) element and the promoter.

His-tagged UbxIa (Ryoo and Mann, 1999), Antp (Jaffe et al., 1997), and Exd (Chan et al., 1997) constructs have been described. An untagged Hth expression vector was assembled by cloning the full-length Hth coding sequence into pET9 (Novagen). A near full-length UbxIVa coding sequence (residues 57–346) was cloned in-frame with the 6× His tag of pQE9 (Qiagen). Antp-Ubx chimeras were prepared by PCR and cloned in-frame into pET14b (Novagen). Conservative mutations within Antp (at residues 276–277) were introduced to create a SacI site. The linker, homeodomain, and C-terminal regions of UbxIa or Antp were cloned in-frame with the rest of Antp using this SacI site. The parts of Antp are defined as the N terminus (residues 1–287), linker (residues 288–295), homeodomain (residues 296–356), and C terminus (residues 357–378), and for UbxIa as the linker (residues 244–284), homeodomain (residues 285–345), and C terminus (residues 346–380). Open reading frames were cloned into the pUAST vector for misexpression in flies (Brand and Perrimon, 1993). All constructs were confirmed by DNA sequencing.

Protein Purification and EMSAs

His-tagged constructs were transformed into BL21 and protein expression was induced for 2 hr with IPTG. The His-Exd and Hth constructs were transformed together, placed under double antibiotic selection, and purified as heterodimers under native conditions using Ni chromatography. Antp, UbxIa, UbxIVa, UUU*, and all the chimeras were purified as described (Thanos and Maniatis, 1996). All proteins were quantitated by the Bradford assay and confirmed by SDS-PAGE and Coomassie blue analysis. EMSAs were performed as described (Gebelein and Urrutia, 2001). DllR and $DllR^{con}$ are identical except that the 5′-AAATTAAATCA-3′ Hox/Exd sequence was replaced with 5′-CCATAAATCA-3′. The amount of protein used in each EMSA was 2.0 pmol of His-Exd/Hth and 0.5, 1.5, and 4.5 pmol of Ubx (Figure 1). For all other EMSAs, the amount of His-Exd/Hth used was 2.0 pmol and the amount of each Hox protein was 0.1, 0.25, 0.5, 1.0, 2.5, and 5.0 pmol. The percentage of probe bound using the highest concentration of Hox factor plus Exd/Hth was determined using phosphorimaging. The ratio of %DllR bound to %$DllR^{con}$ bound × 100 was calculated, and found to be UbxIa, 25%; Antp, 7.7%; UbxIVa, 26%; UUU*, 29%; AAUA, 6%; AAUU, 30%; AUUU, 20%; AAAU, 11%; AUAA, 12%; and AUAU, 16%.

Fly Stocks and Antibody Stainings

Transformants were made in a *yw* background. UAS-Antp (Casares and Mann, 1998) and *Dll304-lacZ* (Vachon et al., 1992) were described. Hox misexpression was driven by arm-Gal4 in the presence of *Dll304-lacZ*. All chimeras were detected using an anti-Antp antibody (Condie et al., 1991). The UbxIa, UbxIVa, and UUU* proteins were detected using FP3.38 (White and Wilcox, 1985). The anti-β-gal (Cappell) and anti-Dll (Vachon et al., 1992) antibodies have been described. In all cases, the expression of the endogenous *Dll* gene paralleled the expression of *Dll304-lacZ*. When each chimera was assayed, a parallel stain was performed for Antp and images were obtained using identical confocal settings to control for expression levels. A similar control was performed with UbxIa when UbxIVa and UUU* were analyzed.

Cell Transfection and Transcription Assays

The Gal4DBD (residues 1–147) was cloned into the pPac expression vector (InVitrogen) by itself or with the Engrailed repressor domain (Gal4-En, residues 1–298), the UbxIa linker (Gal4-link, residues 244–

284), or C terminus (Gal4-Cterm, residues 346–380). To test for repression, a firefly luciferase vector was made containing five UAS Gal4 binding sites upstream of a truncated actin promoter (−250 to +90; Chung and Keller, 1990). S2 cells were transfected in serum-free media (sfm-900) with Cellfectin (InVitrogen). An rsv-renilla luciferase vector was used to control for transfection efficiency (Gebelein and Urrutia, 2001). A total of 2 μg of plasmid per well was used as follows: 1.3 μg of the Gal4 fusion vector, 0.35 μg of UAS-firefly luciferase, and 0.35 μg of renilla luciferase. The dual luciferase assay was used to measure reporter gene expression (Promega). Similar expression levels of the Gal4-Ubx proteins were detected by Western blot analysis using an anti-Gal4DBD antibody (Santa Cruz) and chemiluminescence (Amersham; data not shown).

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References

Brand, A., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401–415.

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.

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. (1995). Homeotic genes and the evolution of arthropods and chordates. Nature *376*, 479–485.

Casares, F., and Mann, R.S. (1998). Control of antennal versus leg development in *Drosophila*. Nature *392*, 723–726.

Chan, S.-K., and Mann, R.S. (1993). The segment identity functions of Ultrabithorax are contained within its homeo domain and carboxy-terminal sequences. Genes Dev. *7*, 796–811.

Chan, S.-K., Ryoo, H.D., Gould, A., Krumlauf, R., and Mann, R. (1997). Switching the in vivo specificity of a minimal Hox-responsive element. Development *124*, 2007–2014.

Chang, C.P., Brocchieri, L., Shen, W.F., Largman, C., and Cleary, M.L. (1996). Pbx modulation of Hox homeodomain amino-terminal arms establishes different DNA-binding specificities across the Hox locus. Mol. Cell. Biol. *16*, 1734–1745.

Chauvet, S., Merabet, S., Bilder, D., Scott, M.P., Pradel, J., and Graba, Y. (2000). Distinct hox protein sequences determine specificity in different tissues. Proc. Natl. Acad. Sci. USA *97*, 4064–4069.

Chung, Y.-T., and Keller, E.B. (1990). Positive and negative regulatory elements mediating transcription from the *Drosophila melanogaster* actin 5C distal promoter. Mol. Cell. Biol. *10*, 6172–6180.

Condie, J.M., Mustard, J.A., and Brower, D. (1991). Generation of anti-Antennapedia monoclonal antibodies and Antennapedia protein expression in imaginal discs. Drosoph. Inf. Serv. *70*, 52–54.

Ferretti, E., Marshall, H., Popperl, H., Maconochie, M., Krumlauf, R., and Blasi, F. (2000). Segmental expression of Hoxb2 in r4 requires two separate sites that integrate cooperative interactions between Prep1, Pbx and Hox proteins. Development *127*, 155–166.

Florence, B., Guichet, A., Ephrussi, A., and Laughon, A. (1997). Ftz-F1 is a cofactor in Ftz activation of the *Drosophila* engrailed gene. Development *124*, 839–847.

Furukubo-Tokunaga, K., Flister, S., and Gehring, W.J. (1993). Functional specificity of the Antennapedia homeodomain. Proc. Natl. Acad. Sci. USA *90*, 6360–6364.

Galant, R., and Carroll, S.B. (2002). Evolution of a transcriptional repression domain in an insect Hox protein. Nature *415*, 910–913.

Gebelein, B., and Urrutia, R. (2001). Sequence-specific transcriptional repression by KS1, a multiple-zinc-finger-Kruppel-associated box protein. Mol. Cell. Biol. *21*, 928–939.

Gellon, G., and McGinnis, W. (1998). Shaping animal body plans in development and evolution by modulation of Hox expression patterns. Bioessays *20*, 116–125.

Gibson, G., Schier, A., LeMotte, P., and Gehring, W.J. (1990). The specificities of Sex combs reduced and Antennapedia are defined by a distinct portion of each protein that includes the homeodomain. Cell *62*, 1087–1103.

Graba, Y., Aragnol, D., and Pradel, J. (1997). *Drosophila* Hox complex downstream targets and the function of homeotic genes. Bioessays *19*, 379–388.

Grenier, J.K., and Carroll, S.B. (2000). Functional evolution of the Ultrabithorax protein. Proc. Natl. Acad. Sci. USA *97*, 704–709.

Guichet, A., Copeland, J.W., Erdelyi, M., Hlousek, D., Zavorszky, P., Ho, J., Brown, S., Percival-Smith, A., Krause, H.M., and Ephrussi, A. (1997). The nuclear receptor homologue Ftz-F1 and the homeodomain protein Ftz are mutually dependent cofactors. Nature *385*, 548–552.

Henderson, K.D., and Andrew, D.J. (2000). Regulation and function of Scr, exd, and hth in the *Drosophila* salivary gland. Dev. Biol. *217*, 362–374.

Jacobs, Y., Schnabel, C.A., and Cleary, M.L. (1999). Trimeric association of Hox and TALE homeodomain proteins mediates *Hoxb2* hindbrain enhancer activity. Mol. Cell. Biol. *19*, 5134–5142.

Jaffe, L., Ryoo, H.D., and Mann, R.S. (1997). A role for phosphorylation by casein kinase 2 in modulating Antennapedia function in *Drosophila*. Genes Dev. *11*, 1327–1340.

Kornfeld, K., Saint, R.B., Beachy, P.A., Harte, P.J., Peattie, D.A., and Hogness, D.S. (1989). Structure and expression of a family of *Ultrabithorax* mRNAs generated by alternative splicing and polyadenylation in *Drosophila*. Genes Dev. *3*, 243–258.

Kuziora, M.A., and McGinnis, W. (1989). A homeodomain substitution changes the regulatory specificity of the deformed protein in *Drosophila* embryos. Cell *59*, 563–571.

Lefstin, J.A., and Yamamoto, K.R. (1998). Allosteric effects of DNA on transcriptional regulators. Nature *392*, 885–888.

Li, X., and McGinnis, W.M. (1999). Activity regulation of Hox proteins, a mechanism for altering functional specificity in development and evolution. Proc. Natl. Acad. Sci. USA *96*, 6802–6807.

Li, T., Stark, M., Johnson, A., and Wolberger, C. (1995). Crystal structure of the MATa1/MATα2 homeodomain heterodimer bound to DNA. Science *270*, 262–269.

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.

Lin, L., and McGinnis, W. (1992). Mapping functional specificity in the Dfd and Ubx homeo domains. Genes Dev. *6*, 1071–1081.

Lopez, A.J., and Hogness, D.S. (1991). Immunochemical dissection of the *Ultrabithorax* homeoprotein family in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA *88*, 9924–9928.

Mann, R.S., and Affolter, M. (1998). Hox proteins meet more partners. Curr. Opin. Genet. Dev. *8*, 423–429.

Mann, R.S., and Chan, S.-K. (1996). Extra specificity from *extradenticle*: the partnership between HOX and exd/pbx homeodomain proteins. Trends Genet. *12*, 258–262.

Mann, R.S., and Hogness, D.S. (1990). Functional dissection of Ultrabithorax proteins in *D. melanogaster*. Cell *60*, 597–610.

McGinnis, W., and Krumlauf, R. (1992). Homeobox genes and axial patterning. Cell *68*, 283–302.

Nasiadka, A., Grill, A., and Krause, H.M. (2000). Mechanisms regulating target gene selection by the homeodomain-containing protein Fushi tarazu. Development *127*, 2965–2976.

Palopoli, M.F., and Patel, N.H. (1998). Evolution of the interaction between Hox genes and a downstream target. Curr. Biol. *8*, 587–590.

Panganiban, G., Irvine, S.M., Lowe, C., Roehl, H., Corley, L.S., Sherbon, B., Grenier, J.K., Fallon, J.F., Kimble, J., Walker, M., et al. (1997). The origin and evolution of animal appendages. Proc. Natl. Acad. Sci. USA *94*, 5162–5166.

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*, 714–718.

Peifer, M., and Wieschaus, E. (1990). Mutations in the *Drosophila* gene *extradenticle* affect the way specific homeo domain proteins regulate segmental identity. Genes Dev. *4*, 1209–1223.

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.

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. Genes Dev. *13*, 1704–1716.

Ryoo, H.-D., Marty, T., Casares, F., Affolter, M., and Mann, R.S. (1999). Regulation of Hox target genes by a DNA bound Homothorax/Hox/Extradenticle complex. Development *126*, 5137–5148.

Scully, K.M., Jacobson, E.M., Jepsen, K., Lunyak, V., Viadiu, H., Carriere, C., Rose, D.W., Hooshmand, F., Aggarwal, A.K., and Rosenfeld, M.G. (2000). Allosteric effects of Pit-1 DNA sites on long-term repression in cell type specification. Science *290*, 1127–1131.

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.

Thanos, D., and Maniatis, T. (1996). In vitro assembly of enhancer complexes. Methods Enzymol. *274*, 162–173.

Vachon, G., Cohen, B., Pfeifle, C., McGuffin, M.E., Botas, J., and Cohen, S. (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.

Weatherbee, S.D., and Carroll, S.B. (1999). Selector genes and limb identity in arthropods and vertebrates. Cell *30*, 283–286.

White, R.A.H., and Wilcox, M. (1985). Distribution of *Ultrabithorax* proteins in *Drosophila*. EMBO J. *4*, 2035–2043.

White, R.A., Aspland, S.E., Brookman, J.J., Clayton, L., and Sproat, G. (2000). The design and analysis of a homeotic response element. Mech. Dev. *91*, 217–226.

Yu, Y., Li, W., Su, K., Yussa, M., Han, W., Perrimon, N., and Pick, L. (1997). The nuclear hormone receptor Ftz-F1 is a cofactor for the *Drosophila* homeodomain protein Ftz. Nature *385*, 552–555.

Zeng, W., Andrew, D.J., Mathies, L.D., Horner, M.A., and Scott, M.P. (1993). Ectopic expression and function of the *Antp* and *Scr* homeotic genes: the N terminus of the homeodomain is critical to functional specificity. Development *118*, 339–352.