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The Hexapeptide and Linker Regions of the AbdA Hox Protein Regulate Its Activating and Repressive Functions

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Summary

The Hox family transcription factors control diversified morphogenesis during development and evolution. They function in concert with Pbc cofactor proteins. Pbc proteins bind the Hox hexapeptide (HX) motif and are thereby thought to confer DNA binding specificity. Here we report that mutation of the AbdA HX motif does not alter its binding site selection but does modify its transregulatory properties in a gene-specific manner in vivo. We also show that a short, evolutionarily conserved motif, PFER, in the homeodomain-HX linker region acts together with the HX to control an AbdA activation/repression switch. Our in vivo data thus reveal functions not previously anticipated from in vitro analyses for the hexapeptide motif in the regulation of Hox activity.

Introduction

Hox genes play fundamental roles in the organization of the animal body plan during development and evolution (McGinnis and Krumlauf, 1992). They encode differentially expressed transcription factors that specify morphogenetic traits along the anteroposterior axis, by locally controlling batteries of subordinate target genes (Graba et al., 1997). Hox proteins share a helix-turn-helix DNA binding motif, the homeodomain (HD), and, consequently, recognize very similar TAAT core sequences, which contrasts with their highly specific biological functions during development. It is now well established that Hox proteins gain specificity by physically interacting with Pbc class cofactors (Mann and Chan, 1996). Association with Pbc proteins increases the DNA binding specificity of Hox proteins: Hox/Pbc complexes recognize a larger motif, TGATNNATNN, where the identity of the central NN nucleotides depends on the particular Hox protein involved (Chan and Mann, 1996; Chan et al., 1997).

Hox/Pbc interactions are now well characterized in vitro, both in biochemical (Chang et al., 1995; Johnson et al., 1995; Phelan et al., 1995) and structural (Passner et al., 1999; Piper et al., 1999) terms. Pbc proteins belong to the TALE (three amino acid loop extension) class of atypical HD-containing proteins, which is characterized by a three amino acid insertion between helices 1 and 2 of the HD. These residues participate in the constitution of a hydrophobic pocket that mediates interaction with Hox proteins, through a short evolutionary conserved sequence, the hexapeptide (HX), lying upstream of the HD in all but the Abdominal-B class of Hox proteins. Structurally, the HX folds into a classical type I reverse turn and is connected to the HD by a short sequence commonly termed the linker region. The variable length and disordered structure of the linker region suggest that it has a passive role in connecting the HX to the HD.

In vitro, the HX promotes the formation of Hox/Pbc complexes with heightened DNA binding affinity and specificity (Sanchez et al., 1997; Shanmugam et al., 1997; Shen et al., 1996; Sprules et al., 2000), suggesting that this domain critically contributes to the selection of Hox target genes during development. The role of the HX in vivo has, however, been poorly investigated so far. One study has addressed the point by analyzing in *Drosophila* the effect of the HX-mutated Labial (Lab) protein on the regulation of a heterologous mouse *Hoxb1* enhancer, *3Xrpt3* (Pöpperl et al., 1995). The authors concluded that the recruitment of Extradenticle (Exd) by the HX neutralizes an inhibitory effect of the HX on Lab DNA binding. This might, however, be a very specialized function of the HX, since Lab is very peculiar in the sense that, unlike most Hox proteins, it does not bind DNA on its own (Chan et al., 1996).

Here we report a detailed analysis of the HX function in the regulation of bona fide target genes during development. We found that the HX is not involved in controlling DNA binding and target gene selection, nor is it necessary for Exd recruitment, but, rather, it controls transregulatory functions of the Hox protein Abdominal-A (AbdA). We also show that an evolutionarily conserved motif in the linker region interferes with the HX, to control a repression/activation switch in AbdA.

Results

Altering the HX and the Linker Region Does Not Affect AbdA Epidermal Functions

AbdA instructs an A2-like identity on abdominal segments A2–A5 and transforms thoracic segments into A2 when ectopically expressed (Sanchez-Herrero et al., 1994). To address the contribution of the HX and the linker region, we generated two variants, AbdA(HXm) and AbdA(PFERm), where the YPWM motif and the

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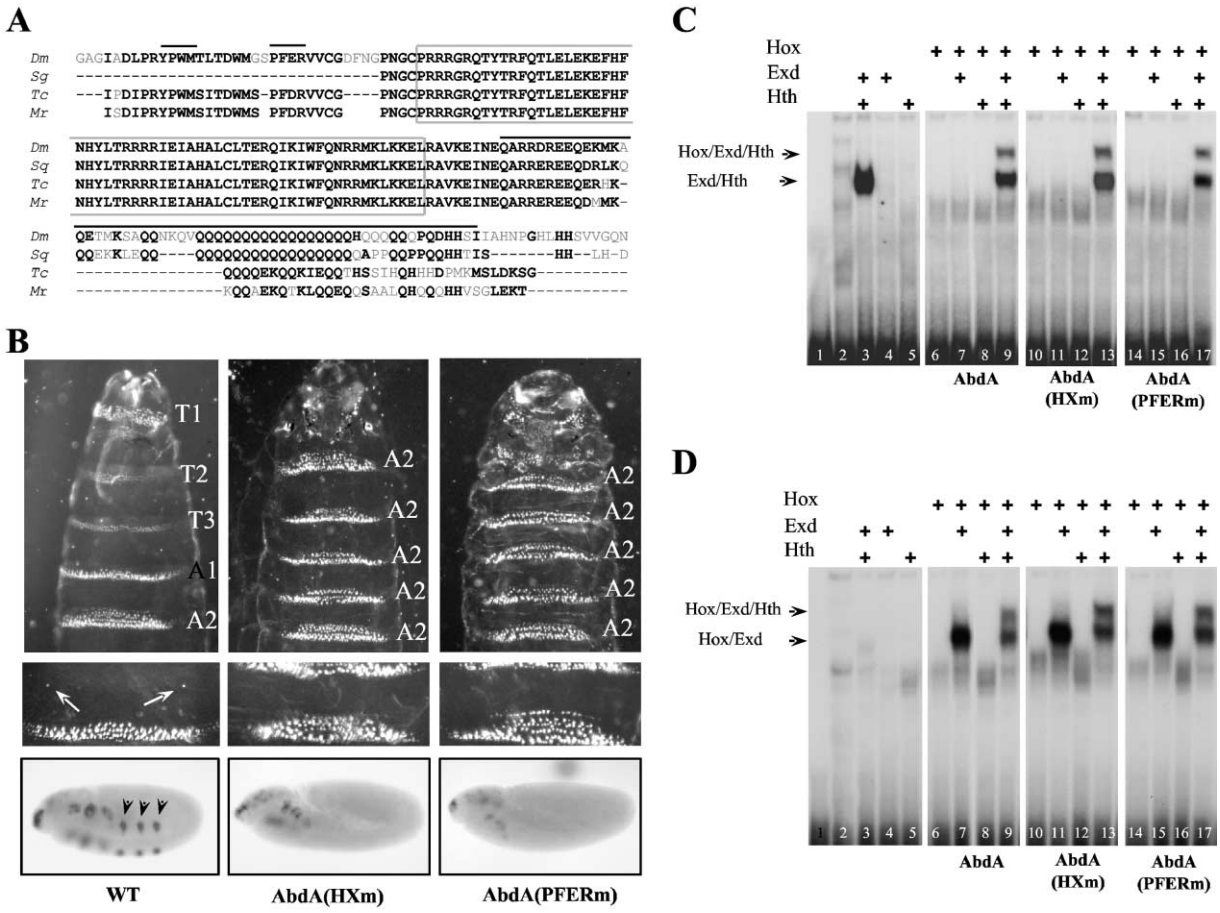

Figure 1. The Evolutionarily Conserved HX and PFER Sequences Are Dispensable for AbdA Epidermal Functions

(A) Conserved sequences in insect AbdA proteins. *Dm*, *Drosophila melanogaster*; *Sg*, *Schistocerca gregaria*; *Tc*, *Tribolium castaneum*; *Mr*, *Myrmica rubra*. Sequence conservation (identity and conservative changes), highlighted in bold, concerns the HX, the linker region, the HD (boxed), and a C-terminal glutamine- and glutamic acid-rich domain. Solid bars above the sequences highlight the sequences mutated (YPWM and PFER) or deleted [poly(Q) domain].

(B) The top panel shows the anterior part of cuticles of wild-type, 69B/AbdA(HXm), or 69B/AbdA(PFERm) animals. Driving epidermal expression of AbdA(HXm) or AbdA(PFERm) by *69B-Gal4* transforms thoracic in A2-like segments. The middle and bottom panels show, respectively, the loss of Keilin's organs (arrows) and repression of *Dll* transcription in thoracic segments (arrowheads) in the same genetic contexts.

(C) EMSA of AbdA, AbdA(HXm), and AbdA(PFERm) in the presence of Exd, Hth, or Exd/Hth on DllR double-stranded oligonucleotides. Five microliters of the programmed lysate were used for each protein and for the mock lysate (lane 2). Small variations in the amount of Exd and Hth proteins are apparent in these assays, as visualized by the slight differences in band intensity corresponding to the Exd/Hth complexes (compare lanes 9, 13, and 17).

(D) Same as in (C) on DllR^{con} double-stranded oligonucleotides.

PFER sequence were mutated into AAAA. PFER lies in the middle of the AbdA linker region, which has been fairly well conserved in the insect phylum (Figure 1A). Ubiquitous epidermal expression of each variant transforms thoracic segments into A2 identity, including changes in the denticle pattern (Figure 1B, top panels), suppression of Keilin's organ formation (middle panels), and repression of the limb-specifying gene *Distalless* (*Dll*; bottom panels). Thus, the HX and the integrity of the linker region are dispensable for AbdA epidermal functions.

The AbdA(HXm) and AbdA(PFERm) Variants Interact with Exd and Form a Trimeric AbdA/Exd/Hth Complex on a *Distalless* Repressor Element

Repression of *Dll* requires a recently characterized repressor element (DllR) that contains binding sites for Hox, Exd, and Homothorax (Hth) (Gebelein et al., 2002), another TALE HD-containing protein that interacts with Exd. To compare the DNA binding activity of parent and variant forms of AbdA and their ability to recruit Exd and Hth, we used the DllR repressor element in electromobility shift assays (EMSA). At the concentration used in our assays, AbdA, AbdA(HXm), and AbdA(PFERm) by themselves or in the presence of Exd or Hth do not significantly bind DllR (Figure 1C, lanes 6–8, 10–12, and 14–16). In combination with Exd and Hth, the three AbdA proteins form trimeric complexes on DllR with the same efficiency (Figure 1C, lanes 9, 13, and 17). DllR contains a divergent Hox/Exd binding site, which could explain why trimeric Hox/Exd/Hth, but not dimeric Hox/Exd, complexes are formed on this element. To compare the Exd binding activities of parent and mutant forms of AbdA, we performed EMSA using a variant of DllR,

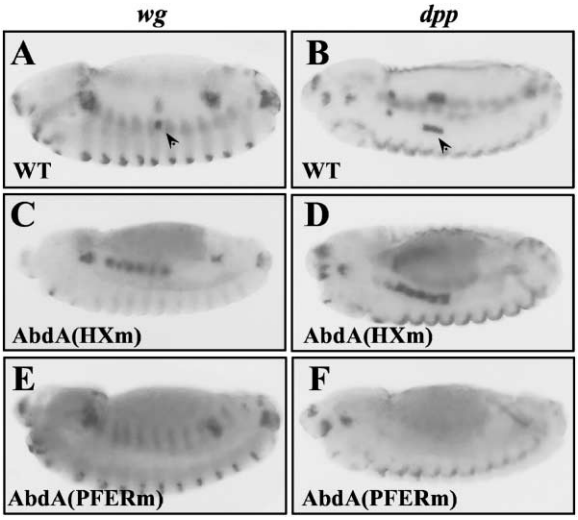

Figure 2. The HX and PFER Motifs Select the Activation/Repression Potential of AbdA in the Regulation of *dpp* and *wg* in the VM

(A and B) The arrows point to *wg* (A) and *dpp* (B) expression in PS8 and PS7 of the midgut VM in wild-type embryos.

(C–F) Effects of HX and PFER mutations on *wg* and *dpp* transcription. *24B-Gal4*-driven mesodermal expression of AbdA(HXm) activates *wg* anterior to PS8 (C) and *dpp* anterior to PS7 (D). Expression of AbdA(PFERm) in the whole mesoderm results in the loss of *wg* (E) and *dpp* (F) expression.

DllR^{con}, bearing the consensus Hox/Exd binding sequence (Gebelein et al., 2002). In these conditions, AbdA/Exd, AbdA(HXm)/Exd, and AbdA(PFERm)/Exd complexes are assembled and display very similar DNA binding affinities (Figure 1D, lanes 7, 11, and 15). In the presence of Exd and Hth, AbdA variants show similar efficiency in forming trimeric complexes (Figure 1D, lanes 9, 13, and 17). The divergence from the Hox/Exd consensus in DllR thus makes Hth required for complex formation. In summary, HX or PFER mutations neither alter the in vitro DNA binding activity of AbdA nor affect its capacity to interact with Exd and Exd/Hth, consistent with the unchanged in vivo ability to impose epidermal A2 identity.

The HX and PFER Motifs Control AbdA Regulatory Activity in the Visceral Mesoderm

The Hox target genes *wingless* (*wg*; Immerglück et al., 1990) and *decapentaplegic* (*dpp*; Capovilla et al., 1994; Manak et al., 1994) are expressed in parasegments (PSs) 8 and 7 of the visceral mesoderm (VM), respectively (Figures 2A and 2B). When ectopically provided in the VM, AbdA activates *wg* anterior to PS8 and represses the endogenous expression of *dpp* in PS7 (Sanchez-Herrero et al., 1994). Providing AbdA(HXm) in the whole mesoderm results in *wg* and *dpp* activation in the anterior VM (Figures 2C and 2D). As *dpp* is a transcriptional target of Ultrabithorax (Ubx) in PS7, AbdA(HXm) could activate *dpp* indirectly through the activation of *Ubx*. We excluded this possibility, since the *Ubx* pattern does not change upon expression of AbdA(HXm) (data not shown). Thus, the HX mutation does not affect the ability of AbdA to induce *wg* but has a drastic effect on *dpp*, which becomes activated, instead of repressed, by AbdA.

Exd is required for AbdA-mediated activation of *wg* in the VM (Rauskolb and Wieschaus, 1994). To test whether it is also needed for *wg* activation by AbdA(HXm), we analyzed the *wg* pattern in embryos expressing the variant ubiquitously and deficient for *hth*, which impairs Exd nuclear translocation and mimics Exd maternal and zygotic loss (Rieckhof et al., 1997). In this context, AbdA(HXm) no longer activates *wg* (data not shown), indicating that Exd is required and acts in an HX-independent manner to assist the Hox protein in the induction of *wg* transcription. Exd is also required for Ubx-mediated activation of *dpp* (Rauskolb and Wieschaus, 1994). Whether *dpp* activation by AbdA(HXm) depends on Exd could not be similarly tested because the loss of Exd function results in a Hox-independent anterior ectopic expression of *dpp* (Rauskolb and Wieschaus, 1994).

We next asked whether the linker region is important for AbdA function in the VM and found that expression of AbdA(PFERm) represses not only *dpp*, as does AbdA, but also *wg* (Figures 2E and 2F). As *wg* transcription requires Dpp signaling, the observed loss of *wg* expression might result from the loss of *dpp* transcription. We provided AbdA(PFERm) and Dpp simultaneously in the whole VM and observed that *wg* expression was not restored (data not shown). These results indicate that altering the linker region by mutating the PFER sequence does not affect the regulation of *dpp*, but impairs the ability of AbdA to promote *wg* transcription.

HX and PFER Mutations Do Not Affect AbdA Binding Site Selection in the Regulation of *dpp*

The regulation of *dpp* by AbdA in the VM is mediated by the dpp674 enhancer (Capovilla et al., 1994; Capovilla and Botas, 1998), which contains seven binding sites for AbdA. Sites 1–4 in dpp419 (the 3′ portion of dpp674) mediate repression by AbdA, while sites 5–7 in dpp265 (the 5′ portion of dpp674) mediate activation (Capovilla and Botas, 1998). Interestingly, dpp265 reveals an activating potential of AbdA on *dpp* transcription that is masked by the prevalence of repression over activation in the regulation of dpp674 or *dpp*. To address the Exd requirement for the activating potential of AbdA, we analyzed the dpp265 pattern in *exd*- or *hth*-deficient embryos. In both cases, we found that dpp265 activity in the AbdA expression domain was not affected (Figure 3A). Thus, while activation of *dpp* by Ubx in PS7 requires Exd, activation (shown here) and repression (Rauskolb and Wieschaus, 1994) by AbdA in PS8–12 are Exd independent.

The characterization of activator and repressor sites in *dpp* provides a unique opportunity to test whether the HX mutation, which turns AbdA from a repressor to an activator, results from a change in binding site selection. The prediction is that AbdA(HXm) could have lost the ability to bind repressor sites 1–4 but kept the capacity to bind activator sites 5–7. We performed DNaseI footprint experiments on dpp674 in the absence of Exd, which is not required for *dpp* regulation by AbdA. The results show that AbdA(HXm) protects sites 1–4 and sites 5–7 as efficiently as does AbdA (Figure 3B). Similarity in footprint patterns, moreover, indicates that AbdA(HXm) does not bind additional sequences with

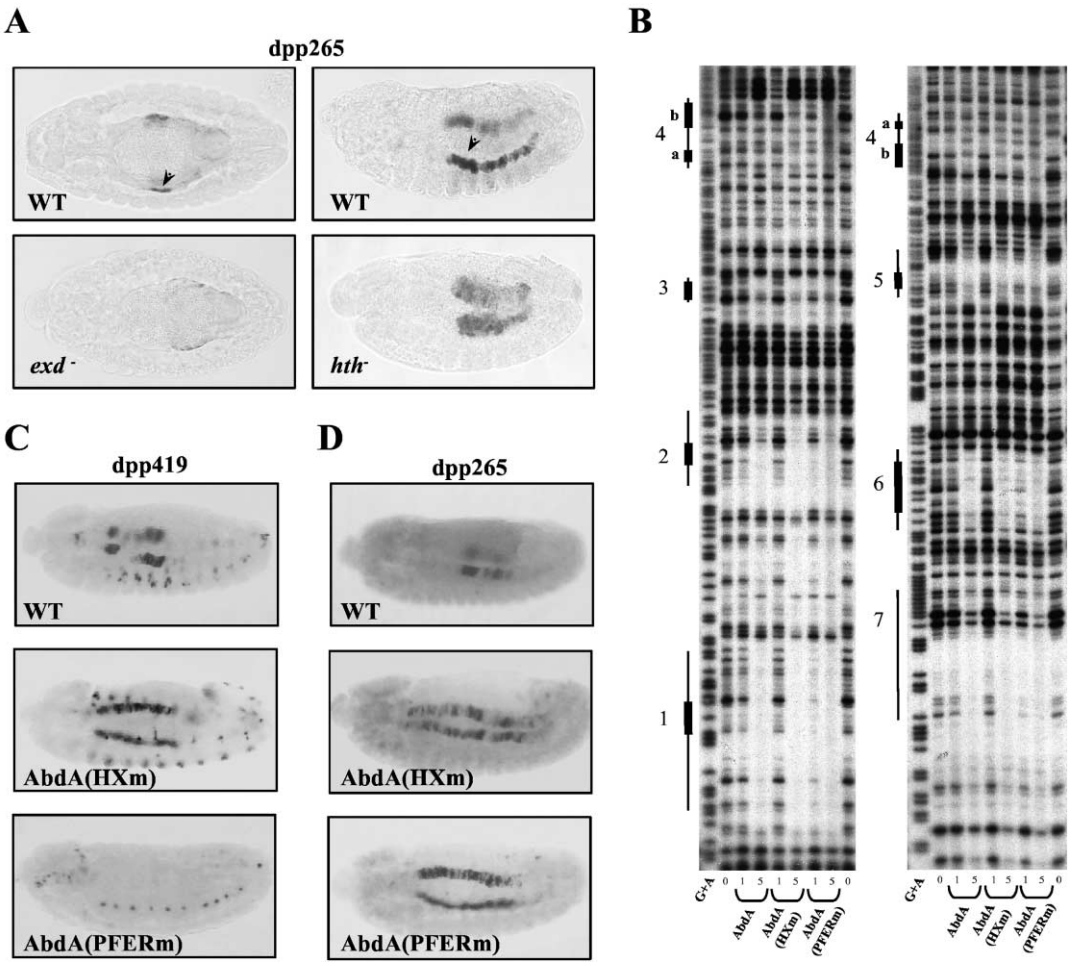

Figure 3. Unaltered In Vitro and In Vivo DNA Binding Properties of the AbdA(HXm) and AbdA(PFERm) Variants

(A) Exd is dispensable for dpp265 activation by AbdA. The activity of dpp265, followed by anti-β-galactosidase staining, is strong in PS7 (arrowhead). Posterior to PS7, in the AbdA expression domain, dpp265 activity is weaker. In *exd* or *hth* mutants, dpp265 activity in the AbdA expression domain is not affected. Embryos lacking maternal and zygotic *exd* function were recognized by their abnormal morphology; *hth* mutants were recognized by the absence of abdominal *lacZ* staining due to a "blue balancer."

(B) In vitro binding of AbdA, AbdA(HXm), and AbdA(PFERm) on dpp674. The AbdA binding sites present in dpp419 and dpp265 are equally well protected in DNase I footprint experiments by AbdA, AbdA(HXm), and AbdA(PFERm). In each panel, the left lane is the G+A chemical cleavage sequence reaction. For each protein, 1 μl (100 ng) and 5 μl (500 ng) of purified proteins were used. Protected and core Hox binding sequences are indicated by bars and boxes and numbered according to Capovilla et al. (1994).

(C) Regulation of the dpp419 enhancer followed by in situ hybridization with a *lacZ* riboprobe. *dpp419-lacZ* recapitulates wild-type *dpp* expression in the VM and is repressed by endogenous AbdA in the posterior midgut. This enhancer is activated by AbdA(HXm) and repressed by AbdA(PFERm).

(D) Regulation of the dpp265 enhancer followed by in situ hybridization with a *lacZ* riboprobe. *dpp265-lacZ*, instead of being repressed, is activated by AbdA in the posterior midgut. dpp265 is activated by AbdA(HXm) and AbdA(PFERm).

regard to AbdA. Thus, the activation/repression switch induced by the HX mutation does not result from intrinsic changes in AbdA DNA binding properties.

These in vitro data do not exclude the possibility that the HX mutation prevents interactions with unknown partners required for target site recognition in vivo. In this case, as *dpp* is no longer repressed by AbdA(HXm), one would expect that the mutant protein has lost the ability to act on dpp419 that contains the repressor sites but still acts on dpp265 that contains the activator sites. We observed that both dpp419 and dpp265 respond to AbdA(HXm) (Figures 3C and 3D), which indicates that, in vivo, the mutant protein binds sites present on both enhancers, as it does in vitro. dpp419 is repressed by AbdA but activated by AbdA(HXm), in agreement with the abovementioned HX function, to prevent AbdA from inducing the *dpp* gene. Taken together, these in vitro and in vivo data establish that the HX mutation has no effect on AbdA DNA binding properties but changes its transregulatory properties.

Although the PFER mutation does not affect the regulatory activity of AbdA on *dpp*, we analyzed the in vitro and in vivo properties of AbdA(PFERm) on dpp265 and dpp419 enhancers. In vitro, the PFER variant protects

all AbdA binding sites at least as efficiently as AbdA and AbdA(HXm) (Figure 3B). In vivo, AbdA(PFERm) also acts on both enhancers, repressing through dpp419 and activating through dpp265 (Figures 3C and 3D). Activation through dpp265 indicates that the variant has not lost its transactivating potential and, therefore, that *dpp* repression results from an active mechanism that requires sequences present in dpp419, but absent in dpp265. Thus, repression of *wg* by AbdA(PFERm) (Figure 2E) also most likely rests on an active mechanism that switched the Hox protein from an activator to a repressor.

The HX and PFER Motifs Are Functionally Linked in Controlling an AbdA Activation/Repression Switch

The in vivo activities gained by the two variants with regard to the wild-type protein indicate that the HX and PFER motifs control an activation/repression switch in the regulation of *wg* and *dpp*. The C terminus of AbdA contains a stretch of glutamine (Q) residues that is well conserved in closely related species (Figure 1A). Poly(Q) stretches have already been shown to act as activation domains in HD-containing proteins (Janody et al., 2001). To ask whether the Q-rich domain is required for the ability of AbdA(HXm) to activate *dpp*, we analyzed the in vivo activity of the AbdA(HXm) variant when deleted of this domain. The ability of AbdA(HXm;ΔQ) to activate *dpp* is clearly diminished, although not completely impaired (Figure 4B). We also found that AbdA(HXm;ΔQ) less efficiently activates *wg* (Figure 4A). Thus, the poly(Q)-rich region behaves as an activator domain, but other regions in AbdA most certainly contribute to its activating potential. A similar situation with alternative activation domains has already been described for Hoxd9 from cell culture experiments (Vigano et al., 1998). We next analyzed the effect of deleting the poly(Q) stretch in AbdA(PFERm) and found that AbdA(PFERm;ΔQ) still represses *wg* and *dpp* (Figures 4C and 4D). This indicates that the poly(Q) region is required for activation only and suggests that a repression domain should be located elsewhere in the protein.

A simple interpretation of these results is that, in the context of the *dpp cis*-regulatory region, the HX inhibits the function of the C-terminal activation domain, while, in the context of the *wg cis*-regulatory region, the PFER sequence blocks the activity of a repression domain that remains to be identified. If the two motifs act independently, one expects additive functions for a variant simultaneously mutant for the HX and PFER motifs, which should then activate *dpp* and repress *wg*. Providing this variant, AbdA(HXm;PFERm), everywhere in the VM results in the activation of both *wg* and *dpp* (Figures 4E and 4F). This indicates that the two motifs do not act independently to select the repressive/activating potential of AbdA. Instead, since AbdA(HXm;PFERm) phenocopies AbdA(HXm), a functional epistatic relationship exists between the two motifs, with the PFER sequence acting upstream and through the HX.

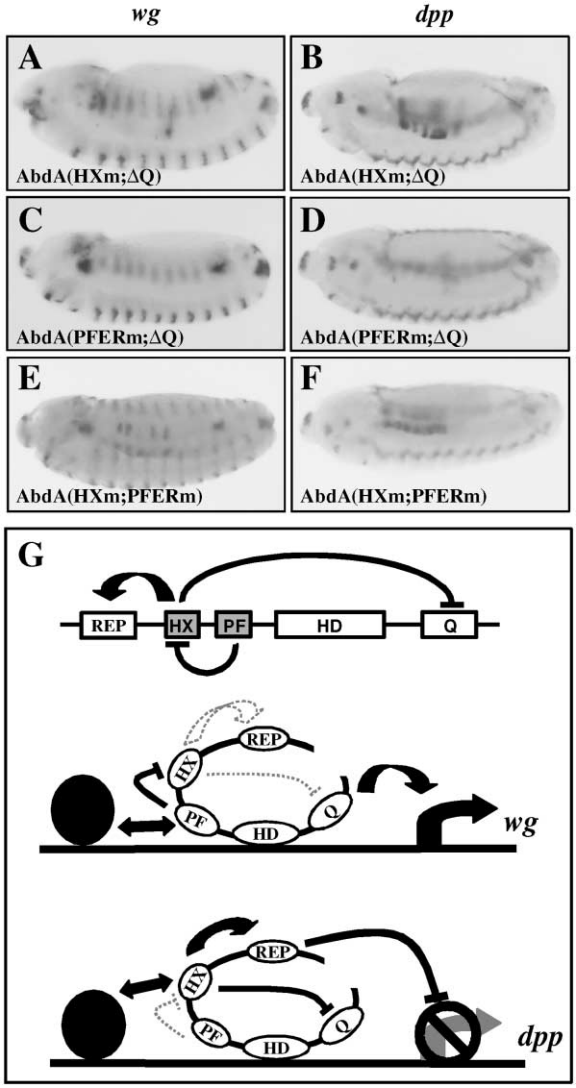

Figure 4. Functional Interactions between the HX, PFER, and a Poly(Q) Domain in Controlling AbdA Activity

(A–D) Localization of a poly(Q) activation domain. Compared with that of AbdA(HXm) (Figures 2C and 2D), the ability of AbdA(HXm;ΔQ) to activate *wg* (A) or *dpp* (B) is diminished. The deletion of the Q-rich domain more strongly affects *wg* transcription. AbdA(PFERm;ΔQ) still efficiently represses *wg* (C) and *dpp* (D).

(E and F) Functional interdependence of the HX and PFER motifs. AbdA(HXm;PFERm) phenocopies AbdA(HXm), activating both *wg* (E) and *dpp* (F).

(G) Model for AbdA activity regulation. Top panel, intramolecular regulatory circuit connecting the HX, PFER, poly(Q), and a potential repression domain. The repression domain has been arbitrarily located in the N terminus but could also be adjacent to the HX or include the HX. In the model proposed, the HX and linker region act as a molecular platform to sense *cis*-regulatory specificity and control the activation/repression potential of AbdA. Middle and bottom panels, selection of the transregulatory mode of AbdA by sensing *wg* or *dpp cis*-regulatory specificity. Interaction of PFER with a positive cofactor of *wg* transcription blocks the HX function, selecting the activating mode of AbdA by favoring the activity of the C-terminal Q-rich activation domain. Interaction of the HX with a corepressor of *dpp* stimulates the function of the HX, thus selecting the repression mode of AbdA by favoring the activity of a repression domain.

Discussion

Extensive in vitro analyses have demonstrated that the HX is responsible for the interaction with Pbc proteins,

leading to the view that this motif imparts Hox DNA binding specificity and therefore assists Hox proteins in the selection of appropriate target genes. Our in vivo data challenge this view in several ways. First, the unaltered capacity of AbdA(HXm) to induce A2-like identities in the thorax and to form dimeric complexes on DNA with Exd shows that the HX is not the only motif of AbdA that is able to recruit Exd. A similar situation has been shown to occur in Ubx (Galant et al., 2002), indicating that other residues in Hox proteins can compensate for the lack of the HX in mediating Hox/Exd interactions. Second, mutation of the HX does not affect binding site selection by AbdA, as shown by the ability of the mutant protein to bind target sequences from *Dll* and *dpp* in vitro, and to control dpp265 and dpp419 in vivo. Accordingly, the HX mutation does not alter target gene selection (in this case, *wg* and *dpp* in the VM) in vivo. Third, the fact that the HX mutation modifies AbdA function in the regulation of *dpp*, which does not depend on Exd, implies that the HX should interact with additional proteins that remain to be identified. Our data thus endow the HX with unexpected functions; this does not preclude that the HX could, however, play a role in target selection in other developmental contexts. We also found that the PFER motif within the linker region fulfils an important regulatory function, which was also unexpected, considering the variable length and disordered structure of this region.

Exd acts in a Hox-independent manner to repress *dpp* in the anterior VM (Rauskolb and Wieschaus, 1994). Anterior expression of *dpp* induced by AbdA(HXm) could therefore result from an interference with the repressive function of Exd, rather than from a direct effect on *dpp* transcription. However, while dpp265 is not derepressed anteriorly in *exd*- or *hth*-deficient animals (Figure 3A) and, therefore, does not contain the sequences mediating repression by Exd, it is activated by AbdA(HXm) (Figure 3D). Thus, Exd and AbdA(HXm) act on different regulatory sequences to respectively repress or activate *dpp* in the anterior VM, which makes it unlikely that activation by AbdA(HXm) results from an interference with the Hox-independent repressive function of Exd. Considering that the HX mutation affects neither DNA binding nor target site recognition in vitro and in vivo, we propose that AbdA(HXm), as does AbdA, controls *dpp* transcription directly.

The function of the HX and PFER motifs in switching AbdA from an activator to a repressor clearly depends on the *cis*-regulatory target sequence, which is illustrated by the distinct effects of the variants on *dpp* and *wg* transcription, and of AbdA(PFERm) on dpp419 and dpp265. Taking these observations together, we propose a model that accounts for how the distinct regulatory modules identified functionally interconnect to specify AbdA activity in the VM (Figure 4G). According to this model, the HX plays a central dual role in repressing the function of a Q-rich activation domain and promoting that of a repression domain whose location remains to be determined. For the regulation of *dpp*, the HX senses *dpp cis*-regulatory specificity to select the repressive potential of AbdA. Conversely, in the regulation of *wg*, the PFER sequence senses *wg cis*-regulatory specificity to select the activating potential of the Hox protein. According to the functional epistatic relationship between the two motifs, suggested by the activity of the doubly mutated AbdA(HXm;PFERm) variant, the PFER sequence would not directly control repressive or activating domains of AbdA but, rather, acts upstream, as an inhibitor of HX function.

Our study demonstrates unappreciated regulatory functions for the HX and for the linker region, both acting together as a platform, sensing *cis*-regulatory specificity to ultimately select the activating or repressing potential of AbdA. Results from other studies suggest that conclusions from our in vivo experiments could be extended to Hox factors other than AbdA. First, our model emphasizes the importance of the *cis*-regulatory context for the control of AbdA activity, consistent with the dependency of the transactivating potential of vertebrate Hox proteins on the DNA binding context (Vigano et al., 1998). Second, the functional importance of the linker region is also suggested by the finding that a phosphorylated residue lying between the HX and the HD is critical for mouse Hoxb7-mediated inhibition of granulocytic differentiation (Yaron et al., 2001). Third, and most importantly, a recent report also identified the linker region as playing a DNA binding-independent role in Ubx-mediated repression of *Dll* (Gebelein et al., 2002).

Finally, our observations might also be relevant for mechanisms that relate molecular changes in Hox proteins to changes in morphology during animal evolution. The HX has recently been proposed to play a major role in conferring homeotic character to HD-containing proteins, as suggested by the simultaneous loss of homeotic function and HX motif in the *Drosophila* pair-rule Fushi-tarazu protein (Lohr et al., 2001). Together with our observations, this suggests that the acquisition of novel developmental properties by HD proteins during evolution presumably relies not only on changes in DNA binding specificity, but also on changes in transregulatory properties. In this context, modifying the regulation of only a subset of Hox targets while leaving others unchanged, by gain or loss of regulatory modules such as the HX and PFER motifs, might provide evolutionary advantages and be causal in morphological diversification. The importance of a tight control of Hox transregulatory properties in evolution has recently gained further support from the evolving capacity of Ubx in controlling the repression of *Dll* in the insect phylum (Galant and Carroll, 2002; Ronshaugen et al., 2002).

Experimental Procedures

Chimeric Genes and Transgenic Lines

AbdA variants were generated according to the splicing by overlap extension (SOE) procedure (Horton et al., 1989). The sequences of primers used are available upon request. AbdA variants were directionally cloned into the pUAS-T vector after EcoRI and XbaI digestion and sequence verified. Plasmid DNA for each construct was used for P element-mediated germline transformation (Rubin and Spradling, 1982). The P insertions were genetically mapped, and at least two balanced lines were established for each variant. In all transgenic lines, similar levels of nuclear AbdA variants were observed (data not shown).

Flies, Egg Collections, Cuticle Preparations, In Situ Hybridization, and Immunostaining

Oregon R was used as a standard. hth^{P2} was kindly provided by R. Mann, and the *UAS-dpp* stock was provided by the Bloomington

Stock Center. The *exd*^{XP11} and *exd*^{YO12} alleles were used to generate female germline mosaics as described by Rauskolb and Wieschaus (1994). *24B-Gal4* and *69B-Gal4* were used as mesodermal and epidermal drivers. The *dpp* enhancers used were described in Capovilla and Botas (1998). Embryo collections, cuticle preparations, in situ hybridizations, and immunodetections to whole embryos were performed according to standard procedures. Digoxigenin RNA-labeled probes were generated according to the manufacturer's protocol (Boehringer-Mannheim) from *wg*, *dpp*, and *Dll* cDNAs cloned in Bluescript (Stratagene). Monoclonal antibodies Dm.Abd-A.1 and mAb Ubx FP3.38 were generous gifts from D. Mattson-Duncan and R. White and were used at 1:1000 dilution and at 20 μg/ml, respectively. The anti-β-galactosidase antibody (Cappel) was used at 1:1000 dilution.

Protein Purification and DNA Binding Assays

His-tagged AbdA, AbdA(HXm), and AbdA(PFERm) were produced and purified from pet15b in BL21(DE3)pLys and used in DNaseI footprint experiments as described in Capovilla and Botas (1998). Labeled probes were obtained from the dpp674 BamHI-XhoI fragment by filling with Klenow the XhoI (sense strand) or BamHI (antisense strand) sites. Proteins [AbdA, AbdA(HXm), AbdA(PFERm), Exd, and Hth] for EMSA were produced with the TNT coupled in vitro transcription/translation system (Promega). Protein production was estimated by labeling the proteins with ^{35}S-methionine and found to be at similar amounts. EMSAs were performed in 20 μl as described in Pöpperl et al. (1995) with radiolabeled DllR or DllR^{con} oligonucleotides (Gebelein et al., 2002).

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