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Internal Regulatory Interactions Determine DNA Binding Specificity by a Hox Transcription Factor

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In developing bilaterans, the Hox transcription factor family regulates batteries of downstream genes to diversify serially repeated units. Given Hox homeodomains bind a wider array of DNA binding sites *in vitro* than are regulated by the full-length protein *in vivo*, regions outside the homeodomain must aid DNA site selection. Indeed, we find affinity for disparate DNA sequences varies less than 3-fold for the homeodomain isolated from the *Drosophila* Hox protein Ultrabithorax Ia (UbxHD), whereas for the full-length protein (UbxIa) affinity differs by more than 10-fold. The rank order of preferred DNA sequences also differs, further demonstrating distinct DNA binding preferences. The increased specificity of UbxIa can be partially attributed to the I1 region, which lies adjacent to the homeodomain and directly impacts binding energetics. Each of three segments within I1—the Extradenticle-binding YPWM motif, the six amino acids immediately N-terminal to this motif, and the eight amino acids abutting the YPWM C-terminus—uniquely contribute to DNA specificity. Combination of these regions synergistically modifies DNA binding to further enhance specificity. Intriguingly, the presence of the YPWM motif in UbxIa inhibits DNA binding only to Ubx–Extradenticle heterodimer binding sites, potentially functioning *in vivo* to prevent Ubx monomers from binding and misregulating heterodimer target genes. However, removal of the surrounding region allows the YPWM motif to also inhibit binding to Hox-only recognition sequences. Despite a modular domain design for Hox proteins, these results suggest that multiple Hox protein regions form a network of regulatory interactions that coordinate context- and gene-specific responses. Since most nonhomeodomain regions are not conserved between Hox family members, these regulatory interactions have the potential to diversify binding by the highly homologous Hox homeodomains.

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Introduction

A central challenge in developmental biology is to link events in patterning and morphogenesis to the gene regulatory hierarchy that drives these processes. Because only a few proteins regulate tissue-specific gene transcription relative to the number of unique tissues that must be generated, each transcription factor active in development must uniquely function in multiple cellular contexts. Thus, this challenge is doubly complex, requiring identification of crucial genes and cis-acting regulatory sequences as well as elucidation of mechanisms that govern context-specific transcription factor function.

The Hox transcription factor family acts near the top of this regulatory hierarchy in all bilaterally symmetric animals. Each Hox protein specifies multiple structures—directing appendage and organ development as well as determining local attributes of tissues in all three germ layers.[1–3] As transcription factors, the crucial contributions of Hox proteins are based on their ability to target different promoters in

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Abbreviations used: UbxIa, Ultrabithorax Ia; UBxHD, Ubx homeodomain; Exd, Extradenticle.

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a tissue-specific manner.[4–6] However, the DNA-binding specificity of Hox–DNA interactions is anticipated to be insufficient to distinguish cognate and noncognate targets in even a single tissue.[7–9] Consequently, we and others have postulated that regions of Hox proteins outside the homeodomain must influence DNA binding to generate the necessary level of sequence selection.[7–11] Furthermore, modulation of nonhomeodomain regulatory regions through posttranslational modifications,[12] protein interactions,[2,13–15] or alternative splicing[16] would provide an opportunity for tissue-specific regulation of target gene selection.

Unfortunately, identification of nonhomeodomain regions capable of modifying binding is a long-standing challenge, hampered both by the predominance of intrinsic disorder outside the homeodomain[10] and by the tendency of full-length Hox proteins to aggregate.[17] As a result, DNA binding studies have frequently been limited to the use of deletion mutants or unpurified proteins produced *in vitro*, preventing quantitative analysis of function in the native protein.[11,18] We have recently devised protocols to overcome these barriers for the full-length *Drosophila melanogaster* Hox protein Ultrabithorax Ia (UbxIa),[10,17] the dominant alternative splicing isoform expressed in the *Drosophila* embryo.[16] By measuring equilibrium DNA binding by purified UbxIa of high activity under conditions where the DNA concentration is more than an order of magnitude less than the dissociation constant[19] and excess nonspecific DNA sequences are absent, we can accurately assess the DNA binding affinity of wild-type and mutant UbxIa, independent of the influence of other factors or competition with nonspecific DNA interactions. Using this approach, we have discovered that most of the UbxIa protein sequence can influence the *affinity* of the homeodomain for the optimal Ubx DNA binding site.[10] Both prediction algorithms and native-state proteolysis experiments demonstrated that most of the sequences that modulate DNA-binding affinity in UbxIa are intrinsically disordered.[10]

Herein, we demonstrate directly for the first time that regions outside the homeodomain of a Hox protein also impact DNA binding *specificity*—altering affinity in a DNA-sequence-dependent manner. DNA binding by full-length UbxIa and the Ubx homeodomain (UbxHD) was monitored for a variety of DNA sequences. Sites examined included the UbxHD optimal binding site[20] and representative Ubx genomic-binding targets selected to vary a wide range of factors: binding site sequence, number and density, the presence of binding sites for the Hox-interacting protein Extradenticle (Exd), the transcriptional outcome of binding (activation *versus* repression), and the developmental stage and tissue in which the enhancer is active.[18,20–24] Although UbxHD binds tightly to all examined sequences, the affinity of full-length UbxIa varies by more than an order of magnitude with DNA sequence, indicating regions outside the homeodomain do improve specificity. We next sought to identify specificity determinants—regions that either impact binding only to a subset of DNA sequences or have opposite effects on binding different DNA sequences. We focused on the I1 affinity-modulating region of Ubx,[10] which contains an 18-amino-acid sequence required for repression of *distalless* by Ubx *in vivo*.[25] We find removal of this sequence alters binding affinity in a DNA-sequence-specific manner, demonstrating that the 18-amino-acid region acts as a specificity determinant. We next divided this region into three sections: the N-terminal 6 amino acids, the central 4-amino-acid Exd interaction motif (YPWM), and the intrinsically disordered C-terminal 8 amino acids, which contain a portion of the mI alternatively spliced microexon.[10,14–16,26–29] Mutation or deletion of each of these three sections has a differing impact on DNA specificity. Furthermore, combination of these regions produces new DNA binding specificity patterns. We propose the I1 region may act as a cellular "antenna," integrating tissue-specific information provided by alternative splicing and protein interactions to direct UbxIa to the appropriate DNA binding sites.

Results

Full-length UbxIa binds DNA with higher selectivity than UbxHD

Previously, we demonstrated that the full-length UbxIa splicing isoform (Fig. 1a) and its homeodomain have significantly different affinities and responses to pH when binding an optimal DNA sequence.[10] Strikingly, most nonhomeodomain regions of Ubx can modulate Ubx–DNA binding *affinity* (Fig. 1e), utilizing a variety of mechanisms.[10] To determine whether nonhomeodomain sequences also enhance Hox–DNA binding *specificity*, we compared the range of affinities observed for UbxIa and UbxHD binding to a variety of DNA sequences, including the UbxHD optimal binding site, termed 40AB,[20] and five representative Ubx genomic binding targets: Dll, Dpp, UA, A1, and Sal (Table 1 and Fig. 2).[18,20–24] DNA binding sequences were selected to maximize the variety of (i) base sequences adjacent to the 5′-TAAT-3′ consensus site or sites, (ii) binding site densities of the promoters regulated by Ubx *in vivo*, (iii) requirements for the Hox interacting protein Exd, (iv) transcriptional outcomes (activation *versus* repression), and (v) the cellular contexts in which Ubx binds the natural sequences, including the developmental stage, germ layer, and tissue. To facilitate analysis, small (<90 bp) sections of these complex cis-acting regulatory regions were used for DNA binding experiments.

Equilibrium mobility shift analysis experiments (gel shifts) were used to assess DNA binding at protein concentrations near the dissociation constant, where binding is most sensitive to DNA sequence, and at DNA concentrations well below this range. Gel shifts allow separation and observation of complexes with

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