Minireview

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Understanding the Homeodomain*

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The homeotic genes of *Drosophila* have long captured the fascination of biologists, in part for the remarkable phenotypes that mutant animals display. Homeotic mutants are capable of placing whole body parts in inappropriate locations, in effect replacing halteres with wings, or antennae with legs, or mouth parts with legs. These phenotypes suggested that the groups of cells whose descendents will make these body parts choose between alternative developmental pathways during the course of normal development and implied that these decisions are directed by the products of the homeotic genes. It follows that the cells whose developmental program can be switched between alternative fates share an inherent homology and that the different homeotic genes that direct their ultimate diversification function in similar capacities. The suggestion that the homeotic genes might share a common ancestry was made several years before any were isolated (1).

The notion that the present day homeotic genes might have arisen by duplication and divergence gained immediate acceptance when their isolation and characterization revealed, in each, a conserved homeobox region (2, 3). The homeobox has since been found to be present in a large family of eukaryotic regulatory proteins (4). The homeobox family now numbers more than 300, continues to grow, and includes genes from organisms broadly representative of both the plant and animal kingdoms. Although it is now clear that most members of this gene family have developmental roles that are unrelated to homeosis, the homeobox remains a hallmark of homeotic genes, and a number of these genes appear to have retained both their precisely ordered tandem arrangement in the genome, as well as their developmental roles in axial patterning across vast evolutionary time (5).

The impressive conservation of nucleotide sequences of the homeoboxes has fostered the efficient and rapid isolation of homeobox-containing genes from diverse sources. Even more impressive is the conservation of the protein segment encoded by the homeobox, the homeodomain. For instance, the *Drosophila* Antennapedia (Antp) and human HoxB7 homeodomains differ at only 1 of 61 residues, and 16 other homeodomains from fly, human, sea urchin, mouse, rat, and frog that are related by sequence to the Antp homeodomain differ at fewer than 7 residues. Such extraordinary conservation must be indicative of conserved functions.

Structural models have been obtained for three homeodomains: 1) a polypeptide representing the *Drosophila* Antp homeodomain and seven additional C-terminal residues; 2) a polypeptide representing the *Drosophila* engrailed homeodomain; and 3) a C-terminal 82-residue fragment of the yeast MATα2 protein that contains the α2 homeodomain and a 21-amino acid C-terminal tail. Each of these protein fragments adopts a stable structure that binds DNA with high affinity. X-ray crystallographic analysis of the DNA-bound engrailed (6) and α2 homeodomains (7) and an NMR spectroscopy-derived structure of the Antennapedia-DNA complex revealed nearly identical structures. Each protein contains three α-helices that are spaced and arranged similarly, and each contacts DNA with a pincer-like grip. In each, the N-terminal arm of 9 residues extends across the DNA binding site making contacts in the minor groove, and residues in the third α-helix contact DNA in the adjacent major groove. Moreover, despite only 27% sequence identity and a 3-residue insertion in α2 between residues 23 and 24 (Fig. 1*A*), the α-carbon backbones between residues 9 and 58 of engrailed and α2 homeodomains align with a root mean square difference of 1.0 Å. These models underscore the similarities of even disparate homeodomains, as well as the modular nature of the proteins of which these segments are a part. Such remarkable conformational identity between distantly related members of the homeodomain family suggests that their salient structural traits and functional properties are shared among all homeodomains.

The N-terminal Arm

Although the NMR and crystallographic data did not resolve the N-terminal residues of either the Antp (residues 0–6), engrailed (residues 0–2), or α2 (residues 0–3) homeodomains, or the side chains of several of the succeeding residues, contacts with several bases in the minor groove were detected. In engrailed, Arg-3 and Arg-5 contacted three or four AT base pairs; in α2, Arg-7 contacted two AT base pairs, and the N-terminal arm of Antennapedia (Antp) was in a position to make similar contacts. The possibility that these are artifactual contacts that form only in the environment of the crystals seems unlikely in view of several related observations. For instance, conservation of Arg at residue 5 (97% among the >300 members of the homeodomain family) implies that Arg-5 has a particularly important role. In addition, minor groove base and phosphate backbone contacts are also indicated by patterns of methylation and ethylation that blocked binding of Antp and fushi tarazu (ftz) homeodomains (8, 9). The contacts inferred from these interference studies are consistent with every minor groove base and phosphate contact observed in the engrailed cocrystal. Finally, the importance of the N-terminal arm-DNA contacts is underscored by the behavior of mutant homeodomains. A ftz homeodomain that lacks the N-terminal 6 residues makes fewer base and phosphate backbone contacts and binds DNA with 130-fold lower affinity than does the complete homeodomain peptide (8). An α2 mutant in which Ala replaces Arg-7 has significantly lower repressor activity *in vivo* (cited in Ref. 7). A POU domain protein that lacks residues 4 and 5 failed to bind DNA *in vitro* (10).

Despite the apparent importance of the minor groove contacts by the N-terminal arm to DNA binding, the contribution that these contacts make to sequence selectivity or specificity of binding is unclear. It is thought that minor groove contacts cannot discriminate between AT and TA base pairs (11) but that the extracyclic NH_2 of the guanine should allow for CG and GC base pairs to be readily distinguished from AT base pairs. The Arg-5 and Arg-7 side chains of the engrailed, α2, and Antp homeodomains that contact AT base pairs in the minor groove presumably would have unfavorable interactions with a guanine base. These considerations may explain the preference of many homeodomain proteins for AT-rich sites, but selecting

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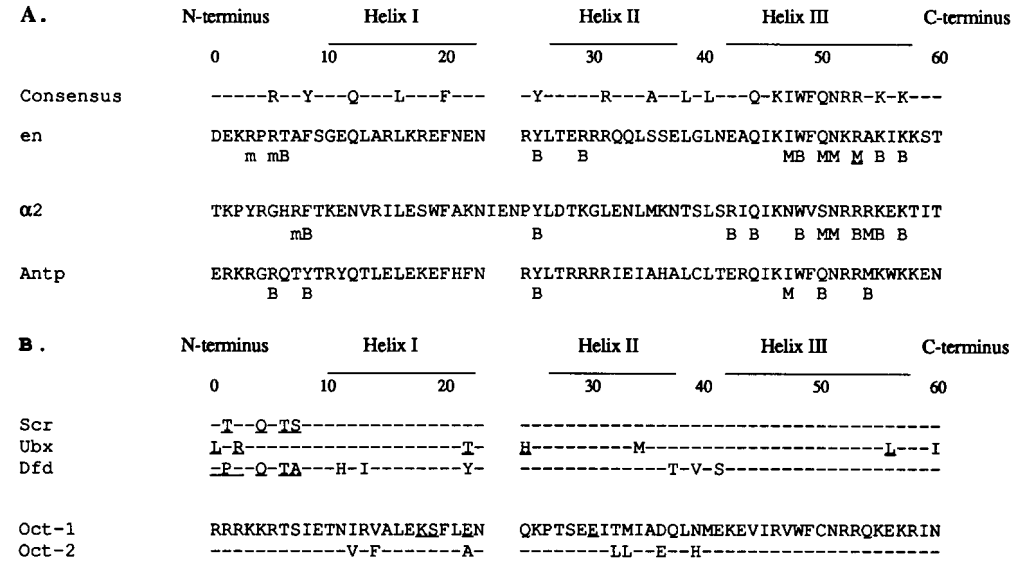

FIG. 1. **Sequence comparisons of homeodomains.** *A*, indicated at the *top* are the α-helices common to engrailed, α2, and Antp homeodomains. They have been aligned with the sequences of the engrailed, α2, Antp, and Oct-1 homeodomains. The "consensus" sequence has been determined by comparing 87 homeodomains and the conserved residues are shown interspersed among positions (indicated by *dashes*) with more sequence variation (4). The numbering is that of Kissinger *et al.* (6). Below the engrailed, α2, and Antp sequences are indicated residues that contact the phosphate backbone (*B*) or that contact bases in the minor (*m*) or major (*M*) groove of DNA. *B*, comparisons of the Scr, Ubx, and Dfd homeodomains to Antp and of Oct-2 to Oct-1 represent common residues as *dashes* and specify different residues with the single letter code. *Underlined* residues indicate those amino acids whose identity alters specificity in chimeric *Drosophila* proteins or are critical to the interaction of Oct-1 with VP16.

among AT-rich sites might not be possible on the basis of such contacts. It is therefore surprising that much of the target specificity of the homeodomain has been found to be in the N-terminal arm.

Drosophila homeotic genes such as *Ultrabithorax* (*Ubx*), *Antp*, *Deformed* (*Dfd*), and *Sex combs reduced* (*Scr*) encode proteins whose homeodomains have very similar sequences (Fig. 1*B*). The number of amino acids that distinguish the Ubx, Scr, and Dfd homeodomains from the Antp homeodomain are 4, 6, and 10, respectively. In contrast, the Antp, Ubx, Scr, and Dfd proteins have few other sequence similarities. These homeodomain proteins are expressed in different regions of the developing fly and are thought to each direct developmental pathways by regulating different sets of downstream target genes. The basis of their target specificity has been investigated by creating chimeric genes in which the homeodomains and/or flanking regions were exchanged, and the capacity of the chimeric proteins to direct different developmental pathways has been assessed *in vivo*. Since gene replacement is not possible in *Drosophila* and since the regulatory regions that control the expression of these genes are too large to manipulate, the assays compared the effects of ubiquituous expression of wild type and chimeric proteins encoded by gene constructs that had been incorporated into the fly genome by P-element mediated transformation. These transgenes were regulated by heat-inducible promoters and their expression, after induction with a heat shock regimen, was presumed to be equivalent in all cells. These assays do not monitor wild type function. Rather, they compare the effects of different forms of these proteins in abnormal settings, and it is uncertain how these findings would correspond to parallel rescue assays. Nevertheless, the homeodomain proteins elicit remarkably specific effects when expressed ubiquituously, illustrating their functional specificity even in ectopic locations and providing a means to assess the relative contributions of their various domains.

For each protein, the homeodomain and the adjacent residues were found to contribute to target specificity (12–16). And in each case, the N-terminal sequence of the homeodomain was discovered to be critical to target selection. Replacing the four distinguishing amino acids in the N-terminal arm of the Antp homeodomain with Scr-specific residues was found to be sufficient to change the specificity of the Antp protein to that of Scr (16). Criteria used in these assays were *Antp*-specific induction of ectopic anterior *teashirt* expression in the embryonic midgut and head epidermis, *Scr*-specific induction of ectopic larval salivary glands, and larval cuticular and adult head transformations which are different in HS-*Scr* and HS-*Antp* animals. Similarly, replacing the 6 distinguishing residues in the N-terminal arm of the Dfd protein with Ubx-specific residues was sufficient to change the specificity of the Dfd protein to that of Ubx (14). Replacing two N-terminal residues was found to be necessary but not sufficient to change the specificity of Ubx protein to that of Antp (15).

If the engrailed-DNA cocrystal structure is an appropriate model for these other homeodomains, then we might conclude that the residues in the N-terminal arm that determine specificity do not interact with DNA directly. Whereas Arg-3 and Arg-5 of engrailed contact DNA and these two arginines are conserved in Ubx, Antp, Dfd, and Scr, it is the residues that surround Arg-3 and Arg-5 that distinguish the target specificity of these other proteins. These considerations suggest that the residues important for specificity determination do not influence DNA binding directly but either influence the conformation of the N-terminal arm or interact, perhaps, with protein cofactors that contribute in some way to sequence selection. The N-terminal arm of engrailed is available for such interactions (Fig. 2) since the side chains of residues 4, 6, 7, 8, and 9 point away from the DNA. An example of association with a cofactor might be the reported interaction of the *Drosophila* homeodomain protein I-POU with Cf1-a, another protein of the POU homeodomain class. This interaction is dependent upon the sequence of the I-POU N-terminal arm (17).

The possibility that direct interactions with DNA are the principal basis for the contribution of the N-terminal domain to binding specificity cannot be ruled out, however. Although the engrailed, α2, and Antp homeodomain structures are remarkably similar throughout the helical portions, their N-terminal arms may not adopt identical conformations. The reported minor groove contacts of the α2 homeodomain differ from those observed for Antp and engrailed, and better resolution of this

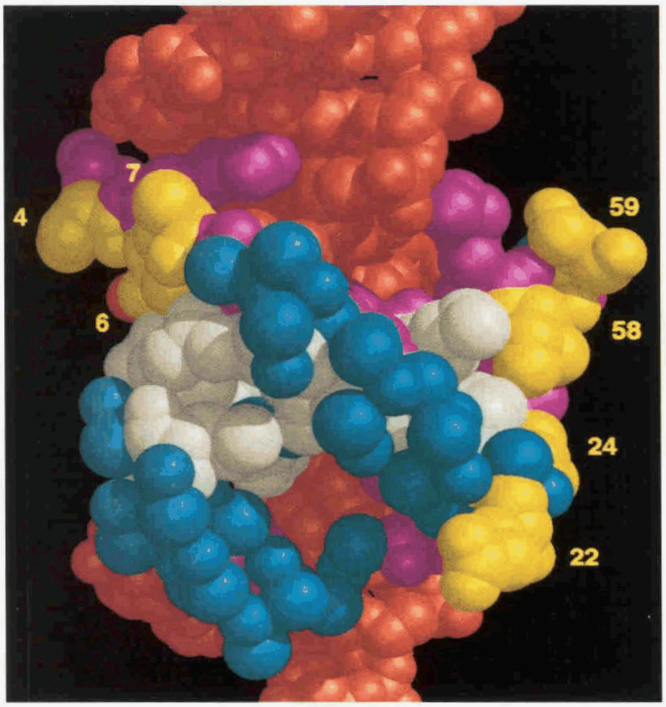

FIG. 2. **Space-filling model of the engrailed homeodomain-DNA complex.** The DNA is shown in *orange*, and the residues of the homeodomain are in *blue* except for those which contact DNA (*purple*), are invariant (*white*), or have been implicated as contributing to specificity (*yellow*). Note the clusters of *yellow* (specificity) residues on the exterior of the homeodomain and the concentration of *white* (conserved) residues in the interior. *Yellow* residues are numbered as in Kissinger *et al.* (6).

region of the homeodomain will be needed to work out these critical details. Since the structures obtained by NMR analyses of the Antp homeodomain indicate that the N-terminal arm adopts a regular structure only when bound to DNA (18), it is possible that the conformation the N-terminal arm adopts could be influenced by the sequence of the binding site or by cofactors with which the protein associates.

The α-Helical Region

Forty-one of the 51 residues that follow the N-terminal arm of the engrailed, α2, and Antp homeodomains are part of α-helical units (Fig. 1). A 13-residue helix 1 is separated from the 11-residue helix 2 by a loop of 5 residues in most homeodomains but is 8 residues long in α2. The 3-residue insertion in α2 does not affect the overall conformation of the homeodomain. A short loop (turn) of 4 residues joins helix 2 to the 17-residue helix 3. These helices pack against each other to form a compact and stable structure. Helices 1 and 2 stack against each other in an antiparallel arrangement, spanning the major groove of DNA at a roughly perpendicular orientation. These helices are separated from the DNA by helix 3, which packs against helices 1 and 2 with its hydrophobic face and fits into the major groove with its hydrophilic face.

Sixteen of the residues in the α-helices are conserved in the homeodomain family. The side chains of 9 of these residues point to the protein's interior and presumably define the conformational relationships of the three helices that are so well conserved. Remarkably, some of the residues that contact DNA are also conserved in many homeodomains. Since the α-carbon backbones of helix 3 in the engrailed and α2 structures fit into the major groove in precisely the same way, it is likely that these residues help to align the homeodomain with the DNA ligand. In so doing, the homeodomains sacrifice an opportunity for sequence selection in order to retain their docking mechanism. We can speculate that contributions from other regions of the homeodomain (such as the N-terminal arm) contribute to sequence-specific association and thereby might compensate for restricting the sequence in helix 3.

The most highly conserved residue in helix 3 (the "recognition" helix) is Asn-51. Asn-51 forms a set of hydrogen bonds with an adenine in the binding site, and along with the set of backbone contacts made by other conserved residues, Asn-51 is thought to position the recognition helix on the DNA. Other residues in the recognition helix that contact DNA are Ile-47, Gln-50, and Arg-53 in engrailed, and Asn-47, Ser-50, and Arg-54 in α2. Residues 47, 50, and 54 are less well conserved, and their variability presumably contributes to specificity of binding. Residue 50 of both engrailed and α2 makes contact with an adenine base in the major groove. Genetic studies have pinpointed residue 50 as a critical residue for controlling specificity, and its identity has been shown to affect base preference in both *in vitro* binding and *in vivo* assays (8, 19–21). Residue 54 apparently contacts DNA when its side chain is sufficiently long. As Ala (in engrailed), it does not make contact but as either Arg (in α2) or Met (in Antp), it does. Residue 54 has the potential to make contact in bicoid, mec3, and POU homeodomain proteins, where it is Arg, Ser, or Gln, respectively. Residues 47 of engrailed (Ile) and Antp (Ile) contact DNA, but residue 47 of α2 (Asn), which also projects into the major groove, does not.

In addition to these residues that contact DNA directly, several others may play an indirect role in the mechanism that guides homeodomain proteins to their target sequences. Examples are the two residues in the loop connecting Ubx helices 1 and 2 (residues 22 and 24) that help to determine whether the protein has Ubx or Antp function (15). This loop can vary in length without affecting the conformation or orientation of helices 1 or 2. The side chains of residues 22 and 24 are exposed to solvent (Fig. 2) and are conceivably available to associate with other proteins. In view of the close proximity of the loop to DNA (the conserved Tyr-25 of engrailed, α2, and Antp contacts the phosphate backbone) it is conceivable that a protein binding to this loop might influence DNA binding. A second example is revealed by the interaction of the ubiquituously expressed homeodomain protein, Oct-1, with the herpes simplex virus protein, VP16. In the presence of a protein HCF, Oct-1 can form a complex with VP16 that confers the capacity to activate transcription. In contrast, the lymphoid-specific Oct-2, whose homeodomain differs from that of Oct-1 at only seven positions, cannot associate with VP16 (22–25). The specificity for this interaction has been mapped to a single residue (residue 22) in helix 1 of the homeodomain (26), and three other residues that are identical in Oct-1 and Oct-2 (Lys-18, Ser-19, and Glu-30) are equally important for association with VP16 (27). Residues 18, 19, 22, and 24 are exposed to solvent in the engrailed, α2, and Antp structures.

DNA Sequence Recognition

With few exceptions, genes encoding homeodomain proteins have been identified on the basis of their mutant phenotype or by the sequence homology of their homeodomain. For these homeodomain proteins, discovering the DNA sequence they target *in vivo* has proven to be difficult. For instance, the many homeotic homeodomains whose sequence closely matches the "consensus" homeodomain sequence reveal remarkably little selectivity *in vitro* (reviewed in Ref. 28). Their promiscuity *in vitro* has frustrated efforts to define natural binding sites and contrasts starkly with the specificity these proteins manifest *in vivo*. The related sequence specificities these proteins share are a likely consequence of their evolutionary kinship. Their shared affinity for sites containing a TAAT motif certainly reflects the presence of conserved residues in their N termini and

recognition helices that contact these bases. Moreover, sequence selectivity via minor groove contacts is probably limited, and the number of opportunities a rodlike α-helix has to contact bases within the helical saddle of the major groove of B-form DNA is also small. Thus, the geometry that is so elegantly constrained by the conserved embrace of the homeodomain-DNA complex severely limits the number of specific contacts that can be made.

Resolutions to this apparent paradox may come in several forms. It is possible that the DNA in the *in vivo* homeodomain-DNA complex is bent in a way that brings additional bases into contact with the recognition helix. It is also possible that the *in vitro* studies have been compromised by the quality of protein preparations used; they were either expressed in *Escherichia coli* as homeodomain fragments, fusion proteins, or full-length proteins and partially purified with or without renaturation, or they were translated *in vitro*. Conclusions about relative affinities with such preparations should be made with caution. Another contributing factor may be that for most of these proteins, binding was characterized with artificial binding sites because *in vivo* binding sites were not known. However, more recent studies with Dfd and Ubx homeodomains found statistically significant differences in affinities for related sequences (29, 30). Although these differences are small in relation to the distinctly separate roles these proteins have *in vivo*, a heterologous yeast system designed to compare Ubx- and Dfd-dependent activation with two such sequences revealed almost 100-fold differences when the binding sites were multimerized (30). A second example of selective trans-activation of related sequences is the differential response of Pit-1 and octomer response elements to the POU homeodomain proteins Pit-1 and Oct-2. Although the Pit-1 and octomer response elements differ at only two positions and are both recognized *in vitro* by Pit-1 and Oct-2, no significant cross-activation was observed *in vivo* (31). *In vivo* amplification of small differences in affinity has been attributed to cooperative interactions between homeodomain protein monomers (30, 32), and it will be important to determine the extent to which such interactions contribute to selective targeting *in vivo* at relevant natural sites.

Homeodomain protein binding proteins are also likely to contribute complexity and specificity to the homeodomain-DNA interaction. Such binding proteins can be inferred from the "anti-ftz" phenotype associated with expression of a ftz polypeptide containing a deletion of the homeodomain (33) and from the presence of amino acids on the surface of the homeodomain that are critical to specificity (Fig. 2). Such homeodomain protein binding proteins may include herpes virus VP16, which interacts with Oct-1, human serum response factor, which interacts with Phox1 (34), and *Saccharomyces cerevisiae* MCM1. The interaction of MCM1 with MATα2 at an operator located upstream of the STE6 gene provides the best understood example of homeodomain protein binding site selection. MCM1 raises the target specificity of α2 by forming a complex only at sites that have the proper spacing and orientation to bind two monomers of α2 and a dimer of MCM1 (35). In this way, relatively poor sequence discrimination by α2 monomers is overcome by cooperative interactions with MCM1 (via a short flexible region adjacent to the α2 homeodomain) and with itself (via its N-terminal domain) (35, 36).

Perspective

This review has focused almost exclusively on the structure, properties, and interactions of the homeodomain *per se*. A more complete analysis would also address the functions of determinants outside the homeodomain, since there is increasing evidence that regions N-terminal and C-terminal to homeodomains can contribute to specificity (15, 37–39), to cooperativity (32, 40), and to level or type of activity (12, 34). In neglecting to place the homeodomain in its appropriate protein context, I do not intend to imply that the homeodomain functions as a separate entity or that all functions of homeodomain proteins must follow directly from DNA binding. Nevertheless, the modular nature of homeodomain proteins has facilitated analysis of their DNA binding properties by providing an opportunity to study the homeodomain in isolation. Since the activities of these proteins as activators and repressors of transcription are likely to be an outcome of more complex interactions with additional proteins and DNA and since these interactions involve other regions of the proteins as well, better preparations of intact, native, and fully modified homeodomain proteins and better assays of homeodomain protein function are needed. Success in developing these reagents and methods will yield a more complete understanding of the manner by which these proteins selectively bind their target sequences and influence the transcription apparatus.

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