Site-specific Heterodimerization by Paired Class Homeodomain Proteins Mediates Selective Transcriptional Responses*

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Alx4 is a paired class homeodomain protein involved in defining anterior/posterior polarity in the developing limb bud. The paired class of homeodomain proteins cooperatively bind palindromic DNA elements as homodimers or as heterodimers with other paired homeodomain proteins. Previous characterization demonstrates that the strength of the cooperativity as well as the preference for targets is dictated largely by the identity of amino acid 50 of the homeodomain. Here we compare and contrast the DNA binding properties of a glutamine 50 paired homeodomain protein, Alx4, and a lysine 50 paired homeodomain protein, Goosecoid. We demonstrate that Alx4 homodimers, Gsc homodimers, and Alx4/Gsc heterodimers each have distinct DNA binding properties, and each can discriminate between highly related palindromic elements. Using reporter gene assays, we show that Alx4 activates transcription in a site-specific manner, and that Gsc is capable of antagonizing Alx4-mediated activation only from promoter elements that support heterodimer formation. These data demonstrate that paired homeodomain proteins with different DNA binding properties are able to form heterodimeric complexes with unique DNA binding and transcriptional activities. Thus, heterodimerization regulates the DNA binding specificity of these transcription factors and may partially explain how paired homeodomain proteins direct specific developmental functions.

The mechanisms involved in vertebrate limb patterning serve as a model for understanding pattern formation throughout the body plan. Classical developmental studies in avian systems have provided a foundation for understanding this process by defining three major signaling centers within the developing limb bud: 1) the apical ectodermal ridge, a region of specialized epithelial cells at the distal margin of the limb bud; 2) the dorsal ectoderm; and 3) the zone of polarizing activity (ZPA), a group of mesenchymal cells in the posterior aspect of the limb bud. These signaling centers are derived from both epithelial and mesenchymal cells and form a series of interdependent feedback loops such that this system also serves as a model for understanding epithelial-mesenchymal interactions (for reviews, see Refs. 1 and 2).

The establishment of anterior-posterior (A/P) polarity in the limb bud is controlled largely by the ZPA. The ZPA was initially defined by studies in which explants of cells from various regions of a donor limb bud were grafted to the anterior margin of a recipient limb bud. When posterior mesenchymal cells of the donor limb bud are grafted onto the anterior margin of the recipient limb bud, the resulting limb displays A/P axis duplications (3). These duplications are marked by the appearance of additional anterior digits with posterior characteristics (preaxial polydactyly). This is a dose-dependent phenomenon; more ZPA cells grafted to the anterior margin result in a greater number of ectopic digits. The ZPA does not contribute tissue to the digits, but instead serves as an organizer that patterns surrounding tissue. Two lines of evidence suggest that the key molecule mediating the polarizing activity of the ZPA is Sonic hedgehog (Shh). 1) Shh expression coincides with cells that have polarizing activity, both in the limb and in other tissues; and 2) direct application of Shh to the anterior margin of a limb bud results in digit duplications in a dose-dependent fashion (4, 5).

We have previously identified and characterized a novel homeodomain protein, aristless-like 4 (Alx4) (6, 7). In wild-type embryos, Alx4 is expressed in the anterior mesenchyme of the limb bud. Mice that are homozygous for a null Alx4 allele exhibit preaxial polydactyly that is associated with ectopic Shh expression along the anterior margin of the limb bud during development. Genes previously demonstrated to be downstream targets of the Shh signaling pathway in the limb bud, such as patched and HoxD13, are misexpressed in the anterior mesenchyme of the limb bud as well (7). These results demonstrate that although Alx4 is not required for normal ZPA formation, it is required to prevent an ectopic anterior ZPA from forming. Although several polydactyous mouse mutants have been shown to misexpress Shh at the anterior margin of the limb bud, the genes responsible have been identified in only two cases. Alx4 mutations are known to be responsible for the defects in Strong’s luxoid, and Gli3 is disrupted in the mutant Extra toes (8, 9). To understand the molecular mechanism by which Alx4 regulates A/P polarity in the limb, a detailed characterization of its biochemical and molecular properties is required.

The most outstanding structural feature of Alx4 is the presence of a homeodomain, an evolutionarily conserved DNA binding motif shared by a large family of eukaryotic transcription factors. Genetic experiments demonstrate that these proteins play fundamental roles rooted in the establishment of cell identity or position. Furthermore, these experiments indicate that, in most cases, the homeodomain is necessary for function and biologic specificity (10–12).

Despite the diverse roles that homeodomain proteins play biologically, early molecular characterizations indicated that most bind a similar 5–6-base pair A-T-rich DNA element (13).
It seems implausible that this mode of DNA binding can accommodate the diverse functional specificity implied by the genetic data (for a review, see Ref. 14; for an alternative view, see Ref. 15). More recently, it has been shown that several classes of homeodomain proteins are able to generate target site specificity by forming heterodimeric DNA binding complexes with other proteins (16–24).

Ax4 belongs to the paired class of homeodomain (prd HD) proteins. One mechanism by which prd HD proteins generate target site specificity is by binding DNA as cooperative homodimers. The target elements consist of palindromic repeats of the sequence 5’-TAAT-3’ (P elements) separated by a variable number of nucleotides. Physical and biochemical experiments have shown that the prd HD is both necessary and sufficient for this activity. Furthermore, these studies demonstrate that a major determinant of the DNA binding properties exhibited by prd HD proteins is the identity of residue 50 within the homeodomain (22). Amino acid 50 resides within the DNA recognition helix of the homeodomain, and crystallographic studies reveal that this residue mediates contacts with the nucleotide bases immediately 3’ to the 5’-TAAT-3’ target site (25). Members of the prd class of HDs have either a serine (Ser-50), lysine (Lys-50) or glutamine (Gln-50) at position 50, and each subclass has unique DNA binding properties (22).

The Ser-50 subclass of prd HD proteins preferentially dimerizes on P elements in which the palindromic half-sites are separated by two intervening nucleotides (5’-TAAT NN ATTA-3’; P2 sites). Representative members of this subclass are encoded by the paired (prd) gene from Drosophila (26) and the vertebrate Pux genes (27). In addition to the homeodomain, this subclass is characterized by the presence of a second DNA binding motif, the paired domain, which expands the repertoire of DNA target elements with which these proteins can interact (28). The Lys-50 subclass of prd HDs selectively forms dimers on P elements separated by three intervening nucleotides (P3 elements), with a strong preference for cytosines 3’ to each core half-site, i.e. 5’-TAAT CCG ATTA-3’ (P3C). The half-site comprising this element (5’-TAAT CC-3’) is also recognized by Lys-50 homeodomain monomers from other classes, such as bicoid (28, 29). The Gln-50 subclass preferentially binds P3 sites as well, but has a less stringent requirement for particular residues 3’ to each TAAT half-site. In addition to the differences in target site specificity, the Lys-50 and Gln-50 prd HDs differ in one other aspect; the Lys-50 subclass binds approximately 15-fold more cooperatively to palindromic P3 elements than does the Lys-50 subclass. Cooperativity is defined approximately 15-fold more cooperatively to palindromic P3 elements separated by three intervening nucleotides (P3). The Lys-50 subclass of prd HDs selectively forms dimers on P elements in which the palindromic half-sites are separated by three intervening nucleotides (P3). The Lys-50 subclass of prd HDs selectively forms dimers on P elements in which the palindromic half-sites are separated by three intervening nucleotides (P3).

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The Lys-50 prd HD protein Goosecoid (Gsc) is expressed in mesenchymal cells of the limb bud and first branchial arch in domains that broadly overlap those of Alx4 (34). This protein is of particular interest for two reasons. First, the gsc protein of Drosophila has been shown to function as a transcriptional repressor and antagonist of the Lys-50 activator orthodenticle (otd) (37). Second, mice homozygous for a targeted loss of function allele of Gsc display defects in structures that are also affected by Alx4 mutations, including the skull, mandible, and limb (37, 38).

Here we present a characterization of the DNA binding and transcriptional regulatory properties of Ax4 and Gsc. We find that Alx4 homodimers, Gsc homodimers, and Alx4/Gsc heterodimers all display unique DNA binding properties. Each of these complexes differentiate between P3 elements based on the nucleotides separating the palindromic half-sites. Thus, the selective homo- and heterodimerization of prd HD proteins converts the generic P3 element into a family of related elements, each capable of providing unique transcriptional responses in the presence of Gln-50 and Lys-50 prd HD proteins. These results have implications for understanding the developmental specificity demonstrated by prd HD proteins.

**EXPERIMENTAL PROCEDURES**

*Gel Shift Assays.—Peptides containing the homeodomains of Ax4 (residues 185–265), Gsc (residues 159–219), Max1 (residues 183–226), and Cart1 (128–195) were expressed as (His)6-tagged peptides in Escherichia coli BL21 and purified to homogeneity by chromatography on Ni2+-nitrilotriacetic acid-agarose as described previously (40). Unc4 protein was prepared similarly and was a generous gift of David Miller and Kim Liptig. Gel shift reactions contained 15 μg Tris, pH 7.5, 75 μM NaCl, 1.5 μM EDTA, 0.3% Nonidet P-40, 0.8 μM of 32P-labeled oligonucleotide at 4 μM spermidine, 4 μM spermine, 1.5 μM dithiothreitol, and 7.5% glycerol. Where indicated (Figs. 3 and 4), low dose gel shifts contained 5 μM protein and high dose gel shifts 20 μM protein. After incubation on ice for 10 min, 32P-labeled probe was added and the mixture was incubated at room temperature for 15 min before separation on 7% polyacrylamide gels that contained 0.5× TBE. In competition experiments, a 20-fold molar excess of the competing oligonucleotide was added during the preincubation phase. In preliminary experiments, DNA binding was shown to be dependent on protein concentration. The sequence of the gel shift probes is shown (top strand with TAAT repeats in bold). P1/2C, 5’-CCTGAGAATAAACCGGAGACTGACA-3’; P2C, 5’-CCTGAGAATACCGGATTAGCTGACA-3’; P3C, 5’-CCTGAGAATACCGGATTAGCTGACA-3’; P4C, 5’-CCTGAGAATACCGGATTAGCTGACA-3’; P5C, 5’-CCTGAGAATACCCTTGAGATTAGCTGACA-3’; P3Cmut, 5’-CCTGAGAATACCCTTGAGATTAGCTGACA-3’; P3A, 5’-CCTGAGAATACCGGATTAGCTGACA-3’; P3G, 5’-CCTGAGAATACCGGATTAGCTGACA-3’; P3T, 5’-CCTGAGAATACCGGATTAGCTGACA-3’; P3TTC, 5’-CC-
RESULTS

Alx4 Preferentially Binds the P3 Element as a Cooperative Dimer—Alx4 is a member of the prd class of homeobox genes. It has been previously demonstrated that prd class HD proteins bind preferentially and with high affinity as cooperative homodimers to palindromic repeats of the sequence 5′-TAAT-3′. The preferred spacing of the palindromes is dictated in large part by residue 50 of the homeodomain, such that prd HDs bearing a glutamine or a lysine at position 50 (Gln-50 or Lys-50) prefer P3 elements (5′-TAAT NNN ATTA-3′), while Ser-50 prd HDs prefer P2 elements (5′-TAAT NN ATTA-3′) (22). Alx4 contains a Gln-50 HD and therefore is predicted to bind P3 elements. To test this prediction, gel shift assays were performed using probes containing TAAT repeats separated by a variable number of nucleotides (Fig. 1B). In these experiments, we used a recombinant Alx4 peptide fragment containing the HD that was expressed in bacteria and purified to homogeneity via nickel agarose chromatography (Fig. 1A). Alx4 bound all of the indicated probes to some extent (Fig. 1C). Migration of monomeric protein-DNA complexes is defined by binding to P1/2C, which contains a single half-site (Fig. 1C, lanes 2–4), and the more slowly migrating species on other probes represents dimeric binding. The prd HD is known to exist as a monomer in solution and interact with DNA in a stepwise fashion resulting in dimeric protein-DNA complexes (22). Thus, the overall binding affinity is a function of the affinity of peptide monomers for the first half-site and the enhanced affinity for a monomeric peptide binding the second half-site. The -fold increase for the second binding event is defined as the cooperativity coefficient, $\tau$ (see “Experimental Procedures”). Measurements of $\tau$ in this and similar experiments indicated that cooperativity is greatest on the P3C site where $\tau \approx 300$; on P2C and P4C sites, $\tau \approx 20$; and binding was noncooperative on P5C.

These results are consistent with values previously reported for other Gln-50 prd HD proteins (22). The preference for P3 elements was further demonstrated by a competition experiment in which binding of Alx4 to P3C was competed by a 20-fold excess of cold P3C probe (Fig. 1D, lane 3), but not P1/2C, P2C, P4C, or P5C (Fig. 1D, lanes 4–7). To ensure that the DNA binding properties observed for the recombinant peptide frag-

TGAGAATATTCTCTACGTACA-3′; P3TGG, 5′-CCTGAGATTT- TTGATTACTGATA-3′.

Cooperativity measurements were made using a Molecular Dynamics PhosphorImager. Dried gels were scanned and the relevant bands quantitated. By definition, $\tau = \frac{K_{d2}}{K_{d1}}$, where $K_{d1}$ and $K_{d2}$ are functions of the following binding reactions.

$$K_{d1} = \frac{P + D}{PD}$$

REACTION 1

$$K_{d2} = \frac{PD + P}{P_D}$$

REACTION 2

P is free protein, D is DNA, PD is monomerically bound DNA, and $P_D$ is dimerically bound DNA. Using the PhosphorImager to quantitate bands representing the relevant complexes, $\tau$ can be calculated via Equation 1.

$$\tau = \frac{K_{d2}}{K_{d1}} = 4[D/P][P/(D/IP)]^2$$  

(Eq. 1)

$\tau$ measurements were made from lanes in which approximately 50% of the probe was shifted and represent an average from three independent experiments with a deviation of less than 15% (for details, see Ref. 22).

Reporter Gene Assays—Three copies of the relevant sequences were cloned upstream of a basal promoter (from the adenovirus E1b gene) and chloramphenicol acetyltransferase (CAT) gene in the plasmid pCMX-Alx4 has previously been described (40). The mouse Gsc cDNA with a hemagglutinin tag was cloned into pCMX (41) to generate the expression plasmid pCMX-Gsc. The Alx4 expression plasmid pCMX-Alx4 has been previously demonstrated that prd class HD proteins bind preferentially and with high affinity as cooperative homodimers to palindromic repeats of the sequence 5′-TAAT-3′. The preferred spacing of the palindromes is dictated in large part by residue 50 of the homeodomain, such that prd HDs bearing a glutamine or a lysine at position 50 (Gln-50 or Lys-50) prefer P3 elements (5′-TAAT NNN ATTA-3′), while Ser-50 prd HDs prefer P2 elements (5′-TAAT NN ATTA-3′) (22). Alx4 contains a Gln-50 HD and therefore is predicted to bind P3 elements. To test this prediction, gel shift assays were performed using probes containing TAAT repeats separated by a variable number of nucleotides (Fig. 1B). In these experiments, we used a recombinant Alx4 peptide fragment containing the HD that was expressed in bacteria and purified to homogeneity via nickel agarose chromatography (Fig. 1A). Alx4 bound all of the indicated probes to some extent (Fig. 1C). Migration of monomeric protein-DNA complexes is defined by binding to P1/2C, which contains a single half-site (Fig. 1C, lanes 2–4), and the more slowly migrating species on other probes represents dimeric binding. The prd HD is known to exist as a monomer in solution and interact with DNA in a stepwise fashion resulting in dimeric protein-DNA complexes (22). Thus, the overall binding affinity is a function of the affinity of peptide monomers for the first half-site and the enhanced affinity for a monomeric peptide binding the second half-site. The -fold increase for the second binding event is defined as the cooperativity coefficient, $\tau$ (see “Experimental Procedures”). Measurements of $\tau$ in this and similar experiments indicated that cooperativity is greatest on the P3C site where $\tau \approx 300$; on P2C and P4C sites, $\tau \approx 20$; and binding was noncooperative on P5C.

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Alx4 activates transcription in a P3C-specific manner. A, 293 cells were cotransfected with 100 ng of pCMX-Alx4 and 100 ng of the indicated reporter gene. 48 h after transfection, cell lysates were assayed for CAT activity. Transfections included the β-galactosidase expression plasmid pCH110 for standardization via Alx4-specific gel shift was detected only on the P3C probe (Fig. 2A, lane 9). Because of the decreased mobility of protein-DNA complexes with full-length Alx4, dimeric and monomeric complexes could not be resolved. The P3C-specific binding observed with the full-length protein is in contrast to the previous series of gel shifts in which the recombinant Alx4 peptide bound all sites tested. This difference is most likely due to the fact that lower levels of Alx4 protein are present in the nuclear extracts. Consistent with this idea, gel shifts of the various P elements at intermediate doses of recombinant peptide indicate that dimeric binding was detected only on the P3C site (Fig. 1C, compare lane 11 to lanes 3, 7, 15, and 19). This suggests that the binding activity detected on sites that lack the P3 spacing require amounts of protein greater than that expressed in transfected 293 cells, and therefore high affinity cooperative dimerization on P3 sites may be the only binding event that occurs in vivo.

Alx4 Is a Transactivator of P3 Elements—To test the possibility that the DNA binding activity described in Fig. 1 can result in transactivation, a series of synthetic reporter constructs were utilized. The various P elements used in DNA binding assays (see Fig. 1B) were cloned in triplicate upstream of a basal E1b promoter driving CAT expression. Transient cotransfections were performed in 293 cells using the P element reporters with or without pCMX-Alx4.

Reporter gene activity was detected only on the P3C reporter (P3C-CAT) (Fig. 2A and data not shown). This activation was dependent on the dose of pCMX-Alx4 to a maximal activation of 100 ng of input plasmid. At higher doses, the level of activation declined, probably due to squelching effects. These results suggest that high affinity, cooperative DNA binding is required for transcriptional activation and that the non-cooperative and weakly cooperative binding detected in vitro are not sufficient to mediate a biologic response.

Nucleotides Spacing the P3 Palindrome Define High and Low Affinity Binding Sites—The P3 elements were previously identified as binding sites for prd HD proteins using a polymerase chain reaction-based site selection technique, which over several rounds of selection and enrichment generates a consensus site (48). However, this method may fail to identify potential target elements with a lower binding affinity, or become biased against some high affinity sites. Since the ability of Alx4 to bind different P3 elements as homodimers is determined, in part, by the intrinsic affinity for different half-sites, we defined the half-site preference for Alx4 by varying the nucleotide immediately 3' to the core TAAT half-site. Using double-stranded oligonucleotides containing P1/2 sites as probes (Fig. 3A, top), we performed gel shifts with two doses of Alx4 peptide. At lower doses (Fig. 3A, lanes 1, 3, 5, and 7), Alx4 bound to P1/2C and P1/2T only. A 4-fold higher concentration of peptide (Fig. 3A, lanes 2, 4, 6, and 8) resulted in binding to all sites, with binding to P1/2C and P1/2T greater than that of P1/2A and P1/2G. Therefore, the preferred half-site can be represented as 5'-TAAT Py-3'.

We went on to compare Alx4 binding to a series of idealized P3 elements in which the nucleotides separating the core half-sites were made palindromic (Fig. 3B, top). This experiment was performed with two concentrations of peptide as well, lanes 7–10 having 4-fold more than lanes 2–5. Consistent with the half-site preferences (Fig. 3A), Alx4 preferentially formed homodimeric complexes on P3C and P3T (Fig. 3B, compare lanes 3 and 5 to lanes 2 and 4), although binding to P1/2A and P1/2G could be detected at higher doses of protein (Fig. 3B, lanes 7 and 9). The same experiment performed using an A/T base pair at the center position instead of C/G yielded similar results (data not shown). These experiments reveal the existence of at least two classes of Alx4 target elements. Consistent with previous results, P3C and P3T elements serve as high affinity sites (22). Additionally, we detected gel shift activity on P3A and P3G, which serve as low affinity sites. The high affinity consensus site is represented as 5'TAAT PyNPy ATT3' and the low affinity consensus 5'-TAAT PyNPy ATT3'. Considering that the cooperativity of binding remains constant on both classes of sites (data not shown), we attribute differences in binding to differences in the affinity for individual half-sites, as demonstrated in Fig. 3A.

The ability of Alx4 to activate a series of reporter genes harboring P3A, P3C, P3G, or P3T elements was tested in transient cotransfection assays. The high affinity sites mediated
activation to levels over 10-fold greater than the low affinity sites and activated at lower levels of pCMX-Alx4 (Fig. 3C).

However, low affinity sites are activated more than 100-fold over background at the highest levels of activator plasmid, suggesting that both high affinity and low affinity P3 binding sites could serve as biologically relevant promoter elements, with the lower affinity sites presumably mediating activation only at sites of high Alx4 expression.

Gsc Antagonizes Alx4 Activation—Although physical interactions between members of the same prd HD subclass have been well described (22, 36, 37), interactions between the subclasses have not. Four lines of evidence led us to explore the possibility that Alx4 might interact with the Lys-50 prd HD protein Gsc. 1) Gsc expression overlaps with that of Alx4 in the limb bud and first branchial arch; 2) genetic experiments reveal that both Alx4 and Gsc play a role in patterning the skull, jaw, and limb; 3) both Alx4 and Gsc have been shown to bind P3C elements as cooperative dimers; and 4) Alx4 acts genetically to repress the expression of Shh in the anterior limb mesoderm, and Drosophila gsc is a transcriptional repressor shown to antagonize the prd HD activator odd (7, 22, 36–39, 42).

To address the biochemical and molecular properties of a potential Alx4/Gsc heterodimeric complex, we first analyzed the DNA binding properties of Gsc alone. Using the same series of P1/2 site probes as before (Fig. 3A), and recombinant Gsc homeodomain purified from bacteria (Fig. 1A), we performed gel shift experiments to define the preferred Gsc half-site. In contrast to Alx4, which bound both P1/2C and P1/2T with high affinity (Fig. 3A), Gsc bound almost exclusively to the P1/2C probe (Fig. 4A). We went on to test the ability of Gsc to bind the panel of idealized P3 sites as well. Consistent with its half-site preference, Gsc preferentially bound the P3C element as a cooperative dimer (Fig. 4B). The cooperativity measured for Gsc homodimers was much less than that for Alx4 (compare Fig. 3B to Fig. 4B; note the relative increase in the monomer-DNA complex with Gsc). On P3C, we determined that $\tau \sim 20$ for Gsc and $\tau \sim 300$ for Alx4, although the total amount of protein complexed with DNA is similar due to the increased half-site binding by Gsc. Therefore, compared with Alx4, Gsc interacts with a more restricted range of P3 sites, exhibits lower cooperativity, but has a higher affinity for the P1/2C site.

We went on to address the DNA binding properties of Alx4/Gsc heterodimers using the four idealized P-elements as probes (see Fig. 3B). Because the Alx4 peptide is larger than the Gsc peptide, heterodimers can be recognized by the appearance of a complex with intermediate mobility. Heterodimers could be detected on all sites tested, but formed preferentially on P3C (Fig. 4A). The cooperativity constant for heterodimer binding on the P3C site ($\tau \sim 100$) was greater than that of Gsc homodimer formation ($\tau \sim 20$); as a consequence, the heterodimer was the preferred species on this site. The failure of Alx4 and Gsc to be co-immunoprecipitated (data not shown) strongly suggests that heterodimerization is dependent on the presence of a DNA binding site. Similar mixing experiments with the Alx4 HD and purified Max1 HD (see Fig. 1A) demonstrated that the ability to form cooperative heterodimers is not a general property of HDs outside of the prd class (Fig. 5B).

Gsc is distinguished from other prd HD proteins in that it functions as a transcriptional repressor. Experiments with gsc from Drosophila demonstrate that the repressor activity is an active process requiring a 9-amino acid domain called the enailed homology one (eh-1) domain (43), which is conserved in mouse, human, and Xenopus Gsc. To determine what affect Gsc has on Alx4 activity, we tested the ability of Gsc to antagonize Alx4-mediated activation in cotransfection assays. Increasing amounts of the expression vector pCMX-Gsc, which encodes full-length Gsc, was transfected along with 100 ng of pCMX-Alx4, an amount shown to strongly activate P3C-CAT (Fig. 2). The results were consistent with the gel shift assays designed to detect heterodimer formation (Fig. 5A). Gsc preferentially antagonized Alx4-mediated activation of the P3C-CAT reporter, as shown by a 20-fold decrease in activity at the lowest dose of pCMX-Gsc (50 ng) tested (Fig. 5, C and D). Likewise, reporter genes containing sites that support weak heterodimer formation are not efficiently antagonized by Gsc. The ability of Gsc to antagonize Alx4 was dependent on the eh-1 domain (data not shown), indicating that this represented active repression and not a simple competition for sites. Furthermore, P3 sites that Alx4 bound with higher affinity also supported higher levels of Alx4-mediated transactivation. This demonstrates that differences in the nucleotides separating the P3 palindromic half-sites provide for a wide range of transcriptional responses to Alx4 and Gsc, and serve as a series of differentially responsive transcriptional control elements.

Hybrid P3 Elements Display Predictable Responses—Although the elements tested thus far provide insights into the biochemical and molecular properties of Alx4 and Gsc, they also suggest that a third class of elements exist, which are hybrids of the high and low affinity elements. One such element, P3TTC, is present in the mouse Gsc promoter and may play a role in autoregulation (44). We also chose to test the hybrid element P3TGG, which bears an optimal Alx4 half-site (P1/2T, see Fig. 3A) and an optimal Gsc half-site (P1/2C, see Fig. 4A). Consistent with the absence of a high affinity Gsc half-site, Gsc bound P3TTC weakly as a monomer (Fig. 6A, lane 4) and did not form homodimers or heterodimers with Alx4 on this site. Gsc bound P3TGG as a monomer in the absence of Alx4 (Fig. 6A, lane 8); however, with the addition of Alx4, heterodimers formed preferentially on this site (Fig. 6A, lane 7). Alx4 bound both elements similarly, although cooperativity measurements reveal that $\tau$ is reduced approximately 3-fold on the P3TTC site (Fig. 6A, compare lanes 2 and 6, and data not shown).

In cotransfection experiments, Alx4 activated reporter gene expression from both hybrid sites (Fig. 6B). Although activation of the P3TGG-CAT reporter was similar to that of P3C-CAT, activation of the P3TTC-CAT was about 5-fold less efficient (Fig. 6B). This result may be attributable to the reduced cooperativity of binding to this site. We next tested the ability of Gsc to antagonize Alx4-mediated activation of these sites. We found that Gsc efficiently antagonized the activation of P3TGG-CAT, but not P3TTC-CAT (Fig. 6C). The results are consistent with the gel shift results presented in Fig. 6A, and suggest that Gsc can antagonize Alx4-mediated activation only if it is able to participate in the formation of a dimeric DNA binding complex.

DISCUSSION

The homeodomain is one of the most highly conserved and widely distributed DNA binding motifs in all of biology (10). Despite binding similar DNA elements as monomers, HD pro-
shift assays were performed using a 5 nM concentration of the indicated peptides and 32P-labeled P3C probe (lanes 1–4). Each lane contains 5P-labeled P3C probe and 5 nM peptide as indicated. C, 293 cells were cotransfected with 100 ng of pCMX-Alx4, 500 ng of the indicated reporter gene, and 0, 50, or 100 ng of pCMX-Gsc as indicated. CAT activity was determined as in Fig. 2, except that the data were graphed such that the maximal CAT activity observed for each reporter is arbitrarily designated 100%. D, data from C graphed such that the maximal CAT activity observed with the P3C reporter is arbitrarily designated 100% and all other values are expressed relatively.

**Fig. 6.** Alx4 and Gsc interactions on hybrid P3 elements. A, gel shift assays were performed using a 5 nM concentration of the indicated peptides and 5P-labeled P3TTC (lanes 1–4) or P3TTG (lanes 5–8). AD indicates Alx4 dimer; AM indicates Alx4 monomer; H indicates Alx4/Gsc heterodimer; and GM indicates Gsc monomer. B, 293 cells were cotransfected with increasing amounts of pCMX-Alx4 as indicated, and 500 ng of the indicated reporter gene. CAT activity was determined as in Fig. 2. C, 293 cells were cotransfected with 100 ng of pCMX-Alx4, 500 ng of the indicated reporter gene, and 0, 50, or 100 ng of pCMX-Gsc as indicated. CAT activity was determined as in Fig. 2, except that data were graphed such that the maximal CAT activity observed for each reporter is arbitrarily designated 100%.

In particular, the prd class of HD proteins have evolved the capacity to form cooperative homodimers to specify DNA target elements. These proteins selectively bind palindromic repeats of the sequence 5'TAAT-3', with different subclasses having a unique preference for the number of bases separating the half-sites (22). We present evidence that the Gln-50 prd HD protein Alx4 binds a broader range of target DNA elements than the Lys-50 prd HD protein Gsc, and that the formation of Alx4/Gsc heterodimers expands the range of sites Gsc can act on. We demonstrate that these DNA binding properties provide for a complex mechanism of gene regulation, such that changes in the nucleotides spacing the core half-sites generate a family of differential response elements for prd class HD proteins.

Cooperative DNA Binding Provides Control—Cooperative binding is a two-step process for achieving high affinity DNA-protein interactions, and each step provides an opportunity for regulation. For example, Alx4 interacts more efficiently with the P3C site than P3A. Since the cooperativity of binding is the same for each site (τ ~ 300), differences in dimeric binding are a consequence of the greater affinity of Alx4 monomers for each P3C half-site. In comparing Alx4 and Gsc, we demonstrate that the overall affinity of each homodimer for the P3C site is similar, despite the fact that Gsc has a greater affinity for each P3C half-site. This is because the increased half-site affinity of Gsc is offset by a corresponding increase in the cooperativity exhibited by Alx4. These examples demonstrate how each step in the binding reaction can be modulated to generate target site specificity.

Previous studies have demonstrated that some prd HD proteins can mediate transcriptional affects through P1/2 sites, albeit with much reduced efficiency compared with P3 sites (22). Our data suggest that Alx4 does not function in this manner, but they do not rule out the possibility that other prd HD proteins, as well as Alx4, could function through half-sites in certain cellular contexts. In the case of the Gln-50 prd HD protein Phox1, binding to a P1/2 site is enhanced by interactions with serum response factor, which binds an adjacent site (19). Taken together, the data suggest that, in native promoters, prd HD proteins likely act on palindromic elements or on half-sites that are juxtaposed to the target site of other DNA binding partners.

**Functional Importance of Residue 50**—Consistent with previous reports, we find that Alx4, a Gln-50 prd HD, binds a
broader range of targets than does Gsc, a Lys-50 prd HD protein. Residue 50 of prd HD proteins has been shown to specify the spacing preference, contribute to cooperativity, and direct the preference for specific half-sites (Figs. 3A and 4A and Refs. 22 and 51). Crystallographic analyses of other HD proteins provide insights into the mechanisms that underlie some of these differences. The engrailed (en) HD, which contains a lysine at position 50, has been crystallized bound to DNA (52), as has a mutant en with a glutamine substituted at this position (en Gln-50) (53). The results indicate that a lysine can mediate direct contacts to the nucleotides 3’ to the core TAAT element, while the corresponding en Gln-50 contacts are water-mediated and indirect. Similar conclusions were drawn from the analysis of the prd Gln-50 structure (25). The difference in the way Gln-50 or Lys-50 mediates base contacts may explain why Gln-50 prd HD proteins bind a broader range of target elements than the Lys-50 subclass; furthermore, it could explain the higher affinity of Lys-50 prd HD proteins for the 5’-TAAT C-3’ half-site.

Each prd HD protein studied thus far demonstrates DNA binding properties typical of other members of its subclass. The degree of conservation both in sequence and DNA binding properties suggest that the differential responsiveness of P3 sites to Alx4 and Gsc may apply to prd Gln-50 and Lys-50 HD proteins in general. In support of this idea, we have demonstrated that the closest relative of Alx4, Cart1, shares its DNA binding properties (36), as does the more divergent Gln-50 prd HD protein Unc4 from Caenorhabditis elegans (data not shown); previous studies with the prd Gln-50 mutant supports this conclusion as well (22).

Site-selective Heterodimerization Generates a Family of Differentially Responsive Promoter Elements—By acting as homodimers or heterodimers, prd HD proteins are able to differentiate between P3 elements based on the nucleotides spacing the core palindromic half-sites. This defines the P3 site as a family of prd response elements, such that genes containing these sites in their promoter can elicit unique transcriptional responses when exposed to specific prd HD proteins in a dose-dependent manner. For example, genes containing the P3C element can be activated at sites of relatively low Alx4 expression while genes containing P3A elements can be activated only at sites of high Alx4 expression. Likewise, Gsc can antagonize some Alx4 target genes when present at low levels (e.g., those that contain P3C sites), others only when present at high levels (e.g., those that contain P3A sites), and others not at all (e.g., those that contain P3TTC sites). Furthermore, a single promoter element can provide target genes with distinct responses to different prd HD protein complexes. For example, the P3T element is highly responsive to Gln-50 prd HD proteins, but poorly responsive to the action of Lys-50 prd HD proteins.

The hybrid sites highlight the flexibility of this system of response elements. P3TTC represents an Alx4 intermediate response element that is non-responsive to Gsc. Conversely, the P3TGG element is highly responsive to Alx4 and is readily antagonized by Gsc. Therefore, in the absence of Gsc, P3TGG-containing genes are more efficiently expressed than P3TTC-containing genes, but, in the presence of Gsc, P3TTC-containing genes are more efficiently expressed. This also suggests that repression by Gsc requires the ability to form homo- or heterodimeric complexes, since sites that Gsc can bind only as a monomer, such as P3TTC, are not efficiently repressed.

By mixing and matching half-sites that Alx4 and Gsc bind with differing affinities, elements that mediate unique transcriptional responses are generated. The fact that some Gln-50 prd HD proteins act as repressors, for example Unc4 (54), adds additional complexity to this network of differential response elements. Our data suggest that a Gln-50 repressor such as Unc4 would be able to antagonize activation from a broader range of P3 elements than does the Lys-50 repressor Gsc.

The ability of the repressor Gsc to complex with the activator Alx4 may provide some insight into understanding how Alx4 functions to repress Shh expression in the anterior mesenchyme of the limb bud. The expression patterns and physical interactions described for Alx4 and Gsc are consistent with a model whereby Alx4 and Gsc act directly at the Shh limb bud enhancer. While Gsc mutant mice do not show A/P patterning defects in the limb, Gsc does function to as an enhancer of Alx4-dependent polydactyly. The existence of additional Gsc-related genes suggests that functional redundancy is possible. A clearer understanding of the role of prd HD proteins in limb patterning may be revealed by a functional definition of the Shh limb bud regulatory elements as well as further genetic analyses.

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