The Paired-domain Regulates DNA Binding by the Homeodomain within the Intact Pax-3 Protein*

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Pax-3 contains two structurally independent DNA-binding domains, a paired-domain and a homeodomain. Their functional interdependence has been suggested by the analysis of the Sp-delayed (Sp†) mouse mutant, in which a glycine to arginine substitution at position 9 of the paired-domain abrogates DNA binding by both domains. This glycine is located in the β-turn portion of a β-hairpin motif, and the requirement for this structure was investigated by mutagenesis at this and neighboring positions. At position 9, only substitution with proline increased DNA binding by the paired-domain and homeodomain above the level observed with the Sp† arginine mutation, suggesting that the β-turn is necessary for the function of both DNA-binding domains. Alanine scanning mutagenesis also identified a number of flanking residues important for DNA binding by both domains, emphasizing the requirement of the β-hairpin for the interaction of Pax-3 with DNA. Furthermore, we show that these mutations reduce binding by the homeodomain at the monomeric level and do not impair dimerization on a TAAT(N)3ATTA consensus motif. In contrast, the wild-type paired-domain was found to prevent dimerization on consensus motifs with 3-base pair spacing of the type TAAT(N)3ATTA. Importantly, both the deleterious effect of the Sp† mutation on homeodomain DNA binding and the loss of dimerization on TAAT(N)3ATTA motifs can be transferred to a heterologous homeodomain from the human phox protein. Moreover, the presence of the paired-domain affects sequence discrimination within the 3-base pair spacer in this context. These analyses establish that the β-hairpin motif is essential for paired-domain and homeodomain DNA binding, and suggest a novel mechanism by which the paired-domain can influence sequence specificity of the homeodomain within the Pax-3 polypeptide.

The paired-domain was originally described as a region of sequence homology between the Drosophila segmentation gene paired and two genes encoded at the gooseberry locus (1), and was subsequently shown to have a DNA-binding function (2). This domain comprises two independent and interactive subdomains, NH2-terminal and COOH-terminal, that make distinctive contributions to sequence recognition (14, 15). For instance, alternative use of a single glutamine codon in the Pax-3 paired-domain leads to the production of isoforms which differ in their ability to utilize the COOH-terminal subdomain for DNA binding (16). Likewise, an alternative splicing event in the Pax-6 gene encodes an isoform which recognizes DNA exclusively through its COOH-terminal subdomain (15). Adding to this diversity in sequence recognition, consensus sequences derived in vitro for the Prd homeodomain contain palindromic recognition motifs of the type TAAT(N)3ATTA to which two homeodomains bind cooperatively (17). Furthermore, regulation of the Drosophila even-skipped gene by Prd requires the cooperative binding of the paired-domain and homeodomain to a target sequence that contains juxtaposed recognition motifs for both domains (18). Consequently, although the paired-domain and homeodomain can bind DNA with high affinity when expressed separately, their presence in the same polypeptide allows them to cooperate in DNA recognition. It is not clear, however, if the DNA-binding properties of Pax proteins which contain both domains are simply determined by the sum of their parts or if interactions between the domains influence DNA recognition.

Our previous analysis of the mouse Sp† mutation has suggested that the paired-domain and homeodomain of Pax-3 may functionally interact (19). In Sp†, a single glycine to arginine substitution at the ninth position of the paired-domain abrogates DNA binding to both domains. The elucidation of the crystal structure for the paired-domain of the Drosophila Prd protein revealed that the glycine residue contributes to a β-turn that joins 2 short anti-parallel β-strands, together forming a β-hairpin motif (20). To determine the specific requirement for the β-turn and to define the role of the β-hairpin motif...
in paired-domain and homeodomain DNA binding, additional mutagenesis was carried out at position 9 and the conserved residues that make up this structure. The current analysis reveals that the integrity of the β-hairpin is essential for the DNA binding activity of both the paired-domain and homeodomain, confirming our original hypothesis that the homeodomain does not function as an independent globular structure within the intact Pax-3 protein. Importantly, sequence recognition by the homeodomain was found to differ in the presence or absence of the paired-domain in both Pax-3 and a chimeric protein containing a homeodomain derived from the human phox protein, indicating that the paired-domain of Pax-3 might influence the sequence specificity of the homeodomain in vivo.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The construction of expression plasmids encoding wild-type Pax-3 (Pax-39Sp) (Pax-3Sp9 and Pax-3Sp9rd has been previously described (19). The wild-type and Sp9 Pax-3 constructs comprise a portion of the Pax-3 cDNA that extends from nucleotides 297 to 1801 (21) cloned into the eukaryotic expression vector pMT2, allowing for expression in COS-7 cells. Both constructs encode the full-length 479-amino acid Pax-3 polypeptide and differ by the presence of a glycine (wild-type) or serine (Sp9) residue at the ninth position of the paired-domain. The Pax-3Sp9rd construct was generated by replacing the Sp9 mutation with a serine codon in codon 9 of the Pax-3Sp9 cDNA. The sequence of the oligonucleotides used to generate the Pax-3Sp9rd construct was as follows:

TTA[N]8-3

using 20 μg of supercoiled plasmid DNA prepared by CsCl equilibrium density centrifugation. Precipitates were left on cells for 16 h, at which time the cells were washed once with phosphate-buffered saline, and then treated for 1 min with 15% glyceral in 1 x HEPES-buffered saline (2 x HS) is 280 mM NaCl, 150 mM KCl, 50 mM K2PO4, 2H2O, 12 mm dithiothreitol, and 50 mM HEPES, pH 7.0 at 37 °C. Whole cell extracts were prepared 24 h later by sonication in 200 μl of ice-cold buffer containing 20 mM HEPES (pH 7.6), 150 mM NaCl, 0.5 mM dithiothreitol, 0.2 mM EDTA, 0.2 mM EGTA, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin) (19, 26). The level of Pax-3 expression was determined by Western blotting using a polyclonal anti-Pax-3 antibody (19) at a 1:5000 dilution and visualized with enhanced chemiluminescence using a donkey anti-rabbit horseradish peroxidase-conjugated secondary antibody (Amersham). Similarity of the level of protein expression from the pCGN plasmids was determined using a monolecal anti-HA antibody (12CA5, BAbCO) at a 1:1000 dilution and a sheep anti-mouse horseradish peroxidase-conjugated secondary antibody (Amersham).

Electrophoretic Mobility Shift Analysis—Whole cell extracts were added to 5 μl of [32P]-labeled oligonucleotide in a 20 μl reaction volume containing 10 mM Tris-HCl (pH 7.5), 2 mM MgCl2, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 5% glycerol (27). In addition, when using the P3OPT oligonucleotide, 1 μg each of p(philD)-p(philD) and sonicated salmon sperm DNA was added as a nonspecific competitor, whereas 2 μg of sonicated salmon sperm DNA was added when the Pax-3 and P3 oligonucleotides were used. Therenat DNA complexes were allowed to form at 20 °C for 30 min, and were then loaded onto 6% polyacrylamide:bisacrylamide (29:1) gels containing 0.5 x TBE (90 mM Tris-HCl, 90 mM boric acid, 2 mM EDTA, pH 8.3), and electrophoresed at 12 V/cm in the same buffer. Gels were dried under vacuum and exposed to a PhosphoImager screen (Fuji) so that quantitation of radioactivity could be done using a Fuji BAS 2000 PhosphoImaging station and then to Kodak X-AT film with an intensifying screen.

Several oligonucleotides were used in electrophoretic mobility shift assays to detect either paired-domain or homeodomain DNA binding activity. The P3OPT oligonucleotide was derived from optimal recognition sequences determined for the Pax-3 paired-domain in vitro (28, 29) and contains the core sequence 5′-TCCCTCCTCCTCCTCCTC-3′. In addition, to reduce nonspecific binding to the P3OPT oligonucleotide, a 20-fold excess of unlabeled P3OPT was added. Oligonucleotides specific for homeodomain DNA binding were previously identified by the SELEX procedure (17) and include P12 (5′-TTTTAGGCCTTTGTTATGTTGTTGTTGTT-3′), P2 (5′-TTTTAATTGTTGTTGTTGTTGTT-3′, and P3 (5′-TTTTAATTGTTGTTGTTGTTGTT-3′). Oligonucleotides were synthesized to have a recessed 3′ end that would permit end labeling with α32PdATP and Klenow DNA polymerase upon annealing.

RESULTS

Mutagenesis at Position 9 of the Pax-3 Paired-Domain—The Sp9 mutation involves an arginine for glycine substitution at the ninth position of the Pax-3 paired-domain (30). Based on the three-dimensional crystal structure solved for the paired-domain of the Drosophila Prd protein, this conserved glycine residue is situated in a β-turn (β1, Fig. 1A) that connects two anti-parallel β-strands (β1 and β2, Fig. 1A), forming a β-hairpin motif at the amino terminus of the protein. This motif makes three phosphate contacts and also contributes to stabilizing the adjacent helix-turn-helix motif (α2 and α3, Fig. 1A), and a second β-turn (β2, Fig. 1A) that occupies the minor groove (10). Consequently, disruption of the β-turn is expected to have a profound effect on DNA binding by the paired-domain as we have previously shown (19). Interestingly, the Sp9 mutation was also found to decrease the homeodomain-specific DNA binding activity of Pax-3 and suggested that the paired-domain and homeodomain may functionally interact within the full-length protein (19). An alternative explanation to this surprising relationship is that the Sp9 mutation disturbs folding of the Pax-3 polypeptide in a manner that does not reflect normal interactions within the wild-type protein. Therefore, it was important to distinguish between a specific structural requirement for Gly-9 in the β-turn and any nonspecific effects attributed to substitution with arginine. To accomplish this, the glycine residue was independently replaced with glutamate,

Expression and Detection of Pax-3 in COS-7 Cells—COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37 °C under 5% CO2. Approximately 1.5 x 106 COS-7 cells were transfected by calcium-phosphate co-precipitation (25)
cysteine, and proline to evaluate the role of amino acid charge, size, flexibility, and hydrophobicity in the SpΔ DNA-binding defect (summarized in Fig. 1B).

Wild-type Pax-3 and position 9 mutants were expressed in COS-7 cells and Pax-3 protein expression was measured by Western blotting using a Pax-3 specific antiserum (Fig. 1C). This analysis indicated that similar amounts of Pax-3 protein were present in the whole cell extracts corresponding to each of the transfectants, and equivalent amounts of wild-type and mutant proteins were therefore used in electrophoretic mobility shift assays (EMSAs). Using the paired-domain specific oligonucleotide P3OPT, it is apparent that the SpΔ substitution (R9) leads to a complete loss of paired-domain DNA binding activity (compare lanes 1 and 2, Fig. 1D). Results from binding studies with P3OPT are consistent with those previously obtained using the e5 sequence from the Drosophila even-skipped protein, where a 17-fold reduction in binding was observed (19). Interestingly, mutation to glutamate also ablates paired-domain DNA binding activity (lane 3 in Fig. 1D), indicating that the net charge of the substituted amino acid is not important in this phenotype. Similarly, substitution with cysteine, which contains a polar but uncharged side chain, abrogates paired-domain DNA binding activity. Furthermore, since glutamate and cysteine have smaller side chains than arginine and can adopt fewer conformations, loss of binding by these mutants suggests that the size of the substituted amino acid is not an important determinant in the loss of paired-domain DNA binding in SpΔ. The only mutant that does form a detectable complex with the P3OPT oligonucleotide is proline (lane 5, Fig. 1D), although it is characterized by an approximately 20-fold lower affinity than wild-type Pax-3 (lane 1, Fig. 1D). Given that proline is the only substitution that may allow the β-turn to form (modeling data not shown), this analysis would suggest that the SpΔ mutation acts primarily through the disruption of this structure.

Is this structural defect also the basis for the loss in homeodomain DNA binding activity? To answer this, the same series of mutants was analyzed in EMSAs using a consensus oligonucleotide derived for homeodomains of the paired class (P2) (17). As we have shown previously, the SpΔ arginine substitution reduces binding to the P2 oligonucleotide approximately 20-fold when compared with wild-type Pax-3 (compare lanes 1 and 2, Fig. 1E). Likewise, both the glutamate (lane 3, Fig. 1E) and cysteine (lane 4, Fig. 1E) mutations cause an approximately 20-fold reduction in P2 binding. Substitution with proline resulted in a mutant that retained approximately 10% of its homeodomain DNA binding activity (lane 5, Fig. 1E). As was observed with P3OPT, the lack of any correlation between amino acid charge, size, or hydrophobicity with the defect in P2 binding, and the reduced severity of the proline substitution indicate that the loss of the β-turn is also responsible for the reduction in homeodomain DNA binding activity.

Alanine Replacement Mutagenesis at Positions 6–10 of the Pax-3 Paired-domain—The β-turn is an essential component of the β-hairpin motif (Fig. 1A). Within the β-hairpin motif, residues 6–10 are perfectly conserved in all paired-domains described to date which suggests that they are essential for paired-domain function (Fig. 2A). As a result, these residues were chosen for independent alanine replacement to further define the requirement for the β-hairpin in DNA binding by Pax-3. For this, the last residue in the first β-strand (Asn-6), the three residues which make up the β-turn (Gln-7, Leu-8, and Gly-9), and the first residue (Gly-10) of the second β-strand were individually mutated to alanine (summarized in Fig. 2A). With the exception of Gln-7, alanine replacement at each position is predicted to alter the local secondary structure (lower panel in Fig. 2A), and should therefore affect the functionality of the β-hairpin motif. To visualize the role of these amino acids within the β-hairpin motif and the possible impact of their replacement with alanine, the location of side chains is shown schematically with respect to the α-carbon trace in Fig. 2B. The side chains for Asn-6 and Leu-8 are both directed toward the helix-turn-helix structure (HTH), which binds in the adjacent major groove (refer also to Fig. 1B). Similarly, replacement of the hydrogen R-group of Gly-10 with the methyl R-group of alanine would occur in the interface with the HTH motif and, as a result, the effect of these substitutions may not be confined to the β-hairpin. In contrast, substitution of the Gly-9 and Gln-7 side chains occurs on the solvent exposed surface of the β-hairpin structure. Consequently, the individual mutations are likely to affect the overall structure of the β-hairpin in different ways.

Each of the alanine-substituted proteins was expressed in COS-7 cells and Pax-3 levels were determined by Western blotting, revealing similar amounts of Pax-3 polypeptide in each whole cell extract (Fig. 2C). Equivalent amounts of Pax-3 protein were then used in EMSAs with the paired-domain specific oligonucleotide P3OPT (Fig. 2D). In this analysis, the mutants can be categorized into three groups based on the
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Paired-domain Mutations Affect Monomeric Binding by the Pax-3 Homeodomain—In the analyses described above, the DNA binding properties of the Pax-3 homeodomain were investigated using the P2 oligonucleotide as it represents the consensus binding sequence for paired class homeodomains containing a single TAAT motif (Fig. 2E). Alanine replacement at Asn-6 and Gly-9 decreased binding to the P2 oligonucleotide approximately 20-fold (lanes 2 and 5, Fig. 2E), whereas mutation of Leu-8 causes an approximately 5-fold reduction in homeodomain DNA binding activity (lane 4, Fig. 2E) when each is compared with wild-type Pax-3 (lane 1, Fig. 2E). In addition, the Gly-10 mutant which retained partial paired-domain DNA binding activity also has a higher degree of homeodomain DNA binding activity when compared with mutations at positions 6, 8, and 9 (compare lane 6 to lanes 2, 4, and 5 in Fig. 2E), although it is still 2-fold lower than the wild-type Pax-3 protein (lane 1 in Fig. 2E). As was observed with P3OPT, alanine replacement of Gln-7 also displays a wild-type level of homeodomain DNA binding activity (lane 3 in Fig. 2D). The results were obtained with an independent series of whole cell extracts. This analysis reveals that mutations at other positions within the β-hairpin motif of the paired-domain can cause defects in binding by the Pax-3 homeodomain, and is consistent with our original hypothesis that the two domains functionally interact. Importantly, the general correlation between paired-domain and homeodomain DNA binding properties in this mutagenic analysis suggests that the β-hairpin is essential for the DNA binding activity of both domains.

Paired-domain Mutations Affect Monomeric Binding by the Pax-3 Homeodomain—In the analyses described above, the DNA binding properties of the Pax-3 homeodomain were investigated using the P2 oligonucleotide as it represents the consensus binding sequence for paired class homeodomains containing a single TAAT motif (17). In this case, dimerization by the homeodomain occurs with 30–40-fold cooperativity over monomeric binding and, accordingly, the predominant complex formed by the position 9 and alanine scanning mutants appears to be the slower migrating dimeric form (Fig. 1E and Fig. 2E). It was therefore important to distinguish between a requirement for the β-hairpin structure in monomeric binding by the homeodomain from any effects the disruption of this structure could have on dimerization. To address this issue, the monomeric homeodomain binding properties of position 9 and alanine-replacement mutants were characterized using an oligonucleotide which contains a single TAAT motif (P1/2) (Fig. 3).

The difference in Pax-3 binding to P1/2 and P2 is illustrated in Fig. 3A where the single, lower-affinity complex formed with the P1/2 oligonucleotide (lane 2) corresponds to the faster migrating of the two complexes observed with the P2 oligonucleo-
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The wild-type phox and chimeric constructs were expressed in COS-7 cells and proteins were detected by Western blotting using a monoclonal anti-HA antibody (Fig. 4B). The size of the proteins detected by the antibody corresponds to those predicted from the primary sequence of phox (25 kDa) and the Pax-3 chimeras (37 kDa) and indicates that the wild-type (phoxPDG9) and Sp4 (phoxPDR9) chimeras are expressed at similar levels (Fig. 4B).

The DNA binding characteristics of the wild-type phox and chimeric proteins were then analyzed by EMSA using the P1/2 oligonucleotide (Fig. 4C). The wild-type phox protein binds to the P1/2 oligonucleotide with high affinity as expected, as does the phoxPDG9 chimera (compare lanes 1 to lanes 2 and 3, Fig. 4C). However, the phoxPDR9 chimera, which harbors the Sp4 mutation, shows an approximately 20-fold loss in P1/2 binding activity (lanes 4 and 5, in Fig. 4C) when compared with the phoxPDG9 chimera and wild-type phox. Therefore, the requirement for the β-hairpin motif at the level of monomer binding can be transferred to a heterologous homeodomain.

The Pax-3 Paired-domain Affects the Sequence Specificity of the Homeodomain—Paird-class homeodomains have been shown to bind to palindromic TAAT motifs separated by 2- (P2) or 3-base pair spacers (P3), and the homeodomain of Drosophila Prd binds P2 and P3 sequences with similar affinity and cooperativity (17). To further characterize the effect of the paired-domain on homeodomain DNA binding, we monitored the effect of wild-type or mutant paired-domains on homeodomain binding to P3 in the context of the full-length Pax-3 protein (Fig. 5). Interestingly, EMSAs of wild-type Pax-3 on the P3 oligonucleotide (lane 3 and 4, Fig. 5A) only detected the lower affinity monomeric complex upon comparison to complexes formed with the P2 oligonucleotide (lanes 1 and 2, Fig. 5A) when using a fixed amount of Pax-3 and oligonucleotides labeled to the same specific activity. In addition, the P3 DNA binding properties of position 9 and alanine scanning mutants (data not shown) were identical to those observed with the P1/2 oligonucleotide (Fig. 3). To determine if this inability to dimerize on the P3 oligonucleotide derives from the presence of the paired-domain or simply represents an inherent property of the Pax-3 homeodomain, the P3 binding properties of a protein (Pax-3prd) lacking the first 92 amino acids of the paired-domain were investigated. Results shown in Fig. 5B indicate that Pax-3prd can dimerize to the same extent on P2 (lane 3) and P3 oligonucleotides (lane 4), whereas intact Pax-3 can only dimerize on P2 (compare lanes 1 and 2, Fig. 5B). Therefore, the presence of the paired-domain in the Pax-3 protein precludes dimerization of the homeodomain on the P3 oligonucleotide.

The failure of Pax-3 to dimerize on the P3 oligonucleotide was somewhat surprising given that in vitro selection of consensus motifs for the closely related Pax-6 homeodomain revealed its preference for a P3-based motif (32). The Pax-6 derived P3 oligonucleotide was therefore used in EMSAs with Pax-3 to determine if its unique sequence composition affected the ability of Pax-3 to dimerize. Remarkably, this oligonucleotide shows a complete failure to bind to the Pax-3 homeodomain when compared with P2 (compare lanes 1 and 2, Fig. 5C).

This result is even more striking when using phox, phoxPDG9, and phoxΔprd (lanes 3–8, Fig. 5C) for the following reason: the presence of glutamine at position 50 of the phox homeodomain allows it to dimerize with approximately 10-fold greater cooperativity on P3 motifs than paired-class homeodomains containing a serine at this position (17, 31). Despite this difference, the phoxPDG9 chimera fails to bind to the Pax-6 derived P3 oligonucleotide (lane 6, Fig. 5C) when compared with its binding to P2 as a control (lane 3, Fig. 5C). Moreover, the ability of both the phox and phoxΔprd proteins to efficiently dimerize on

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this oligonucleotide (lanes 7 and 8, Fig. 5C), when compared with their monomeric binding to P2 (lanes 4 and 5, Fig. 5C), indicates that the presence of the paired-domain is responsible for the failure of the phox homeodomain to bind the Pax-6-derived P3 sequence effectively.

The Pax-6-derived oligonucleotide (P3.S in Fig. 5D) differs from the original P3 sequence in several ways: it only contains 4 base pairs flanking the palindromic homeodomain recognition site, and both the flanking and spacer sequences differ from that of P2 and P3 (Fig. 5D). To determine if such differences impact upon sequence recognition in the presence of the paired-domain, a series of P3-based oligonucleotides were synthesized that involved the stepwise replacement of sequences in P3.S with those present in P3 (P3.3 and P3.5 in Fig. 5D). In this analysis, the phox homeodomain was chosen because of its greater propensity to dimerize on P3-based sequences. Accordingly, the predominant complex formed between phox and the P3, P3.5, and P3.3 oligonucleotides is dimeric (lanes 1–3, Fig. 5E), and the slightly lower affinity of P3.5 and P3.3 can be attributed to the presence of a non-consensus purine residue following the TAAT motif on the complementary strand (17). As was observed with P3.S (lane 6, Fig. 5C), the phoxPDG9 chimera also fails to bind the P3.5 and P3.3 oligonucleotides (lanes 5 and 6, Fig. 5C). In contrast, phoxPDG9 does bind to the P3 oligonucleotide efficiently (lane 4, Fig. 5E) and indicates that the differences in the spacer sequence between P3 and P3.3 can account for the loss of homeodomain DNA-binding. However, the sequence differences present in these P3 oligonucleotides do not restore homeodomain dimerization to the phoxPDG9 chimera, and this is consistent with the behavior of Pax-3 (Fig. 5C and data not shown). Finally, the restoration of dimerization on P3, P3.3, and P3.5 by deletion of the paired-domain in phox3prd (lanes 7–9, Fig. 5E) indicates that it is indeed the paired-domain which confers these additional sequence constraints and prevents homeodomain dimerization on P3-based sequences.

**DISCUSSION**

The paired-domain has been highly conserved throughout evolution and defines the nine members of the mammalian Pax gene family (3). Like Prd and the two gene products of the *Drosophila* gooseberry locus, the murine Pax-3, -4, -6, and -7 proteins also contain a homeodomain. In the *Caenorhabditis elegans* PAX-6 gene, alternative transcript initiation creates two mRNAs that encode proteins containing both domains, or only the homeodomain (33, 34). Hence, the presence of the paired-domain and homeodomain in separate polypeptides would indicate that they can function independently. In addition, when expressed separately, the paired-domain (20, 28, 35) and homeodomain (17) bind to distinct, *in vitro*-derived consensus sequences with high affinity, allowing the crystallization of the DNA-bound complexes (20, 36), which would seem to establish their structural independence. Despite this apparent independence, rescue of *paired* null mutants requires the presence of both domains within the same polypeptide and mutations in either domain cause similar phenotypes (37). Likewise, mutations in the paired-domain or homeodomain of human PAX-3 both give rise to Waardenburg Syndrome type I (38), suggesting that each domain is necessary for normal Pax-3 function. At the biochemical level, this possibility was suggested by analysis of the mouse Pax-3 Spd̃ mutant in which a glycine-arginine substitution within the paired-domain abrogates the DNA binding activity of both domains (19). The current study extends this observation and establishes that the integrity of the β-hairpin motif in the Pax-3 paired-domain is necessary for DNA binding by both the paired-domain and homeodomain. An important consequence of their presence in the same polypeptide is that the recognition of sequences that would normally be bound by the homeodomain is impaired by the presence of the paired-domain and this is likely to affect sequence recognition by the Pax-3 homeodomain *in vivo*.  

Crystalization of the paired-domain of *Drosophila* Prd identified two globular subdomains, NH₂-terminal and COOH-terminal, each of which contains three α-helices that form a classical ITHT motif (20). In addition, the NH₂-terminal subdomain is characterized by a novel β-hairpin motif that comprises 2 anti-parallel β-strands that are separated by a β-amino acid β-turn, and this motif contributes extensively to the overall docking of the paired-domain on DNA. The 2 β-strands clamp the DNA-phosphate backbone and stabilize a second β-turn which engages the DNA minor groove, and the β-hairpin also interacts with the ITHT motif that occupies the major-groove adjacent to the phosphate clamp (Fig. 1A). In Spd̃, the arginine mutation replaces a glycine residue that is necessary for the maintenance of the β-turn, and its disruption would most likely affect the function of the β-hairpin as a whole, accounting for the abrogation of paired-domain DNA-binding. The present study establishes that replacing Gly-9 in the Pax-3 paired-domain with amino acids (Arg, Glu, Cys, and Ala) of different charge, size, flexibility, and hydrophobicity has a similar phe-
nototypic consequence. The ability of proline to partially restore paired-domain DNA binding indicates that these mutations act primarily through disruption of the \( \beta \)-turn. Furthermore, it is also clear that the defect in homeodomain binding that occurs in association with the \( Sp^d \) mutation involves disruption of this structure and does not simply result from the presence of a positively charged arginine residue. The integrity of the \( \beta \)-turn is therefore necessary for the activity of both Pax-3 DNA-binding domains and suggests that homeodomain does not function independently within the intact protein.

Of the 9 amino acids which make up the \( \beta \)-hairpin motif, only those at positions 6–10 (Asn-Gln-Leu-Gly-Gly) and 12 (Phe) are conserved in all paired-domains described to date, suggesting that they have a critical role in paired-domain function. Mutation of Asn-6 was found to severely impair DNA binding by both the paired-domain and homeodomain and identifies it as a key residue within the first \( \beta \)-strand. Given that the Asn-6 side chain makes a phosphate contact and may also contribute to the stabilization of the adjacent HTH motif (20), the defect in DNA binding to the paired-domain would reflect the loss of these interactions in the presence of alanine. Substitution of Leu-8 and Gly-10 with alanine is also predicted to affect the local secondary structure, and may disturb the interaction with the HTH motif where their side chains normally reside (20). Consistent with this prediction, the Leu-8 mutant has no detectable paired-domain DNA binding activity, although it does retain a fraction of its homeodomain DNA binding activity. The Gly-10 substitution exhibits even greater homeodomain DNA binding activity and detectable binding to the paired-domain, in keeping with the more conservative nature of alanine replacement at position 10. Interestingly, substitution of Gln-7 has no apparent effect on DNA binding by either domain. Although the presence of alanine is not expected to disrupt the secondary structure and would still allow contact with the phosphate backbone through the peptide bond amide group, the Gln-7 side chain normally makes an additional phosphate contact (20). It therefore seems apparent that this contact is not essential for docking of the paired-domain on DNA, although it is possible that the Gln-7 mutant may reduce binding to suboptimal paired-domain recognition motifs where the phosphate contact makes a greater overall contribution to DNA binding. These data suggest that the structural requirement at this position is more important than the phosphate contact for DNA binding. Significantly, the correlation between paired-domain and homeodomain DNA binding activities indicates that the integrity of the \( \beta \)-hairpin is important for DNA binding by both domains, supporting the notion of an underlying functional interdependence.

The derivation of optimal binding sequences for paired-class homeodomains established that they cooperatively dimerize on TAAT(N\(_2\))\(_3\)ATT motifs (17). Consequently, analyses using the P2 oligonucleotide do not resolve the effect of paired-domain mutations on monomer or dimer binding. It was therefore possible that disruption of a specific paired-domain structure could have a deleterious effect on homeodomain dimerization and not on homeodomain DNA binding per se. However, the nature of this requirement was resolved by establishing that mutations which reduce paired-domain DNA binding activity impair monomer binding by the Pax-3 homeodomain (Fig. 3). The transfer of the \( Sp^d \) defect to the phox homeodomain confirms this finding and also establishes that a heterologous homeodomain can be rendered functionally dependent on the paired-domain. In addition, the recent characterization of the \textit{Drosophila even-skipped} gene has identified a regulatory element that requires the cooperative binding of the paired-domain and homeodomain of Prd to function (18). Although this establishes that the paired-domain and homeodomain can cooperate in DNA binding, our analyses suggest that the two domains cannot function independently, but must cooperate to enable homeodomain DNA binding.

How is this functional dependence expected to affect sequence recognition by the Pax-3 homeodomain? A possible answer comes with the finding that wild-type Pax-3 failed to dimerize on a series of oligonucleotides that comprise a palindromic homeodomain recognition motif with a 3-base pair spacer (P3) (Fig. 5 and data not shown). The fact that deleting the paired-domain restores dimer formation on P3 oligonucleotides is both consistent with previous analyses in which the isolated paired homeodomain exhibited similar cooperativity on P2 and P3 sequences (17), and suggests that the paired-domain precludes dimer formation on P3 motifs. This possibility is made even more likely with the analysis of the phox chimera for the following reason: the presence of glutamine at position 50 of the phox homeodomain enables much higher cooperativity on P3 sequences than serine 50 homeodomains, which typically exhibit only 40-fold cooperativity (17). Despite this enhanced dimerization potential, the presence of the paired-domain in the phox chimera completely prevents dimerization. This behavior is also supported by the results of \textit{in vitro} oligonucleotide selection using a Prd polypeptide that contained both a paired-domain and homeodomain (39), or the homeodomain alone (17). Although both assays lead to the identification of the P2 sequence, the P3 motif was no longer selected when the paired-domain was present (39). This suggests that P3 motifs may not be efficiently recognized by Pax proteins \textit{in vivo}, and that the interactions which allow dimerization of intact Pax-3 on P2 sequences must be fundamentally different than those defined in the crystal structure of the Prd homeodomain complexed to a P3 oligonucleotide.

Although the underlying mechanism involved in the cooperative interaction of the Pax-3 paired-domain and homeodomain remains to be defined, our findings parallel those involving other homeodomains, in which interactions with a second DNA-binding domain are required to confer appropriate target gene specificity. In the case of the yeast MAT-a1 and MAT-a2 proteins, this specificity arises through the binding of a MAT-a2-derived amphipathic \( \alpha \)-helix to the MAT-a1 homeodomain (40). In isolation, the MAT-a1 homeodomain has no specific DNA binding activity (41). Similarly, a hydrophobic pentapeptide motif present in certain Hox proteins is required for cooperative interaction with a second homeodomain-containing protein, Pbx1 (42). Despite each of these examples being mechanistically different, such functional interactions invariably confer novel sequence specificity to the DNA-binding partner, and are likely to play an important role in selection of target sequences by Pax-3 \textit{in vivo}.

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