CROSSTALK BETWEEN THE PAIRED DOMAIN AND THE HOMEODOMAIN OF PAX3: DNA BINDING BY EACH DOMAIN CAUSES A STRUCTURAL CHANGE IN THE OTHER DOMAIN, SUPPORTING INTERDEPENDENCE FOR DNA BINDING

Sergio Apuzzo, Aliaa Abdelhakim, Anouk S. Fortin and Philippe Gros*

Department of Biochemistry and McGill Cancer Center, McGill University, Canada

*To whom correspondence should be addressed: Philippe Gros, Ph. D., Department of Biochemistry McGill University, 3655 Sir William Osler Promenade, Montreal, QC, Canada, H3G-1Y6. Phone: 514-398-7291; FAX: 514-398-2603; Email: sapuzz@po-box.mcgill.ca, philippe.gros@mcgill.ca

Running Title: Protease Sensitivity Studies of Pax3
ABSTRACT

The Pax3 protein has two DNA binding domains, a Paired Domain (PD) and a paired-type Homeo Domain (HD). Although the PD and HD can bind to cognate DNA sequences when expressed individually, genetic and biochemical data indicate that the 2 domains are functionally interdependent in intact Pax3. The mechanistic basis of this functional interdependence is unknown and was studied by protease sensitivity. Pax3 was modified by the creation of Factor Xa cleavage sites at discrete locations in the PD (Xa55, Xa66, Xa71, Xa100, Xa114, Xa131), the HD (Xa252, Xa259), and in the linker segment joining the PD and the HD (Xa172, Xa189, Xa216) in individual Pax3 mutants. The effect of Factor Xa insertions on protein stability, and on DNA binding by the PD (P3OPT, P6CON) and the HD (P2, P1/2) was measured using specific target site sequences. Accessibility of inserted Factor Xa sites to protease cleavage was analyzed in mutants that retained wild type DNA binding to PD and HD DNA targets. Independent insertions at positions 100 in the linker separating the first from the second helix-turn-helix motif of the PD, and at position 216 immediately upstream the HD were found to be readily accessible to Factor Xa cleavage. The effect of DNA binding by the PD or the HD on accessibility of Factor Xa sites inserted in the same or in the other domain was monitored and quantitated for multiple mutants bearing different numbers of Xa sites at each position. In general, DNA binding reduced accessibility of all sites, suggesting a more compact and less solvent-exposed structure of DNA-bound vs. DNA-free Pax3. Results of dose response and time course experiments were consistent, and showed DNA binding by the PD not only caused a local structural change in the PD, but also caused a conformational change in the HD (P3OPT binding to Xa216 mutants); similarly, DNA binding by the HD also caused a conformational change in
the PD (P2 binding to Xa100 mutants). These results provide a structural basis for the functional interdependence of the two DNA binding domains of Pax3.

Abbreviations: PD, paired domain; HD, homeodomain; EMSA, electrophoretic mobility shift assay; EDTA, ethylenediamine tetracetic acid; SDSPAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; kDa, kilodaltons; HA, Haemophilus influenzae hemagglutinin A.
INTRODUCTION

Pax3 is a member of a family of 9 transcription factors (1), defined by a DNA binding module, the Paired domain (PD), that was first identified in the Drosophila protein Paired (Prd)(2). Pax proteins play critical roles during normal embryonic development, and inactivating mutations cause major defects in development of skeleton, muscles, nervous system, eyes, kidneys, and the immune system (3-12). Pax3 is expressed in developing somites, neural tube as well as neural crest cell derivatives and plays a role in the proliferation, migration and differentiation of cells involved in neurogenesis and myogenesis (13,14). The Pax3 mouse mutant (splotch, Sp) displays spina bifida and exencephaly, and lacks limb muscles; likewise, mutations in human PAX3 cause Waardenburg syndrome (WS), a condition characterized by pigmentary disturbances, cranio-facial abnormalities and sensorineuronal deafness (14-21).

PAX3 also plays a role in cell transformation as shown by the translocation t(2; 13) (q35; q14) involving PAX3 (22) and forkhead-related transcription factor (FKHR) (23) associated with the solid tumor alveolar rhabdomyosarcoma. The translocation leads to the expression of a fusion protein containing the N-terminal DNA binding domain of PAX3 and the C-terminal activation domain of FKHR (23).

Several members of the Pax family, including Pax3, encode a second DNA binding domain, the paired-type homeodomain (HD)(1,24). Also conserved in the Pax family is the presence of an octapeptide (OP) motif in the segment linking the PD and the HD, as well as a proline-serine-threonine (PST) rich C-terminal domain. Both the OP and PST domain are involved in protein:protein interactions to recruit additional transcription factors required for transcription of target genes. The three-dimensional structures of the DNA bound PD of Prd (25)
and Pax6 (26) reveal that it is bipartite consisting of an N-terminal (PAI) and a C-terminal (RED) subdomains, each formed by a 3 helical fold with the 2 most C-terminal helices forming a helix-turn-helix (HTH) motif which makes DNA specific contacts in the major groove of DNA. The sublinker, connecting the two subdomains of the PD adopts an extended conformation on DNA and makes base contacts in the minor groove. Unique to the PAI subdomain is the \(-\)-turn and \(-\)-hairpin, which precede the three helical fold and participate in DNA binding (25,26). The DNA sequences recognized by the PD are of two classes: binding to Class I sequences require both the PAI and RED subdomains while binding to class II sequences only requires the PAI subdomain (27,28). Some isoforms of Pax6 and Pax8 produce by alternate splicing isoforms that bind PD targets exclusively through the RED subdomain (29,30).

The crystal structure of the DNA bound form of the paired-type HD (pt-HD) consists of a 3 helical fold containing a HTH motif; helix3 makes specific contacts in the major groove of DNA and confers DNA binding specificity of the HD (31-34). Like other HDs, the extended N-terminal arm motif, preceding the three helical fold, is also used to make specific DNA contacts, but in the minor groove of DNA (32-34). The pt-HD class of HDs can bind the TAAT motif but can also uniquely dimerize on palindromic sequences of the type TAAT-(N 2-3)ATTA (32,35). The identity of residue 50 in helix3 determines both DNA specificity and dimerization potential in this class of HDs (32,35). When covalently linked to a PD the pt-HD of Pax proteins (Ser50) only permits the dimerization of the HD on palindromic sequences with a two nucleotide spacer (32,35).

Although the PD and HD can bind to cognate DNA sequences when expressed individually, genetic and biochemical data indicate that the two domains are functionally interdependent in the intact Pax3 protein. The \textit{Splotch-delayed (Sp\(^d\))} mouse mutant bears a single
G42R mutation in the PD which abrogates DNA binding by the PD, but also impairs DNA binding by the HD. Deletion of helix2 of the PAI subdomain in the context of the Spd mutation has been shown to restore HD DNA binding (36). Studies in chimeric PAX3 proteins have shown that the PD can modulate DNA binding specificity and dimerization potential of heterologous HDs (37). On the other hand, a mutant PAX3 variant from a WS patient bearing a mutation at position 53 of the HD (R53G) shows not only loss of DNA binding by the HD but also by the PD (38). More recent biochemical studies by cysteine scanning mutagenesis, and site-specific modification of single cysteine mutants with sulfhydryl reagents have shown that modification of a single cysteine in the PD (Cys82) disables DNA binding by the PD but also by the HD(39). Conversely, modification of a single cysteine at position 263 of the HD (V263C) of the HD abrogates DNA binding by both domains(39).

The mechanistic basis of this functional interdependence, including the protein subdomains involved, remains poorly understood but is likely to be relevant for target site selection by Pax3 in vivo. One plausible mechanism is that DNA binding by one or both of the DNA binding sites of Pax3 causes conformational changes at or near the other binding site to alter its properties. Thus, we wanted to get insight into the conformations adopted by the Pax3 protein when DNA-free and when bound to PD or HD targets. A number of physico-chemical approaches have been used to monitor the effect of substrate binding on protein conformation, including differential immunoreactivity with specific antibodies (40), tryptophan fluorescence (41-45) and site-specific modification of cysteine residues (46,47). Protease sensitivity has also been used extensively to monitor conformational changes in proteins (48-52). Although partial proteolytic digestion with enzymes such as trypsin, chymotrypsin and papain has been used in such studies, delineating the cleavage sites is complicated by the necessity to identify proteolytic
fragments by epitope mapping with specific antibodies and/or peptide sequencing (53). Another implementation of this method involves creating recombinant proteins bearing single heterologous protease cleavage sites (such as Factor Xa) inserted at pre-determined positions in individual mutants. Proteolytic products can be identified using antibodies against antigenic epitope also engineered at convenient positions. In this approach, conformational changes can be studied in a set of recombinant proteins which structural and functional integrity has been ascertained.
MATERIALS AND METHODS

Mutagenesis. The construction of the pMT2 expression plasmid containing the entire protein-encoding region of wildtype Pax3 cDNA has been previously described (39). This pMT2/Pax3 construct encodes for all 479 amino acids of the murine Q+ isoform of Pax3 (54). This cDNA was modified by the in-frame addition of antigenic epitope derived from the human c-Myc protein (c-Myc epitope, EQKLISEEDL) at the amino terminus as well as a polyhistidine tail (His6) and an HA hemagglutinin epitope (YPYDVPDYAS) and a termination codon at the carboxy terminus of the protein. This was accomplished by PCR-mediated mutagenesis with mutagenic primers: P3-Myc (5‘)-
CTCGAATTCTATGGAGCAGAGTATAATCAGCGAAGAGGATCTCACCAACGCTGGCCGG
CGCTGTGCCAGGATG-(3’) and P3-HA (5‘)-
TTTAGCGGATCCGAATTCTTAGTGATGGTGATGGTGATGTTCCGCGCGTAATCTGG
AACGTCATATGGATATCCGAACGTCCAAGGCTTACTTTG-(3’). Both primers were engineered with EcoR I restriction sites at their ends. The resulting 1.5 kb PCR product was digested with EcoR I and ligated into the corresponding site of mammalian expression vector pMT2, and the resulting construct was designated pMT2/Myc-Pax3-HA.

Factor Xa cleavage sites (IE/DGR; see Table 1) were introduced at different positions in Pax3 by 2 procedures. The wild type sequence was mutated (without addition of extra amino acid residues) to convert near matches to IE/DGR via PCR-mediated mutagenesis. Individual mutants were created in 2 independent reactions using complementary oligonucleotide pairs defining each mutation (listed in Table 3) as well as P3-Myc and P3-HA; mutated DNA fragments were annealed, repaired and the full-length cDNAs were synthesized. Mutants Pax3Xa55, Pax3Xa71, Pax3Xa114, Pax3Xa131, Pax3Xa252 and Pax3Xa259 were created in this
fashion and were introduced in pMT2. Mutants Pax3Xa66, Pax3Xa172 and Pax3Xa189 were created by insertion mutagenesis. For this, pMT2/Myr-Pax3-HA plasmid was digested with BsmI, XbaI or ClaI and single or multiple Xa cleavage sites were introduced using double stranded oligonucleotides with cohesive ends (Tables 1 and 3). These oligonucleotides have a sequence just long enough to encode for one or a few Xa protease sites when placed in frame with the rest of the Pax3 encoding region. For mutants Pax3Xa100 and Pax3Xa216, the Pax3 cDNA was modified to introduce unique KasI and AflII sites at nucleotide positions 595 and 940, respectively, using mutagenic primers listed in Table 3. These modified Pax3 cDNAs were subcloned into the EcoRI site of pBluescript (lacks KasI, AflII sites), and one or several factor Xa sites were independently introduced at the KasI or AflII sites by insertion mutagenesis to create mutants Xa100(2), Xa100(4), Xa216(1) and Xa216(2) (Tables 1 and 3). In all cases, the presence of the factor Xa mutations and the integrity of the rest of the Pax3 sequences were verified by nucleotide sequencing. The accessibility of restriction sites used for cloning was verified by restriction enzyme fragmentation.

Expression and Detection of Pax3Mutants. The expression plasmids were used to transiently transfect COS7 Monkey cells. One million cells were plated in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum and were transfected by the calcium phosphate co-precipitation method using 15 μg of plasmid DNA doubly purified by ultracentrifugation on cesium chloride density gradients. Calcium-DNA precipitates were placed onto the cells for 5 h and then treated with HBS (0.14 M NaCl, 5mM KCl, 0.75 mM Na2HPO4, 6mM dextrose, 25mM HEPES, pH 7.05) containing 15% glycerol for 1 min. The cells were then washed and placed in complete DMEM. Whole cell extracts were prepared 24 h following
glycerol shock by sonication in a buffer containing 20mM HEPES (pH 7.6), 0.15 M NaCl, 0.5 mM DTT, 0.2 mM EDTA, 0.2 mM EGTA and a cocktail of protease inhibitors: aprotinin, pepstatin, and leupeptin used at 1mg/ml, and phenylmethysulfonyl fluoride used at 1mM. These extracts were stored frozen at −70°C until use. To assess Pax3 mutant protein expression and stability, aliquots of whole cell extracts were analyzed by electrophoresis on acrylamide-containing SDS gels (SDS-PAGE), followed by electrotransfer onto nitrocellulose membranes and immunoblotting. Immunodetection was performed with mouse monoclonal anti-HA antibody (BabCO, Berkeley, CA) at a dilution of 1:1000 and visualized by enhanced chemiluminescence using a sheep anti-mouse horseradish peroxidase conjugated secondary antibody (Amersham). Following anti-HA probing the membranes were submerged in stripping buffer (100mM 2-mercaptoethanol, 2% sodium dodecyl sulphate, 62.5mM Tris-HCl pH6.7) and incubated at 50°C for 30 minutes. The membranes were then washed with TBST buffer (10mM Tris-HCl pH8, 150mmNaCl, 0.1% Tween20) at room temperature. Following blocking, the membranes were probed with mouse monoclonal anti-Myc antibody (BabCO, Berkeley, CA) at a dilution of 1:1000 and visualized by enhanced chemiluminescence using a sheep anti-mouse horseradish peroxidase conjugated secondary antibody (Amersham).

**Electrophoretic Mobility Shift Assay.** Electrophoretic mobility shift assays were performed as previously described(39). Each protein:DNA binding reaction was carried out using approximately 10µg of total cell extracts from transiently transfected COS-7 monkey cells, and 10fmol (0.06µCi) of radioactively labeled double stranded oligonucleotides containing either PD or HD recognition sites. The final concentration of labeled oligonucleotide in the binding reaction was 0.5 nM. Whole cell extracts were incubated with 32P-labeled PD specific probes in a
volume of 20µl containing 10mM Tris-HCl (pH 7.5), 50mM KCl, 1mM DTT, 2mM spermidine, 2mg/ml BSA and 10% glycerol. Whole cell extracts were also incubated with 32P-labeled HD specific probes in a volume of 20µl containing 10mM Tris-HCl (pH 7.5), 50mM NaCl, 1mM DTT, 2mM MgCl2, 1mM EDTA and 5% glycerol. To reduce non-specific binding, 1 µg of poly(dI-dC)poly(dI-dC) was included in binding studies with PD specific probes, while 2µg of heat-inactivated salmon sperm DNA was added to binding reactions involving HD specific probes. Following a 30 minute incubation at room temperature, samples were electrophoresed at 12V/cm in 6% acrylamide:bis-acrylamide (29:1) gels containing 0.25 or 0.5X TBE (1X TBE is 0.18 M Tris-HCl, 0.18 M boric acid, 4mM EDTA, pH 8.3). Gels were dried under vacuum and exposed to Kodak BMS film with an intensifying screen. PD specific sequences P6CON (5’)-TGGAATTCAGGAAAAATTTTCACGCTTGAAGTTCCACAGCTCGAGTA-(3’) (25) and P3OPT (5’)-TGGTGGTCACGCCTCATTGAATATTA-(3’) (53,54) and HD specific sequences P2 (5’)-’GATCCTGAGTCTAATTGATTACTGTAC-(3’) (30) and P1/2 (5’)-GATCCTGAGTCTAATTGAGCGTCTGTAC-(3’) (30) were synthesized as complementary oligonucleotide pairs and were designed in order to have recessed 3’ ends for end labeling with [~32P] dATP (3,000 Ci/mmol; Perkin Elmer Canada Inc.) using the Klenow fragment of DNA polymerase.

**Factor Xa treatment of Xa Mutants.** For time course studies, 10µg of total cell extracts from transiently transfected COS-7 monkey cells was incubated with or without double stranded oligonucleotides (final concentration of 2µM) corresponding to PD and HD binding sites, in a final volume of 10µl. The whole cell extract was incubated with DNA for 30 minutes at 20°C followed by addition of 0.2µl of 1µg/µl (0.2µg) of Factor Xa protease (NEB Biolabs). The
proteolysis reaction was carried out for pre-determined periods of time (2-180 min) at 20°C and was stopped by the addition of 5µl of Laemmli sample buffer (2% (w/v) SDS, 10% (v/v) glycerol, 62.5 mM Tris-HCl pH6.8, 100mM DTT, 0.05% bromophenol blue). For the dose-response assays, 10µg of total cell extracts from transiently transfected COS-7 monkey cells was incubated for 30 minutes at 20°C with oligonucleotides (final concentration of 2µM) corresponding to PD and HD binding sites or non-specific oligo, in a final volume of 20µl.

Binding reactions done with PD probes (and with a non-specific oligo) contained 10mM Tris-HCl (pH 7.5), 50mM KCl, 1mM DTT, 2mM spermidine, 2mg/ml BSA, 10% glycerol and 1 µg of poly(dI-dC)poly(dI-dC). Binding reactions done with HD probes (and with a non-specific oligo) contained 10mM Tris-HCl (pH 7.5), 50mM NaCl, 1mM DTT, 2mM MgCl2, 1mM EDTA, 5% glycerol and 2µg of heat-inactivated salmon sperm DNA. Various concentrations of Factor Xa protease, ranging from 0 to 200ng Xa protease/µg whole cell extract, were then added to the reaction mixture, and proteolytic cleavage was allowed to take place for 15 minutes at 20°C. The reaction was stopped by addition of 10µl of Laemmli sample buffer. For both assays the proteolytic degradation products were separated by SDS-PAGE on 12% polyacrylamide gels, followed by transfer onto nitrocellulose membranes. Immunodetection of Pax3 products were carried out using anti-HA antibody followed by anti-Myc antibody as described above. Films were used to perform densitometry studies to quantify the amount of chemiluminescence using a Fuji LAS - 1000.
RESULTS

Construction of Pax3 Mutants Bearing Factor Xa Protease Cleavage Sites

A large body of biochemical and genetic data indicate that the Paired domain (PD) and the Homeo domain (HD) of Pax3 are functionally interdependent with strong cooperativity between the two sites. The structural basis for this interdependence remains unclear and was investigated. A major objective of this study was to determine whether or not DNA binding at either the PD or HD of Pax3 causes a conformational change at the other site, thereby providing a possible structural basis for their reported functional interdependence. Possible conformational changes were assessed by protease sensitivity by monitoring accessibility of cleavage sites strategically inserted in the PD and HD of Pax3. A Pax3 cDNA was modified by the in-frame addition of hemagglutinin (HA) and c-Myc epitope tags at the carboxy (C) and amino termini (N) of the protein, respectively (Fig. 1), to facilitate detection of specific proteolytic fragments by immunoblotting. This cDNA was used to insert Factor Xa cleavage sites (IEGR, IDGR; cleavage immediately C-terminal of R) by site-directed mutagenesis. Using the known three-dimensional structures of Pax6 and Paired as molecular templates, Factor Xa sites were introduced either in the PD, in the HD, in the flexible linker separating the PD and the HD, as well as in solvent exposed segments near the end of individual helices in the HTH motifs of the PD and the HD (Figs. 1 and 3). Two strategies were used for mutagenesis. First, near-matches in the wild type Pax3 sequence mapping to the highly conserved HTH modules of the PD and HD were independently converted to I(E/D)GR (Table I) to minimize adverse structural changes possibly impairing DNA binding. Targeted in this group of 6 mutants were helices 1 (1, Xa55), 2 (2, Xa71), 4 (4, Xa114), and 5 (5, Xa131) of the PD as well as the C-terminus of helix 2.
(Xa252) and the N-terminus of helix 3 (Xa259) of the HD (Table 1, Fig. 1). Secondly, and to maximize accessibility to proteolytic cleavage, several solvent-exposed, and less conserved linker segments were also targeted for insertion of 1 or several Xa sites (Table 1, Fig. 1). Targeted in this group of 10 mutants were the linker separating helix 1 and 2 of the PD (Xa66, 1 and 2 sites), the segment linking the first and second HTH motifs of the PD (Xa100, 2 and 4 sites), and the fragment separating the PD and the HD (Xa172, 1 and 3 sites; Xa189, 1 and 2 sites; Xa216, 1 and 2 sites). Multiple Xa sites were inserted at individual locations not only to maximize accessibility to protease fragmentation, but also to provide validation of observed effects.

**DNA Binding Properties of Pax3Xa Mutants**

Wild type Pax3 along with the various Pax3Xa mutants were introduced in the pMT2 expression plasmid, followed by transient transfection into COS-7 Monkey cells. Immunoblotting of whole cell extracts with either anti-HA or anti-cMyc monoclonal antibodies indicate similar stability and comparable levels of expression of all mutants in COS-7 cells, with the notable exception of mutants Xa252 and Xa259 (Fig. 2). Reduced levels of expression for Xa252 and Xa259 were noted in multiple transfections and for independent DNA preparations suggesting that mutations at these 2 positions in the HD may alter protein folding, or processing possibly reducing half-life. The effect of introducing Factor Xa sites on DNA binding properties of the PD and HD of Pax3 in the various mutants was examined by electromobility shift assays (EMSA). DNA binding by the PD was examined using oligonucleotide probes P3OPT (55,56) and P6CON (27), previously shown to reveal binding determinants present in both the amino (PAI) and carboxy (RED) subdomains of the PD (Fig. 2). Mutants at position 55 (Xa55), 66
(Xa66(1,2)) and 71 (Xa71) were found to be severely impaired for DNA binding to P3OPT and P6CON highlighting the critical role of the N-terminal HTH domain (PAI) for DNA binding by the PD. HD Xa mutants Xa252 and Xa259 also appeared compromised for DNA binding by the PD; however, interpretation of DNA binding results for these mutants were complicated by their low level of expression in COS-7 cells. The effect of inserting Factor Xa sites on DNA binding properties of the HD were evaluated using a target sequence (P2) containing the sequence TAAT(N)₂TAAT previously shown to support cooperative dimerization of Pax3 (32). In addition, an oligonucleotide containing half of this sequence (half site, P1/2) and revealing monomeric Pax3 binding by the HD was used (32). Results shown in Fig. 2 indicate that DNA binding by the HD was also impaired in the Xa66 mutant, while mutants Xa55 and Xa71 show impaired monomeric binding to P1/2 but retain some dimerization potential on P2. Importantly, all other mutants (positions 100, 114, 131, 172, 189 and 216) retained WT binding activity towards the 4 oligos tested (summarized in Fig. 3), indicating that Factor Xa insertions in the RED sub-domain of the PD are not detrimental to function by contrast to mutations at the homologous positions of the other, PAI subdomain. Finally, all insertions in linker segments did not affect DNA binding.

**Accessibility of Inserted Xa Sites to Proteolytic Cleavage**

The accessibility of the inserted factor Xa sites to proteolytic cleavage was investigated in mutants showing wild type DNA binding activity. Briefly, whole cell extracts from COS-7 cells expressing either WT or individual Pax3-Xa mutants were incubated with factor Xa protease (0.2μg) and at predetermined time points (2 to 180 min) digestion was stopped and samples were analyzed by SDS-PAGE and immunoblotting with anti-HA (Fig. 4) or anti-cMyc
(Fig. 5) antibodies. The size of the predicted cleavage products immunoreactive with each antibody are shown in Table 2. Under these conditions, wild type Pax3 (WT) was almost completely resistant to factor Xa cleavage, with the full length 55kDa immunoreactive species being the prominent band at all time points (empty arrowhead; Figs. 4 and 5). Additional minor bands were detected either prior to addition of the protease (35kDa, 0 min) or at very late time points (30kDa, 15kDa, 90-180 min). Although these immunoreactive fragments are derived from the full length protein, their appearance is likely caused by non-specific cleavage by factor Xa at sub-optimal sites, or cleavage at specific sites by additional proteases contaminating either the cell extract or the commercial factor Xa preparations. For reasons discussed below (see Discussion), these fragments were not considered in our analysis. Stripping the blot and reprobing with anti-cMyc antibody (Fig. 5) confirmed the resistance of WT Pax3 to digestion by factor Xa under conditions tested. Analysis of mutants Xa114, Xa131, Xa172(1/3), and Xa189 (1/2) showed results similar to WT Pax3, and indicated almost complete resistance of these proteins to digestion by factor Xa (supplementary data, Figure S1), with little if any predicted proteolytic fragments immunoreactive with the HA antibody detected even after 180 min of incubation. Similar results were obtained using the anti-cMyc antibody to analyze digestion products (supplementary data, Figure S2). These results indicate that the C-terminal RED sub-domain and the linker domain immediately downstream the PD are probably not solvent exposed and assume a compact conformation under the conditions tested (Fig. 3). In contrast, mutant Xa100(2) yielded the expected 44kDa C-terminal HA-immunoreactive cleavage product upon incubation with factor Xa (filled arrowhead, Fig. 4); The 44kDa HA fragment was abundant at the earliest time point tested (2 min) and digestion was largely completed by 10 mins. Similar rapid cleavage of Xa100(2), as demonstrated by disappearance of the full length 55kDa protein,
was verified by immunoblotting with the anti-cMyc antibody (Fig. 5) although the N-terminal 12kDa cMyc reactive digestion product was not retained on the gel. Increasing the number of Xa sites from 2 to 4 in mutant Xa100(4) produced similar outcome with even more rapid and more complete cleavage at the targeted site. Thus, cleavage at position 100 is specific in these mutants and strongly suggest that the linker separating the PAI and RED subdomains of the PD is solvent exposed and protease accessible. Analysis of mutants Xa216(1) and Xa216(2) showed similar results. Both of the specific C-terminal HA-reactive 32kDa and N-terminal cMyc-reactive 26kDa digestion products appeared at 2 min, and digestion of the full length protein was almost complete by 20-45 mins (Figs. 4 and 5). These results indicate that the protein segment immediately upstream the HD is solvent exposed and accessible to protease cleavage (Fig. 3).

**Effect of DNA Binding on Protease Sensitivity of Pax3 Mutants Xa100 and Xa216**

The effect of Pax3 binding to PD (P3OPT) and HD (P1/2, P2) target sequences on the conformation of each domain was analyzed by monitoring the effect of DNA binding on accessibility of Xa cleavage sites present in mutants Xa100 (PD) and Xa216 (HD). Also included in these experiments were WT Pax3, as well as Pax3-Xa mutants previously observed to be resistant to factor Xa cleavage in time course studies (supplementary data). Briefly, cell extracts expressing Pax3 proteins were incubated with or without target DNA, followed by addition of factor Xa and detection of HA (Fig. 4) and cMyc (Fig. 5) immunoreactive cleavage products appearing over time. The extent of protection from proteolytic fragmentation was further quantitated after densitometry of the immunoblots and is expressed as the fraction of intact full length Pax3 remaining following 20 minutes of incubation with factor Xa (Fig. 4B, 5B). For the WT Pax3, binding to PD or HD target sequences had no effect on digestion profiles, as expected,
with full length Pax3 remaining the predominant species throughout the incubation period.
Likewise, DNA binding by the PD or the HD of mutants Xa114, Xa131, Xa172, Xa189 did not affect their previously noted resistance to factor Xa cleavage (Fig. 4B, 5B and Figs. S1 and S2 in supplementary data). This suggests that DNA binding by either domain in these mutants does not cause a conformational change that increases solvent exposure of the respective Xa bearing segments. By contrast, incubation of mutant Xa100(2) with P3OPT increased resistance to proteolysis (persistence of 55kDa protein), suggesting that DNA binding to the PD causes a conformational change reducing solvent accessibility of the PD. In addition, monomeric binding to P1/2 and in particular dimerization of on P2 both also caused a dramatic increased resistance to proteolytic cleavage of Xa100(2) (from 10% to 40% intact protein), suggesting that DNA binding by the HD in this mutant also causes a conformational change in the PD. Identical results were obtained with Xa100(4), although the increased susceptibility to cleavage caused by the 4 consecutive Xa sites at position 100 was maintained in this mutant for all DNA binding conditions tested. In the case of mutants Xa216(1) and Xa216(2) mutants that bear Xa sites immediately upstream of the HD, DNA binding by the HD, in particular dimerization on P2, caused a strong increased resistance to proteolysis (from 25% to 85% intact protein) suggestive of a conformational change at that site. In addition, binding of both mutants to the PD target sequence (P3OPT) also increased resistance to proteolysis, suggesting that DNA binding by the PD also causes a conformational change in the HD. In all cases, results of immunoblotting with anti-HA (Fig. 4) and anti-cMyc antibodies (Fig. 5) were in complete agreement (Fig. 4B, 5B). Together, results from Xa100 and Xa216 are remarkably similar and suggest that DNA binding at either the PD or HD causes a conformational change at both sites. This change appears to
reduce the amount of solvent exposed area in the protein, suggesting a more compact
conformation of the DNA-bound protein.

Specificity of the protective effect of PD and HD target sequences on accessibility of the
Xa sites in Xa100 and Xa216 mutants was investigated in dose-response studies. In these
experiments, the Pax3 mutants were incubated with PD or HD oligonucleotide probes and DNA-
Pax3 complexes were allowed to form; following this, increasing amounts of factor Xa protease
was added to the reaction mixture which was further incubated for 15 minutes, and the
appearance of specific proteolytic cleavages products was monitored by SDS-PAGE and
immunoblotting with the anti-HA antibody (Fig. 6A). In these studies, 2 additional control
probes were tested to further validate the specificity of the DNA effect observed in time course
studies: a second, independently derived PD oligonucleotide P6CON, and a PD and HD non-
specific oligo which was used as a negative control. Typical immunoblots are shown in Fig. 6A,
and quantitation of the protective effects of the DNA probes by densitometry is shown in Fig. 6B
(\% of uncleaved Pax3 at concentration indicated by arrow in Fig. 6A). These experiments
showed that incubation of mutants Xa100(2) and Xa216(2) with PD oligonucleotides P6CON
and P3OPT reduced sensitivity of both proteins to increasing doses of factor Xa. Likewise,
incubation of both proteins with HD probes P1/2 and P2 similarly increased resistance to factor
Xa fragmentation. The effect of the 2 PD probes and 2 HD probes on protease sensitivity was
specific and not seen in control DNA-free conditions (data not shown), and upon incubation with
a non-specific target sequence (Fig. 6A/B). Therefore, results of dose response experiments are
consistent with those obtained in time course experiments, and show that DNA binding to the PD
and to the HD of Pax3 cause a conformational change both locally as well as in the other DNA
binding site.
DISCUSSION

The effect of inserting factor Xa sites in different domains of Pax3 provides information on the importance of the targeted sub-domains in DNA binding by the protein. In the PD, insertion of factor Xa sites at the 3 locations tested in the N-terminal PAI sub-domain caused loss of PD DNA binding. Since helix 3 makes critical base and phosphate contact in the major groove of DNA and is mutation-sensitive, it was not targeted for insertions. Nevertheless, we observed that mutations in helix 1 (Xa55), in the linker separating helices 1 and 2 (Xa66), and in helix 2 (Xa71) all impaired binding to PD oligos. Insertion of Xa sites may either disrupt important phosphate contacts made between these helices and DNA (Fig. 1) and/or may destabilize the whole PAI domain. By contrast, 2 insertions created in helices 1 (Xa114) and 2 (Xa131) of the C-terminal RED domain had no major effect on DNA binding to PD sequences. These results highlight the critical role of the PAI domain in DNA binding by the PD, and are in agreement with a) the high degree of primary amino acid sequence conservation of this domain (compared to the RED subdomain) in the Pax/Prd gene family (25,27-29), b) the clustering of inactivating WS1 mutations to the PAI subdomain of PAX3 (38), and c) the fact that Pax proteins can bind DNA exclusively through their PAI subdomain (28). We note that mutations Xa55 and Xa71 in the PAI domain impaired DNA binding to PD oligos only, while the Xa66 mutant showed impaired DNA binding to both PD and HD sequences. This behavior is similar to WS1 mutants G48R/S and P50L (upstream helix 1 of PAI), respectively, and has been suggested to reflect functional interdependence of the PD and HD in DNA binding (38). Interestingly, insertion of 2 or even 4 Xa sites in the linker joining the PAI to the RED (Xa100) had no effect on DNA binding; this linker is well conserved amongst Pax proteins, and sequences immediately downstream the insertion site make extensive phosphate and base-specific contacts in the minor
groove of DNA (Fig. 1). As expected, the 3 insertions (Xa172, Xa189, Xa216) in the poorly conserved linker joining the PD and the HD, including one within the octapeptide motif (Xa189) conserved in other Pax proteins (HSIDGILG; (57)), had no major impact on DNA binding of the corresponding mutants to PD and HD sites. With respect to the HD, 2 mutations inserted either downstream helix 2 (Xa252) or upstream the major DNA binding helix 3 (Xa259) appeared to either strongly diminish or abrogate monomeric or dimeric DNA binding to HD target sequences (Fig. 2). Although this conclusion is supported by both the high degree of conservation of the targeted sequences in the Paired-type HDs of the Pax family (Fig. 1), and the fact that many WSI mutations map to the HD of Pax3 (38), our inability to express high levels of these mutants precluded a more detailed analysis.

Evaluating the accessibility of inserted Xa sites to protease cleavage can readily provide insight into the solvent exposure of the corresponding Pax3 sub-domains. Results of time course studies were very clear and showed that of the 7 sites in which insertions preserved DNA binding only 2, Xa 100 and Xa216, were readily accessible to proteolytic cleavage at the earliest time points of analysis (Fig. 4). In agreement with the proposed structural model of the PD (Fig. 7A), these results showed that the PAI to RED subdomain linker (Xa100 mutant) is clearly exposed to solvent. Although this domain is not believed to play a critical role in DNA binding, current structural models suggest that it is in close proximity to DNA (25). In addition, alternative splicing of a glutamine residue at position 108 of Pax3/Pax7 is known to alter DNA binding specificity of the PD (54,58). Therefore, mutants Xa100 should be ideally suited to monitor structural changes associated with DNA binding by the PD. By contrast, downstream insertions into helices 1 (Xa114) and 2 (Xa131) of the RED domain were completely resistant to protease cleavage, possibly suggesting that the HTH motif is either compact or buried in the core of the
protein or both (Fig. 7A). PD-HD linker insertions mapping far upstream of the HD (Xa172, Xa189) were resistant to protease cleavage. Considering the observed accessibility of neighboring position 216, this is somewhat surprising; however, it is important to note that Xa sites at positions 172 and 189 were engineered immediately upstream a hydrophobic residue (L, I, respectively), a situation known to reduce the efficacy of cleavage by the factor Xa protease (59).

The effect of binding to PD and HD oligos on accessibility of all inserted Xa sites was investigated in time course studies (Figs. 4/5, S1/S2). In all cases, DNA binding did not increase accessibility of Xa sites, suggesting that DNA binding as a whole does not dramatically increase solvent exposure in the protein. However, this conclusion only applies to the subdomains analyzed, and is also limited by the fact that the primary amino acid sequence context of individual sites is not identical, possibly influencing protease cleavage irrespective of the three-dimensional structure (DNA-free or DNA-bound) of this subdomain. On the other hand, DNA binding by the PD reduced accessibility of the Xa100 site, reflecting a conformational change to a more compact and less solvent accessible position of the PAI to RED linker. The effect was specific, was seen in independent mutants bearing different number of Xa sites inserted, was noted upon binding to independent PD site and was not seen upon binding to an unrelated target site sequence. These results are in accordance with CD spectroscopy studies with Pax5 and Pax8 PDs that reveal increased helical content in the DNA bound form of the PD (60). Likewise, DNA binding by the HD also resulted in decreased accessibility of the HD N-terminal linker (Xa216), reflecting a more compact structure of the DNA bound state of this domain. These results are in agreement with X-ray crystallography and NMR studies of HD proteins that reveal an increased order of the HTH motif, including the N-terminal arm and the recognition helix, of the DNA
bound form of Pbx(61-64), Antennapedia(65), engrailed(66) and Oct-1(67) proteins. Increased resistance to protease cleavage of the Xa216 site was more pronounced upon incubation with P2 than with the half site P1/2. This could reflect different stability and steady state level of conformationally similar HDs in the DNA bound state (supported by the EMSA data; Fig. 2) or could reflect 2 distinct conformations of DNA-bound HDs in Pax3 monomers and dimers complexed to P1/2 and P2, respectively.

Importantly, DNA binding by the PD also caused a conformational change in the HD (P3OPT binding to Xa216 mutants); likewise, DNA binding by the HD also caused a conformational change in the PD (P2 binding to Xa100 mutants). These results provide for the first time a structural basis for the functional interdependence of the two DNA binding domains previously noted in the study of a) PD and HD DNA binding properties of PAX3 mutants from WS1 patients (38), and b) the effect of PD and HD DNA binding on site specific modifications of Pax3 mutants bearing single cysteine residues in each domain (39). These results further suggest that the PD and HD of Pax3 can functionally interact for the final selection of target site sequences in vivo. Finally, results in Fig. 5 indicate that dimerization of Pax3 on P2 results in a conformation that appears distinct from that created in the same mutant upon binding to the PD oligo. Indeed, comparison of the digestion profile of P2-bound vs. P3OPT-bound Xa216 mutants identifies a novel non-specific but cMyc immunoreactive cleavage product (shaded arrow, Fig. 5), which is present in the former but absent in the latter set of digest. This non-specific cleavage product is detected only upon binding of Xa216 mutants to P2 and not to P1/2, strongly suggesting that it is caused by dimerization of Pax3 on P2, as opposed to monomeric binding to the half site.
The results of protease sensitivity studies presented here are in agreement with the structural model for combined PD and HD binding to chimeric target sequences proposed for the *Drosophila Prd* protein by Jun and Desplan (25). This model, based in part on the sequence arrangement of binding sites selected *in vitro* by sequential amplification from random DNA oligomers (SELEX procedure), suggests that the N-terminus of the PAI domain is closely apposed to the N-terminus of the HD when bound to the combined PH0 site (68). Their model predicts that the HD and PD bind to opposite sides of the DNA helix, with helix 2 of the PAI domain in very close proximity of the N-terminal extension of the HD, which was targeted for Xa site insertion in the present study. This model may be used to explain the interaction of the PD of Pax3 with the N-terminal arm of the HD of Msx1 (69). Therefore, the critical position of the N-terminal extension of the HD identified in these studies agrees with our observation that DNA binding by either the PD or HD causes a structural change at that site. Finally, the importance of the N-terminal extension of the HD and of helix 2 of the PAI domain in Pax3 function is highlighted by previous studies from our group showing that a) alteration of the N-terminal HD extension abrogate the ability of the PD to modulate DNA binding specificity of the HD (37), and b) that deletion of helix 2 of the PAI domain restores DNA binding by the HD in the context of an otherwise inactivating mutation (SpΔ) in the PD (37).
FIGURE LEGENDS:

FIGURE 1:

Insertion of Factor Xa Protease Sites in the Paired Domain and in the Homeo Domain of Pax3. Schematic representation of the paired domain (PD) and homeo domain (HD) including position of helices and strands (arrows) deduced from the known structures of the Drosophila Prd, and mammalian Pax6 proteins (4,25,26). The amino acid sequence of Pax3 (positions 34-278) is shown, with invariant residues in members of the Pax family identified below. The type of DNA contacts made by these residues (p, phosphate: m, minor groove: M, major groove) is indicated. Pax3 sequences targeted for modification to create factor Xa cleavage sites are boxed. The position of the cMyc and hemaglutinin (HA) epitope tags inserted in frame at the amino and carboxy termini of Pax3, respectively, is shown.

FIGURE 2:

Paired Domain And Homeo Domain DNA Binding Properties Of Wild Type Pax3 And Pax3 Mutants Modified By Insertion Of Factor Xa Protease Sites. Wild type (wt) and mutant Pax3 cDNAs bearing single or multiple factor Xa protease sites inserted at the position indicated at the top, were cloned into pMT2 expression plasmid, and total cell extracts from transiently transfected COS7 Monkey cells were separated by SDS-PAGE (12% acrylamide) and transferred to nitrocellulose membranes. Immunodetection of Pax3 (top 2 panels) was with mouse anti-cMyc (Myc) and anti-HA (HA) monoclonal antibodies and an HRP-conjugated secondary antibody. Extracts from control non-transfected cells (mock) and from cells expressing...
individual Pax3 mutants were used in electrophoretic mobility shift assays to evaluate the DNA binding properties of Pax3 Xa mutants against paired domain (PD) (P6CON, P3OPT) and homeo domain (HD) specific binding sites (P2, P1/2). Protein-DNA complexes were formed using total COS7 cell extracts and were resolved on 6% acrylamide non-denaturing gels, as described in Materials and Methods.

FIGURE 3:

Summary of DNA Binding Properties and Accessibility of Protease Xa Sites of Individual Pax3 Mutants. Shown is a schematic representation of the PD and HD of Pax3 according to structural features described in Figure 1. The sites of modifications to create factor Xa sites are indicated by arrowheads, together with the name of the mutant. The DNA binding properties of each mutant towards PD (P6CON, P3OPT) and HD targets (P1/2, P2) is summarized below the schematic diagram (-, absent; +++, wild type). Gray arrowheads identify mutants that show important or complete loss of DNA binding; Filled (black) and empty arrowheads identify mutants that retain wild type DNA binding for all sequence targets, and in which the created factor Xa protease sites are either accessible (filled arrowheads) or inaccessible (empty arrowheads), under experimental conditions described in Figure 4.

FIGURE 4:

Accessibility to Protease Cleavage of Factor Xa sites Inserted in Selected Pax3 Mutants: Effect of DNA Binding. Ten micrograms of whole cell extracts from COS7 Monkey cells transfected with either wild type Pax3 (WT) or with Pax3 mutants Xa100(2), Xa100(4), Xa216(1) and Xa216(2) were incubated with factor Xa protease (0.2µg), either in the absence
(no DNA) or after a 30 minutes incubation with PD (P3OPT) and HD target sites (P1/2, P2) under DNA binding conditions. At pre-determined time points (0 to 180 min, indicated at the top) aliquots were removed and analyzed by SDS-PAGE on 12% acrylamide gels and by immunoblotting with anti-HA antibody. Shown immediately to the left of each Pax3 series are empty arrowheads that indicate the position of the full length intact Pax3 products, and filled (black) arrowheads that show the position of major predicted, HA epitope-bearing factor Xa cleavage products (see Table 2). The position and size, in kDa of the molecular mass markers are displayed as dashes on the left and numbers on the right, respectively. All immunoblots probed with anti-HA antibody including those of Figure S1 were scanned by densitometry (Fig. 4B).

The intensity of the immunoreactive intact full length WT protein and of individual Pax3 mutants was determined at both the “0” time point and at the “20 min” time points. The amount of intact protein remaining at 20 min (compared to 0 min) was determined for all mutants and for all DNA binding conditions and is expressed as the fraction of intact protein (expressed as a percentage). Several mutants showing inaccessible factor Xa cleavage sites (Xa114, Xa131, Xa172/3, Xa189/2) were also included in the analysis as negative controls.

**FIGURE 5**

*Accessibility to Protease Cleavage of Factor Xa sites Inserted in Selected Pax3 Mutants:*  
*Effect of DNA Binding.* Wild type Pax3 and the different Pax3 mutants were incubated with factor Xa under different conditions and digestion products were analyzed by immunoblotting as described in the legend to Figure 4, except that blots were probed with an anti-cMyc antibody directed against the amino terminal cMyc epitope present in all mutants. All immunoblots probed with anti-Myc antibody including those of Figure S2 were scanned by densitometry (Fig. 4B).
The intensity of the immunoreactive intact full length WT protein and of individual Pax3 mutants was determined at both the “0” time point and at the “20 min” time points. The amount of intact protein remaining at 20 min (compared to 0 min) was determined for all mutants and for all DNA binding conditions and is expressed as the fraction of intact protein (expressed as a percentage). Several mutants showing inaccessible factor Xa cleavage sites (Xa114, Xa131, Xa172/3, Xa189/2) were also included in the analysis as negative controls.

FIGURE 6:

*Effect of DNA Binding on Accessibility of Factor Xa Sites in Pax3 Mutants Xa100 and Xa216: Dose-Response Experiments* (A) Whole cell extracts (10µg) from COS7 Monkey cells transfected with Pax3 mutants Xa100(2), or Xa216(2) were incubated 30 minutes with either PD-specific (P3OPT, P6CON) or HD-specific (P1/2, P2) oligonucleotides or with a non-specific (NS) oligonucleotide. The buffer conditions for PD vs HD DNA binding were different (see Materials and Methods), and the negative control was included in each case. Increasing amounts of factor Xa protease (from 0 to 200ng/µg of whole cell extract) were added followed by a 15 minutes digestion period. Proteolytic fragments were separated by SDS-PAGE and detected by immunoblotting using the anti-HA antibody, as described in the legend to Figure 4. The positions of the full length intact Xa100(2) and Xa216(2) proteins (empty arrowheads), and of the major predicted, HA-immunoreactive product (filled arrowhead, see Table 2) are shown. (B). For quantifying the effect of DNA binding on accessibility of factor Xa cleavage sites in dose-response studies, the immunoblots in (A) were scanned by densitometry. The intensity of the immunoreactive intact Xa100(2) and Xa216(2) full length proteins was determined in the absence of factor Xa, and after digestion with a factor Xa amount indicated by the arrow at the
top of each immunoblot series. The amount of intact protein remaining after factor Xa digestion was determined for Xa100(2) (black) and Xa216(2) (gray) and for all DNA binding conditions and is expressed as the fraction of intact protein (expressed as a percentage).

FIGURE 7:

Structures of the Paired Domain and Homeo Domain Bound to DNA. The structure of the DNA-bound Paired Domain (A) is adapted from that published for Pax6 (4,26). The structures of the HD bound to DNA either as a monomer (B) or as a dimer (C) are taken from that published for the HD of the Drosophila Prd protein (33). The DNA strands are shown as gray ribbons through the sugar phosphate backbone, and bases are shown as protrusions from the ribbons. The Pax3 segments are in green, with the positions of discrete sites targeted for modification to factor Xa sites identified as causing loss-of DNA binding (red), as having no effect on DNA binding and being either accessible (purple) or not (blue) to factor Xa protease fragmentation.

SUPPLEMENTARY FIGURE 1:

Accessibility to Protease Cleavage of Factor Xa sites inserted in Additional Pax3 Mutants: Effect of DNA Binding. Results are shown as described in legend to Figure 4.

SUPPLEMENTARY FIGURE 2:

Accessibility to Protease Cleavage of Factor Xa sites inserted in Additional Pax3 Mutants: Effect of DNA Binding. Results are shown as described in legend to Figure 5.
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Table 1: Factor Xa cleavage sites introduced in individual Pax3 mutants

| Xa Mutant | Wild type sequence | Xa Mutant Sequence |
|-----------|--------------------|--------------------|
| Xa55b     | IRHK               | IEGR               |
| Xa71      | VISR               | IEGR               |
| Xa114     | VEKK               | IEGR               |
| Xa131     | WEIR               | IEGR               |
| Xa252     | LAQR               | IDGR               |
| Xa259     | TEAR               | IEGR               |
| Xa66(1)c,d| ▼                  |HGIR HSIEGRGIR      |
| Xa66(2)   | ▼                  |HGIR HSIEGRGIR      |
| Xa100(2)  | ▼                  |PGA PGAGIEGRGAGIEGRGAI |
| Xa100(4)  | ▼                  |PGA PGAGIEGRGAGIEGRGAI |
| Xa172(1)  | ▼                  |ADLE ADLIEGRLE      |
| Xa172(3)  | ▼                  |ADLE ADLIEGRLE      |
| Xa189(1)  | ▼                  |IDGI IDEGRIDGI      |
| Xa189(2)  | ▼                  |IDGI IDEGRIDGI      |
| Xa216(1)  | ▼                  |LKIDGR SIKKR        |
| Xa216(2)  | ▼                  |LKIDGR SIKKR        |

a Introduced amino acids are underlined and engineered Xa protease sites are indicated in bold.

b Mutants are designated according to the Pax3 amino acid position at which individual Factor Xa cleavage sites were introduced by site-specific modification of wildtype sequence.

c These mutants were created by insertion mutagenesis at the position indicated by an arrow.

d The number of Factor Xa sites introduced at that position is indicated in parenthesis.
Table 2: Expected size of Factor Xa proteolytic fragments in individual Pax3 mutants

| Pax3 Mutants     | aAmino Terminal Fragment (1) | bCarboxy Terminal Fragment (2) |
|------------------|-------------------------------|--------------------------------|
| Xa55             | 6.7                           | 49.5                           |
| Xa71             | 8.6                           | 47.6                           |
| Xa114            | 13.8                          | 42.4                           |
| Xa131            | 15.9                          | 40.2                           |
| Xa252            | 30.5                          | 25.7                           |
| Xa259            | 31.4                          | 24.8                           |
| Xa66(1)          | 8                             | 48.9                           |
| Xa66(2)          | 8                             | 49.6                           |
| Xa100(2)         | 12.6                          | 44.3                           |
| Xa100(4)         | 12.6                          | 44.3                           |
| Xa172(1)         | 20.8                          | 36.1                           |
| Xa172(3)         | 20.8                          | 36.1                           |
| Xa189(1)         | 22.7                          | 34.3                           |
| Xa189(2)         | 22.7                          | 34.7                           |
| Xa216(1)         | 25.5                          | 31.6                           |
| Xa216(2)         | 25.5                          | 31.6                           |

a Expected specific amino terminal proteolytic fragment (kD) detectable using anti-cMyc antibody.

b Expected specific carboxy terminal proteolytic fragment (kD) detectable using anti-HA antibody.
Table 3: Oligonucleotides used for Site-directeda and Insertion Mutagenesis

| Oligonucleotide | Sequence |
|----------------|----------|
| Xa55           | GGAGTATTATCAACGCGCCCGCTGCCAACCATATCGAAGCAGAATAGTGAGATGGCCGACG |
|                | GTGCGCATCTCACTATACTCGTATAGGTTGGGCAAGGCGCCGCTGATAAATACCTCC |
| Xa71           | GGCATTCGCCTGCAATTTGAAGGCTGACAGCTGCGTGCC |
|                | GGACACGGAGACTGCGAATCTGGGCAAGGTAGAGG |
| Xa114          | AAACCCAAGCGTACAACTCCCGGAATCGAGGGAGCTAGGAATAGACGGCC |
|                | CTGGTATTCTCCATAATTAGCTGCCATGTCGAGGTGTTT |
| Xa131          | ACCCCGGGATGTTCTAATTTGAAGCAGAGCAAAATTAGCTCAAGGAC |
|                | GTGCTGAGCAATTGCTGCTGCCGATCTCCCTGCTGTAAT |
| Xa252          | ATTTACACCCAGGAGGAGATCGCGCCGGCGAGCCTGCGAGG |
|                | GCCCCAGAGGGGCTGCTGAGG |
| Xa259          | GCCAGAGGGCAAGCTTATACCGAGCCGGGAGCTGGCAGG |
|                | GTGCTGAACAAATCGTCCGAGG |
| KasI (100)b     | GGCTCCATCCGACCTGCGCCATCGCGCGGC |
|                | GCTGCGCGCCGCTATCGGAGG |
| AflII (216)c   | TCTGACACCTGATTTACCGCTTAAGAGGAGAGCAGGCCAG |
|                | CCTGCGCTGCTTCTCCTTAGCGCGTAAATCAGGTCAGA |
| Xa66 (1)d      | ATCCATAGAAGGTAGAG |
| Xa66 (2)       | ATCCATAGAAGGTAGAG |
| Xa100 (2)      | GGCACCGCatagatAGAGGCGCCATAGAAGGTAGAG |
| Xa100 (4)      | GGCACCGCatagatAGAGGCGCCATAGAAGGTAGAG |
| Xa172 (1)      | CTAGATATCGAAGGTCGT |
| Xa172 (3)      | CTAGATATCGAAGGTCGTCTAGATATCGAAGGTCGT |
| Xa189 (1)      | CGATATCGAAGGTCGAAGGTCG |
| Xa189 (2)      | CGATATCGAAGGTCGAAGGTCG |
| Xa216 (1)      | TTAAGATCGAGGTCGATCGAAGGTCG |
| Xa216 (2)      | TGAAGATCGACGCTGAGGTCG |

aNucleotide substitutions leading to amino acid changes are indicated in bold, and those that introduce silent restriction sites are underlined.

bThese oligos were used to introduce a unique KasI restriction site at position 595 of the wildtype Pax3 cDNA sequence.

cThese oligos were used to introduce a silent and unique AflII restriction site at position 940 of the wildtype Pax3 cDNA sequence.

dOligonucleotide sequences used (double stranded) for insertion mutagenesis in the creation of the corresponding Xa mutants as listed in Table 2.
Figure 1

**PD**

**HD**
Figure 2
Figure 3

PD

P6CON  -  -  -  +++  +++  +
P3OPT   -  -  -  +++  +++  ++
P1/2    -  -  -  +++  +++  ++
P2      ++  -  +  +++  +++  ++

HD

P6CON  +++  +++  +++  -  -
P3OPT   +++  +++  +++  -  -
P1/2    +++  +++  ++  -  -
P2      +++  +++  +++  -  -
Figure 4

A

WT

Xa100 (2)

Xa100 (4)

Xa216 (1)

Xa216 (2)

B

Intact Pax3 (%) vs WT, Xa114, Xa131, Xa100 (2), Xa100 (4), Xa172, Xa189, Xa216 (1), Xa216 (2)
Figure 5

A

B

Intact Pax3 (%)

MT Xa114 Xa131 Xa100 (2) Xa100 (4) Xa172 Xa189 Xa216 (1) Xa216 (2)

no DNA P3OPT P1/2 P2

0 20 40 60 80 100 120

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Figure 6

A

Xa100(2)  Xa216(2)  Xa100(2)  Xa216(2)

BS

P3OPT

P6CON

B

Intact Pax3 (%)

Xa100(2)

Xa216(2)
Figure 7
Figure S1
Crosstalk between the paired domain and the homeodomain of PAX3: DNA binding by each domain causes a structural change in the other domain, supporting interdependence for DNA binding

Sergio Apuzzo, Aliaa Abdelhakim, Anouk S. Fortin and Philippe Gros

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