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Modulation of PAX6 Homeodomain Function by the Paired Domain*

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The abbreviations used are: PD, paired domain; HD, homeodomain; SDS, Sodium doecyl sulfate; EMSA, electrophoretic mobility shift assay; PST, Proline, Serine and Threonine rich transactivation domain.

Running title: PAX6 PD and HD function
ABSTRACT

PAX6 is required for proper development of the eye, CNS and nose. PAX6 has two DNA-binding domains, a glycine-rich region that links the two DNA-binding domains, and a transactivation domain. There is evidence that the different DNA-binding domains of PAX6 have different target genes. However, it is not clear if the two DNA-binding domains function independently. We have studied the effect of structural changes in the paired domain on the function of PAX6 mediated through its homeodomain. The R26G and I87R mutations have been reported in different human patients with clinically different phenotypes and are in the N- and the C-terminal halves of the paired domain, respectively. Surprisingly, we found that the I87R mutant protein not only lost the transactivation function but also failed to bind DNA by either of its DNA-binding domains. In contrast, the R26G mutant protein lost DNA-binding through its paired domain but had greater DNA-binding and transactivation than wild-type PAX6 on homeodomain binding sites. Like R26G, the 5a isoform showed higher DNA binding than wild-type PAX6. This study demonstrates that the two sub-domains of the paired domain influence the function of the homeodomain differentially, and also provides an explanation for the difference in phenotypes associated with these mutations.
INTRODUCTION

Pax6 is considered the master control gene for morphogenesis and evolution of the eye (1). It is an evolutionarily conserved gene in both vertebrates and invertebrates. The Pax6 genes cloned from representatives of at least eight animal phyla are structurally and functionally similar (2), and ectopic expression of mouse and squid Pax6 in Drosophila results in ectopic eye formation (3, 4). Pax6 is expressed in the developing eye, nose, pancreas, and central nervous system (5-10). Loss of PAX6 function leads to severe brain abnormalities, microencephaly, early postnatal death, and absence of eyes and nose in rodents (11, 12) and humans (13). In addition, PAX6 is also essential for the differentiation of α−cells and the formation of the islets in the pancreas (14, 15). Heterozygous mutations in the PAX6 gene are responsible for several naturally occurring phenotypes including aniridia.

The PAX6 protein can be subdivided into several distinct domains. It has two DNA binding domains (a paired domain (PD) at the N terminus and a paired like homeodomain (HD) in the middle) a glycine-rich region that links the two DNA-binding domains and a transactivation domain at the C terminus (5, 16). There are two major alternatively spliced forms of PAX6 that differ by the presence or absence of 14 amino acids within the PD that are coded by an exon known as 5a (17). Studies suggest that the target genes of PAX6 can be regulated by three types of DNA binding sites: (i) those identified by the PD, e.g, mouse N-CAM (18), (ii) those identified by the HD, e.g, rhodopsin (19), and (iii) those identified by cooperative interaction of the paired domain and homeodomain, e.g, neural cell adhesion molecule L1 (20).

The PD of PAX6 can bind to a broad range of DNA sequences with high specificity. The PD actually consists of independent amino-terminal and carboxy-terminal subdomains, and each subdomain can identify and bind to distinct DNA
sequences (21, 22). However recent crystallographic studies using a 26 bp consensus PAX6-DNA binding site and full-length PD peptide revealed that the docking arrangements of the N-subdomain and C-subdomain are very similar (23). However, it is not clear how the PAX6 paired domain recognizes so many different DNA sequences. PDs can also bind DNA by interacting with other DNA-binding domains such as the HD (24, 25).

In the PAX6 protein, the paired-type HD is about 80 amino acids downstream of the PD. Studies have shown that highly diverged HDs have indistinguishable DNA-binding preferences (26). Several mechanisms have been suggested for the specificity of HD function. These include protein oligomerization mediated by dimerization domains outside of the HD, protein-protein interactions of homeoproteins with other factors, association of the HD with other DNA-binding domains, and cooperative dimerization of pairedclass HDs (26). However, which of these mechanisms are utilized by PAX6 is not known.

That the PD of PAX6 can bind to a broad range of DNA sequences and many HDs recognize similar sequences has hindered the identification of target genes of PAX6. Functional and DNA-binding studies using PAX6 missense mutations with the observed developmental defects may be useful in determining how PAX6 recognizes DNA in the target genes and factors that influence binding site selection.

In the present report we analyzed two PD missense mutations reported in human patients: R26G is in the N subdomain and I87R is in the C subdomain of the PD of PAX6 (Fig. 1). The patient with the R26G mutation had Peter’s anomaly, and the Patient with I87R had a much more severe phenotype and is described in detail in Tang et al. (27). According to crystallographic studies of a PD bound to a PAX6 consensus binding site,
neither of these mutations are involved in direct DNA contact (23). We analyzed the transactivation and DNA binding of these proteins with the HD DNA-binding sites P2 and P3 and with three different PD binding sites. We have also used the 5a isoform of PAX6 to study the effect of structural changes in the PD on the DNA-binding function of the homeodomain. Our results demonstrate that the structural changes in the two subdomains of PD influence the DNA binding of the homeodomain of PAX6 differentially and provide a possible explanation for the differences observed in phenotype between the two mutants.
EXPERIMENTAL PROCEDURES

Cell Culture  NIH/3T3, a murine fibroblast cell line, was maintained in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum. HLEB3, a human lens epithelial cell line (28), was maintained in Eagle's minimal essential medium supplemented with 20% fetal calf serum.

Plasmid Constructs  Rc-CMV-PAX6 expression plasmids were constructed by using a PCR cloning strategy. The construction of the R26G and I87R mutants is described elsewhere (27). Plasmids containing the +5a isoform of PAX6 were used to generate the pRc-CMV-5a construct. P2-luc or P3-luc plasmids were generated by inserting two copies of the P2 or P3 sequences at the SalI site in the plasmid pluc-S (29).

Transfections  HLEB3 and NIH/3T3 cells were plated at a density of 4-6 x 10^5 cells per 60-mm or 2 x 10^5 cells per 35-mm tissue-culture dish 24 h before transfection. Transfections for all cells were performed with plasmid DNA coated with the polycationic lipid lipofectamine (Life Technologies, Inc.) according to the manufacturer's instructions. NIH/3T3 cells were transfected at 60-80% confluency using 15 µl (30 µg) of Lipofectamine per 60-mm dish. HLEB3 cells were transfected at 70-80% confluency using 6 µl (12 µg) of Lipofectamine per 60-mm dish. For transfections with the pRc-CMV-PAX6 expression vectors, each 35-mm dish was transfected with 0.2 µg of P2-luc or P3-luc, 0.1 µg of pRc-CMV effector plasmid, and 0.2 µg of pSV2 βgal plasmid (Promega Corp.) as an internal control.

Luciferase and β-Galactosidase Assays  Cell extracts were prepared after 24-48 h of transfection. Luciferase activity was measured at room temperature using a luciferase assay kit (Tropix, Bedford, MA). Briefly, 50-µl aliquots of substrate A were placed into 75 x 12 mm luminometer sample tubes (Sarstedt). Then, 10 µl of individual cell extracts
and 50 µl of substrate B were added sequentially into the tubes, which were then placed immediately into a luminometer for measurement. The luminometer was set to measure the luciferase signal for 10 s with a 2-s delay. β-galactosidase activity was detected using a Galacto-Light Plus chemiluminescent assay kit (Tropix) according to manufacturer’s protocol. The measurement protocol was the same as for the luciferase assay. Luciferase activities were normalized relative to β-galactosidase activity.

**Electrophoretic Mobility Shift Assays (EMSAs)** Crude nuclear extracts were prepared from transfected NIH/3T3 cells as described in detail previously (30). The protein concentrations of all nuclear extracts were measured by the Bradford assay and adjusted to equal protein concentrations by dilution with the nuclear extraction buffer. *In vitro*-transcribed and -translated proteins were generated using the TNT coupled reticulocyte lysate system (Promega Corp.) using the pRc-CMV constructs described above. The amount of PAX6 protein expressed or translated was quantitated by western blot analysis with antibodies against the linker region of PAX6 as described earlier (30). The bands on the western blots were quantitated by using a Personal Densitometer Scanner 1.30 and Image Quant 3.3 software (Molecular Dynamics). Occasionally, quantitation of *in-vitro* synthesized 35S labeled proteins was further confirmed by drying the SDS-PAGE gels and analyzing by using a Storm 840 PhosphorImager (Molecular Dynamics) and Image Quant 5.0 software (Molecular Dynamics).

EMSAs for paired domain DNA-binding were performed as described earlier by Singh et al.(30) and for homeodomain DNA-binding as described by Wilson et al.(26). The gel was dried and then analyzed with a PhosphorImager followed by autoradiography on Kodak X-Omat Autoradiography films. For supershift assays, the binding reaction mixture was incubated with the polyclonal antibody against PAX6 at room temperature for 15 min before it was loaded onto the gel.
RESULTS

Loss of DNA binding to paired domain binding sites by the mutant PAX6 proteins

R26G and I87R. In our earlier report (27), we show that R26G and I87R mutant proteins retain only partial DNA-binding activity when the consensus PAX6 DNA-binding site (P6CON) is used. We further investigated this finding by using two other PAX6 PD binding sites, CD19-2(A-ins) (29) and the binding site found in the promoter region of mouse N-CAM gene (18). The N-CAM binding region is structurally different from the other two sites because it is bipartite containing two TGCTCC motifs separated by 9 bp (Table I). Nuclear extracts used for EMSAs were prepared from transiently transfected NIH/3T3 cells containing equal amounts of PAX6 and mutant PAX6 proteins as determined by western blot analysis (Fig. 2A). As reported earlier (27), R26G and I87R retained partial DNA binding activity when the P6-CON PD-binding site was used (Fig. 2B). However, no detectable binding of mutant proteins to the CD19-2(A-ins) and N-CAM binding sites was observed. A recent report describing the crystal structure of PAX6 bound to optimal PAX6 binding site P6CON showed that neither I87 nor R26 make direct contact with DNA at this site (23). This raises the possibility that PAX6 may bind to different PD binding sites by making DNA contact with different sets of amino acids.

Different effects of R26G and I87R on the Transcriptional Activation of a Reporter Gene bearing an HD binding site. Although it is assumed that the HD and PD bind independently of each other on their own respective recognition sites, we decided to further investigate this by using PD PAX6 mutants and HD DNA-binding sites in a reporter assay. We used both the consensus HD binding sites P2 and P3 for the study. NIH/3T3 cells were transfected with P2-luc or P3-luc reporter plasmid and mutant or wild-type expression plasmids and assayed for luciferase activity. PAX6 activated the transcription of the reporter gene approximately ten fold over the basal activity with
either of the HD binding sites (Fig. 3A). Interestingly, R26G showed higher transcriptional activity than did wild-type PAX6, whereas I87R did not produce any significant transcriptional activation (Fig. 3A). Similar results were obtained with the P3-luc reporter (Fig. 3B). To rule out the possibility that lower transactivation by the I87R mutant protein may have been due to a lower quantity of I87R mutant protein, western blot analysis was carried out in parallel with 10 and 20 µl of extracts from the cells transfected with the highest concentration of expression plasmid using the anti-PAX6 antibodies (Fig. 3C). The results indicated that all the constructs used express similar amounts of proteins.

Modulation of Transcriptional Activation by Coexpression of Mutant and Wild-type PAX6. Next, we determined the effect of expressing wild-type and mutant proteins together, as would be the case in aniridia patients. For a control we transfected the expression construct PAX6(1-240), which has a partial HD and an intact PD and consequently can bind DNA only on PD DNA-binding sites but not HD binding sites (data not shown). When the expression constructs were transiently transfected with the P2-luc reporter alone, PAX6 and the R26G mutant showed 8-fold and 16-fold induction, respectively, over the control and the I87R and PAX6(1-240) mutants did not show induction (Fig. 4). When the R26G mutant constructs was expressed in the presence of wild-type PAX6, 12-fold induction was seen. Minimal interference in activation by PAX6 was seen with the PAX6(1-240) mutant. However, when the I87R mutant was co-expressed with wild-type PAX6, there was a 40% decrease in the induction of reporter plasmid transcription. This decrease may be due to the squelching effect of the intact PST domain in the I87R mutant protein. In an earlier report (27), we showed that overexpression of the PST domain reduces transactivation by PAX6. But why was the
squelching effect not seen with the R26G mutant? On the basis of transactivation data, it appears that although the I87R mutant has an intact HD it may not bind to the DNA properly, and hence no transactivation was seen. However, I87R mutant has an intact PST domain, it was capable of squelching transcription of wild-type PAX6. In contrast, the R26G mutant showed significantly higher transactivation, indicating that it was capable of DNA binding and collaborating with the cofactors necessary for transcription.

**Different Effects of R26G and I87R Mutations on HD DNA binding.** Because transactivation is dependent on DNA binding, we performed gel shift assays to determine the DNA-binding ability of the mutant proteins through the HD. We used oligonucleotides containing optimal HD binding sites P2, P3, and P1/2 (24, 26, 31) (Table I) and *in vitro* transcribed and translated proteins for this assay. The relative amounts of translated protein was determined by a denaturing 10% SDS-polyacrylamide gel (Fig. 5C) followed by quantitation with a PhosphorImager as described in experimental procedures. Increasing amounts of reticulocyte lysate containing translated proteins were used. As P2 and P3 are bipartite, two molecules of PAX6 can bind to these sites. As expected, both monomer and dimer DNA-protein complexes were resolved (Fig. 5A and B). The specificity of the both monomeric and dimeric complexes was determined by competition with unlabeled nucleotide, antibody ablating, P1/2 and truncated PAX6 protein (data not shown). We found that in comparison to PD binding sites a much higher amount of PAX6 protein was required to produce significant binding (Fig. 5A and B) to either P2 or P3. When the binding patterns for the R26G and I87R mutants were analyzed, the R26G mutant showed significantly higher binding (compare
lanes 2-5 with lanes 7-10 in Fig. 5A) than did wild-type PAX6, but more surprisingly the I87R mutant did not show any binding (lanes 12-15 in Fig. 5A, and lanes 9-12 in Fig 5B). Sometimes, at higher amounts of protein (4 and 6 µl), a faster migrating band below the monomeric band was seen; it appeared to be generated by protein degradation.

In vitro synthesized proteins and in vivo translated proteins may differ in their properties. Therefore, gel-shift experiments were also performed with the nuclear extracts described in the experiment for Fig. 2. Six microliters of nuclear extract was used with the $^3$P-labeled P2 probe. Two microliters of extract was used for the CD19-2(A-ins) probe, which was used as a control to determine the position of the monomer PAX6 band (Fig. 6, lane 2). As with the in vitro synthesized proteins, no significant binding was seen with I87R mutant protein (Fig. 6, lane 9) whereas the R26G mutant showed significantly higher binding both as a monomer and as a dimer (Fig. 6, lane 10). An excess of unlabeled oligonucleotides (lanes 11-13) or anti PAX6 antibodies (lanes 14-16) was able to ablate the bands, confirming their specificity, as a non specific band below the PAX6 band was not affected. Similar results were obtained with P3 as binding probe (data not shown). Although these data are in agreement with the transactivation data, higher HD binding by the R26G mutant and the complete absence of HD binding in the case of the I87R mutant were surprising, as these missense mutations are in the PD. The data demonstrated that the I87R mutation resulted in complete loss of function mutation in the in vitro assays, whereas the R26G mutant lost its function through the PD and had enhanced function mediated through its HD. This could be why the I87R mutation results in a more severe phenotype in aniridia patient.
**Higher DNA-binding affinity of the 5a isoform of PAX6 for HD sites.** Like the R26G mutation, which is in the N-subdomain, the 5a isoform of PAX6 is generated by insertion of 14 amino acids within the N subdomain and therefore changes its structure. Because the R26G mutant showed higher DNA-binding, we wanted to determine if other sequence changes in the PD influence DNA binding through the HD. As described earlier, gel-shift assays were performed with increasing amounts of *in vitro* transcribed and translated proteins and $^{32}$P-labeled oligonucleotides containing the P2 and P3 binding sites. As with the R26G mutant, the 5a isoform showed higher DNA binding (Fig. 7A and 7B).

**Higher DNA Binding Abilities of the R26G Mutant and the 5a Isoform** To further characterize the differences in DNA binding among wild-type PAX6, the R26G mutant, and the 5a isoform, we performed protein-DNA binding kinetic studies as described by Underhill et al. (24), Vogan et al. (32), and Singh et al. (30). These experiments included saturation analysis using increasing concentration of different proteins with a saturating amount of probe and a constant amount of protein with increasing amount of probe. In the experiments involving increasing concentrations of proteins, 1-5 µl of reticulocyte lysate containing equal amounts of PAX6 and R26G mutant protein was incubated with 50 fmol of double-stranded DNA probe (P2 or P3) in a 15-µl reaction mixture and resolved on 5% acrylamide gels. The specific bands on the gel were then quantitated. The results of these experiments are displayed in Fig. 8A and 8B. The relative differences were calculated as described previously (30) using total binding. The fold difference in $K_D$ between R26G and PAX6 was 3.6 when P2 was used as probe and 2.1
when P3 was used as probe. The fold difference in $K_D$ between 5a and PAX6 was 3.7 when P2 was used as a probe and 2.2 when P3 was used as a probe. The kinetic data also revealed that monomers were preferentially formed at lower concentrations of PAX6 or mutant proteins. However, this binding did not increase proportionately, and saturation was achieved with a four fold increase in protein, whereas dimeric binding, although relatively low at lower concentrations, increased proportionately, and saturation was not achieved within the amount of protein used in the experiments. Although, we had enough points in the linear range to calculate the binding. Our data are in agreement with an earlier report (26) that demonstrated cooperative dimerization of paired class HD. However, we used full-length protein which has a paired-class HD in the middle of the protein. Saturation analysis was also performed with the nuclear extracts. The binding results indicate that the R26G mutant had more than three fold higher affinity for HD binding site as compared to PAX6 (data not shown). Overall, the data showed that mutations in the two subdomains of the paired domain influence the function of the HD in opposite ways.
DISCUSSION

The data presented demonstrate that structural changes in the PD of PAX6 influence the function of its HD. The data also show that the nature of the influence is dependent on the location of the structural change. This differential influence may be the reason for the difference seen in the phenotype of the two patients carrying these mutations (27).

Previous analysis of Pax6 indicated that the two domains can act independently. In *Caenorhabditis elegans*, two types of Pax6 mRNAs are generated: one encoding a protein containing both domains and one encoding a protein with only the HD(34, 35). The presence of the HD alone in a Pax6 protein indicates that it can function independently. In addition, *in vitro* binding studies have indicated that the PD and HD bind to different consensus sequences, and each domain has been crystallized with its consensus sequence (17, 23, 36-39). There is evidence from gene regulation studies that the two DNA binding domains have different target sequences. For example, certain genes like *N-CAM*, *sine oculis*, and members of the crystallin gene family have cis elements that are recognized by the PD of PAX6 (18, 40, 41), whereas certain other genes like rhodopsin (19) and L1 cell adhesion molecule gene (42) have cis elements that are recognized by the HD of PAX6. Recent study indicated that expression of rat glucagon gene is influenced by Pax6, most likely due to its homeodomain binding on a cis element in the promoter region (43, 44).

In contrast to the above findings, studies by Underhill and Gros (24) on the mouse splotch-delayed mutation (Sp^d_) demonstrate the interdependence of the two DNA-binding
domains in PAX3. The Spd mutation is a glycine to arginine substitution at position 9 of the PD which is located at the β turn of a β-hairpin motif. Subsequent studies showed that structural conservation of this position is essential for DNA binding function of PAX3 by either of its DNA-binding domains (31).

The HDs of the paired class have diverged considerably from those of the Drosophila antennepedia and ultrabithorax classes (45). Other HD classes bind target sites as monomers, whereas paired-HD polypeptide binds to DNA as a dimer in vitro (26). In fact, studies with P2 and the full length PAX3, which like PAX6 also has a paired like HD, showed that not only dimerization occurred but the binding reaction was cooperative (24), as expected. Similarly, in our binding experiments with P2 or P3 and PAX6, the kinetics of binding indicates that full-length PAX6 dimerization is cooperative. This dimerization was better than wild-type PAX6 with both the 5a isoform and R26G mutant proteins.

PAX proteins can be divided into six distinct subfamilies according to their sequence similarities in the PD (33). Each subfamily has binding preferences for distinct binding sites. Members of the subfamily represented by Pax6 show differential binding to the two distinct types of sequences preferred by the members of the Pax5 and Pax3 subfamilies (21), indicating that the conformation of the PD region in different subfamilies may be different. It is conceivable that different conformations of the PD may influence the recognition function of the homeodomain differently.
A large number of genes involved in developmental regulation contain highly conserved HDs. The great degree of functional specificity between different HD containing proteins cannot be explained on the basis of the modest DNA-binding specificity of the HDs (26). Therefore, some of the HD containing proteins may rely on interaction with protein sequence outside the HD to generate specificity. PAX proteins containing paired DNA-binding domains are an example (46, 47). Our results and earlier studies (24, 31) provided a clear indication that PD structure influences the HD and is likely to be involved in regulating specificity and affinity (24, 31).

Although several missense mutations have been identified in the PAX6 gene from the patient data available so far, the absence of missense mutations in the homeodomain is remarkable (for a list of mutations in PAX6, see the web site (53). Two possible explanations for this are that missense mutations in the HD are lethal and therefore not detected and that certain amino acid substitutions introduced in the HD by missense mutations do not result in apparent phenotypes and hence remain undetected. The Pax6 gene is highly conserved, and despite structural analysis of several human samples, no polymorphisms in the HD or PD coding regions have been detected, ruling out the possibility of silent mutations.

Aniridia is considered a haploinsufficiency disease and occurs as a result of loss of function of one allele of PAX6 (5, 48). However, the phenotypic variability in the expression of aniridia cannot be explained by haplosufficiency alone. Our present study in combination with other reports (30, 49-52) suggests that there are multiple factors that
may contribute to the variability seen in the expression of aniridia. As more and more missense mutations with known phenotypes are analyzed for DNA binding and function, the mechanism of PAX6 DNA-binding will become clearer, and it may be possible to determine the effect of a mutation at a particular position.

Acknowledgements

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REFERENCES


53. [http://www.hgu.mrc.ac.uk/Softdata/PAX6](http://www.hgu.mrc.ac.uk/Softdata/PAX6)
**Figure Legend**

**Fig. 1. Functional domains of PAX6 and the predicted structure of the PD of PAX6.** The positions of missense mutations and the insertion site for exon 5a are shown. PD, paired domain; G, Glycine rich linker region; HD, Homeodomain; PST, Proline, Serine and Threonine rich transactivation domain. Numbers on top indicate the position of amino acid at the end of each domain.

**Fig. 2. Differential binding of the PAX6 mutants R26G and I87R to various PAX6 paired domain binding sites.** NIH/3T3 cells were transfected with PAX6, mutant R26G and I87R expression constructs. The expression of each protein was verified by western blot analysis (A). Nuclear extract from each construct was resolved on a denaturing 10% SDS-polyacrylamide gel, and immunoblotting was performed by using anti-PAX6 antibodies. In vitro, synthesized PAX6 protein was used as a control. Nuclear extracts containing equal amounts of protein were then used for binding analysis of $^{32}$P-labeled P6CON, CD19-2 (A-ins), or N-CAM PAX6 PD binding sites by EMSA (B). Nuclear extracts from the cells transfected with the expression vector alone (Re-CMV) were used as control. As expected, PAX6 bound to all the binding sites with high affinity, whereas the mutants R26G and I87R bound to only P6CON and with much lower affinity.

**Fig 3. Differential transcriptional activation of a luciferase reporter gene bearing an HD binding site.** NIH/3T3 cells were transiently transfected with the given amounts of expression constructs and 0.1 µg of reporter construct containing the P2 (A) or P3 (B) HD binding sites. The total concentration of plasmids was kept constant by addition of
empty expression vector. The luciferase activity of the reporter constructs is shown as mean + S.D. of three separate transfection experiments. Western blot analysis was performed to verify the amount of protein expressed by various expression constructs (C). Extracts (10 and 20 µl) from cells transfected with 0.2 µg of expression plasmids were resolved on a 10% SDS-polyacrylamide gel and immunoblotted by using anti-PAX6 antibodies. The results showed that all the expression constructs expressed almost equal amounts of protein.

**Fig. 4. Modulation of transcriptional activation by coexpression of mutant and wild type PAX6.** NIH/3T3 cells were transiently transfected with 0.2 µg of PAX6, I87R, R26G, and PAX6 (1-240) individually or with 0.1 µg of PAX6 and 0.1 µg of either PAX6 (1-240) or I87R or R26G together with P2-luc reporter plasmid. The luciferase activity of the reporter construct is shown as mean + S.D. of three separate experiments.

**Fig. 5. Differential binding of the PAX6 mutants R26G and I87R to the HD binding sites P2 and P1/2 (A) and P3 (B).** Increasing amount of the wild-type and mutant PAX6 proteins synthesized in vitro were used in an EMSA to bind HD binding sites. Because of the bipartite nature of P2 and P3 both monomeric and dimeric bands are detected and are marked by arrows. The P1/2 site contains only one homeodomain binding sequence and was used to determine the position of the monomeric band. (C) SDS-PAGE of in vitro translated wild-type and mutant PAX6 protein.
Fig. 6. DNA binding specificity of wild-type and mutant PAX6. Nuclear extracts described in Fig. 2 were used to test binding with the homeodomain binding site P2. Two microliters of nuclear extract containing wild-type PAX6 was used with PD binding site CD19-2(A-ins) (lane 2) as control to mark the monomeric band. For HD binding site P2, 6 µl of nuclear extracts was used. A nonspecific band seen with P2 was not affected by the addition of a hundred fold excess of unlabeled oligos or by the anti PAX6 antibodies, whereas the specific bands were competed out or ablated. Position of monomeric and dimeric bands are marked by arrows.

Fig. 7. DNA binding of wild-type PAX6 and the 5a isoform of PAX6 to HD binding sites P2 (A) and P3 (B). Increasing amounts of PAX6 or 5a isoform protein synthesized in vitro were used in an EMSA to bind HD DNA binding sites. The P1/2 site which contains one homeodomain binding sequence, was used to determine the position of monomeric band. The 5a isoform has 14 additional amino acids and migrates slower than PAX6. Position of monomeric and dimeric bands are marked by arrows.

Fig. 8. Kinetic analysis of P2 and P3 HD binding sites with increasing amounts of PAX6, 5a isoform of PAX6, and PAX6 mutants. Differences in binding between wild-type and the 5a isoform or mutant proteins are shown. To simplify the calculation of the fold differences in $K_D$ values, total binding was used, as both P2 and P3 are occupied by individual protein molecules. To reflect the difference in the kinetics of monomer and dimer formation, values for each kind of binding are also shown. Increasing concentrations of wild-type and mutant proteins were individually incubated with the $^{32}$P-
labeled probe as described in Experimental Procedures. The protein-DNA complexes were separated from free DNA by gel electrophoresis and the amounts of free DNA and protein-DNA complexes were quantitated with a PhosphorImager and Image Quant program as described in Experimental Procedures. For calculation and plotting of the graphs, CA-Cricket graph III software was used. The experiments were repeated three to four times and data from a representative experiment are shown.
### TABLE I

Sequences of HD oligonucleotides used for DNA-binding of PAX6 PD mutants

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<th>Oligonucleotide</th>
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<tr>
<td>P2</td>
<td>5'-TCGAGGGCATCAGGATGCTAATTGATTAGCATCGATCGGG-3'</td>
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<tr>
<td>P3</td>
<td>5'-TCGAGGGCATCAGGATGCTAATTGAATGAGCATCGATCGGG-3'</td>
</tr>
<tr>
<td>P1/2</td>
<td>5'-GATCCTGAGCTAATTGAGGCTTGTACAGATC-3'</td>
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Sequences of PD oligonucleotides used for DNA-binding of PAX6 PD mutants

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<tr>
<th>Oligonucleotide</th>
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<tbody>
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<td>P6CON</td>
<td>5'-TGGAATTCCAGGAAAAATTTCACGCTTGAGTTCCAAGCTCAGTGGTA-3'</td>
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<tr>
<td>CD19-2(a-ins)</td>
<td>5'-TACTCGAGCTGGCAGTTAGGCAGTTAGCCAATTCTTGGAGATAATTTTCCA-3'</td>
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<tr>
<td>N-CAM</td>
<td>5'-GCAATTTGTCTGCTTGCTGCTGATGGCTCCGCCGCCTCC-3'</td>
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<tr>
<td>Probe</td>
<td>P6 CON</td>
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<tr>
<td>Rc-CMV</td>
<td>PAX6</td>
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<td>PAX6</td>
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<td>Free probe</td>
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![Complexes and Free probes](image-url)
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