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Functional Properties of Natural Human PAX6 and PAX6(5a) Mutants

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Abstract

Purpose—*Pax6* is essential for development of the eye, brain, and pancreas. Two major products of *PAX6* are specific DNA-binding proteins, PAX6 and PAX6(5a). PAX6(5a) contains a short insertion influencing its DNA-binding activity. Heterozygous mutations in *PAX6* result in abnormal eye development implicating haploinsufficiency. Deletions of one *PAX6* allele result in aniridia characterized by severe ocular phenotypes. Approximately 10% of *PAX6* mutations encode missense mutations. These mutations usually cause less severe abnormalities than does aniridia. The moderate phenotypes raise the possibility that different ocular tissues are differently sensitive to specific mutations. To test this hypothesis, we probed functional properties of individual mutated Pax6 proteins in a variety of conditions.

Methods—Mutations in PAX6 and PAX6(5a) were introduced by site-directed mutagenesis and tested by transfections in four cell lines using reporters containing three different Pax6 binding sites. Pax6 binding to DNA was studied by electrophoretic mobility shift assays.

Results—Functional studies of PAX6 and PAX6(5a) and their eight natural missense (G18W, R26G, A33P, S43P, G64V, I87R, V126D and R128C) and two nonsense (R317X and S353X) disease-causing mutants revealed unexpected pleiotropic effects in gene regulation, not predicted by the PAX6-DNA crystal structure. Transactivation by PAX6 and PAX6(5a) was dependent on the location of mutation, type of DNA-binding site, and cellular environment.

Conclusions—This work provides evidence that activation by PAX6 and PAX6(5a) is modulated by specific cellular environments. It is likely that moderate phenotypes associated with PAX6 missense mutations originate from abnormal protein function in a restricted number of ocular cell types.

Pax6 is a highly conserved member of a Pax family of genes encoding transcription factors.¹ Nine mammalian *Pax* genes (*Pax1* to *Pax9*) share an N-terminal 128 amino acid DNA-binding domain called the paired domain (PD). The PD of Pax proteins can be structurally and functionally separated into two independent DNA-binding subdomains, PAI and RED.² Pax6 also contains an internal, paired-type homeodomain (HD), allowing different modes of Pax6 binding to DNA. Mammalian Pax6 genes encode predominantly two forms of the Pax6 protein, Pax6 and Pax6(5a).^{3–5} Pax6 contains a canonical PD. In contrast, Pax6(5a) contains a 14 amino acid insertion within the PAI domain (see Fig. 1A). Pax6 plays critical roles in the organogenesis of the brain, visual, and olfactory systems, as well as peptide hormone gene expression in the pancreas.^{6–10} Numerous studies have shown that Pax6 is essential for morphogenesis of the eye from its earliest stages and subsequent formation of all major ocular tissues.^{7,11,12}

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In humans, heterozygous mutations in *PAX6* cause a wide spectrum of ocular defects¹³⁻²² and subtle changes in the olfactory epithelium and brain.²³ Mutations generating truncated *PAX6* proteins and deletions of one allele typically result in aniridia. The prominent feature of aniridia is iris hypoplasia, often combined with cataracts, glaucoma, nystagmus, and foveal and optic nerve hypoplasia.^{24,25} Missense mutations generating single amino acid substitutions, representing approximately 10% of total mutations, cause less severe abnormalities (e.g., foveal hypoplasia, Peters' anomaly, congenital cataracts, and autosomal dominant keratitis).^{10,26} A rare case of a human *PAX6* compound homozygote resulted in anophthalmia and lethal brain defects.¹⁴ Hence, it has been proposed that a *PAX6* gene dosage effect is responsible for phenotypes associated with mutations in one or both alleles of *PAX6*.^{8,10}

Molecular studies of natural *PAX6* mutants are useful in understanding the molecular mechanisms of *PAX6* and other *PAX* proteins; however, earlier studies have focused only on a single or a few mutants tested in a single common cell line. Notably, two missense mutations, R26G and R128C, tested in P19 cells, reduced *PAX6* but increased *PAX6*(5a)-activation potential, suggesting that a specific mutant can act as a hypermorph.²⁷ Crystal structure of Pax6 PD in complex with DNA provides a model to understand the function of individual structural motifs comprising the PD and may ultimately predict impact of disease-causing single amino acid substitutions.²⁸

In this report, we sought to determine transactivation and DNA-binding properties of 10 representative natural *PAX6* and *PAX6*(5a) mutants with different reporters using a variety of DNA-binding mechanisms in different cell types. The results revealed an unexpected range of reduced or increased transactivation properties of each mutant tested. Our data indicate that cellular environment might influence whether an individual mutation acts as loss- or gain-of-function or neutrally. The results also suggest that some mutations not only impair the respective subdomain but also the overall conformation of Pax6.

Materials and Methods

Oligonucleotides were purchased from Invitrogen (Frederick, MD), and mouse monoclonal antibody against Pax6 from Developmental Biology Hybridoma Bank (Iowa City, IA). 293T, an adenovirus-transformed human embryonic kidney epithelial cell line, was obtained from ATCC (Manassas, VA); mouse P19, undifferentiated embryonal carcinoma cells, were provided by Thomas Harris (Department of Molecular Genetics, Albert Einstein College of Medicine); CHO-K1, a Chinese hamster ovary cell line, was provided by Jonathan Backer (Department of Molecular Pharmacology, Albert Einstein College of Medicine); and N/ N1003A, a rabbit nontransformed lens epithelial cell line, was provided by John Reddan (Oakland Eye Research Institute, Rochester, MI).

Expression Plasmids and Site-Directed Mutagenesis of DNA

Expression plasmids for *PAX6* and *PAX6*(5a), and their mutants (G18W, R26G, A33P, S43P, G64V, I87R, V126D, R128C, R317X, and S353X) were made in the CMV-based vector, pKW10^{29,30} using a mutagenesis kit (QuickChange; Stratagene, La Jolla, CA). The mutants and their phenotypes are shown schematically in Figure 1.

Electrophoretic Mobility Shift Assays

Three double-strand oligonucleotide (5'-3') probes corresponding to standard Pax6 binding sites were prepared (upper strands, area of Pax6 consensus binding site is italic): P6CON, TTCAGGAAAAATTTTCACGCTTGAGTTCACAGCTCGAGT; 5aCON, AAATCTGAACATGCTCAGTGAATGTTTCATTGACTCTCGAGGTC, and HDCON (P3 site) TCGAGGGCATCAGGATGCTAATCTGATTAGCATCCGATCGGG. Electrophoretic

mobility shift assays (EMSAs) with 1 ng of 5'-end-labeled probes were conducted as described earlier.³¹ The respective amounts of extracts used were normalized according to the Western immunoblot data.

Protein Expression and Western Blot Analysis

A panel of 22 Pax6 wild-type and mutated proteins, expressed in vitro in the TnT system driven by the SP6 promoter (Promega, Madison, WI) and in transiently cotransfected 293T, CHO-K1, and COP-8 cells, were analyzed to evaluate the expression levels and the integrity of the proteins. Nuclear extracts from cultured lens cells were used as the positive control. Protein concentrations were determined with a Coomassie Blue protein assay (Bio-Rad Laboratories, Hercules, CA), and 50 µg of protein was loaded in each lane of a 10% discontinuous SDS-PAGE gel (Bio-Rad). The protein was transferred to nitrocellulose and incubated with 1:2000 dilution of anti-Pax6 serum. Bound antibodies were detected with 1:2000 horseradish peroxidase (HRP)-linked antimouse IgG, as described earlier.¹²

Reporter Genes

Six, four, and four copies of the PAX6 PD, PD5a, and HD consensus binding sites, P6CON, 5aCON, and HDCON, respectively, were cloned 5' of the E4 TATA minimal promoter in pGL3 as described elsewhere.³⁰

Cell Cultures, Transfections, and Dual Luciferase Assays

All except for N/N1003A cells were grown in DMEM with 10% fetal bovine serum supplemented with 20 µg/mL gentamicin, in a 5% CO₂ incubator at 37°C. For N/N1003A, rabbit serum (Sigma-Aldrich, St. Louis, MO) was used. For P19 cells, the medium was supplemented with 2 mM L-glutamine. CHO-K1, 293T, P19, and N/N1003A cells were seeded at a density of 40% to 50% confluence in six-well plates the day before transfection. The medium was changed 2 to 3 hours before the cells were transfected by the calcium phosphate coprecipitation procedure. At 60% to 80% confluence, cells were transfected with 5 µg of firefly luciferase reporter DNA and different concentrations of expression vectors encoding PAX6/PAX6 mutants or PAX6(5a)/PAX6(5a) mutants, empty vector pKW10, or both. An internal control plasmid, pCMV *Renilla* luciferase, was included in all transfections. All the mutations were also tested in CHO-K1 cells cotransfected with the different plasmids, pSV40 and pTK *Renilla* luciferase (Promega), used for the normalization to control for possible indirect effects. The cells were kept in medium with 10% serum for 48 hours after transfection. Cells were passively lysed, and luciferase activity was measured at room temperature with a dual luciferase reporter assay kit (Promega). Firefly luciferase activities were normalized relative to *Renilla* luciferase activity. Each experiment was conducted in triplicate and repeated at least twice. The results shown in Figure 5 were calculated as ratios between the activity of mutated Pax6 protein (mutated PAX6 or mutated PAX6(5a)) divided by the activity of corresponding wild-type protein.

Results

Naturally Occurring Human Missense and Nonsense PAX6 Mutations and Their Binding to DNA

Functional studies of natural mutants of PAX6 with distinct developmental defects are useful in understanding the molecular mechanisms of PAX6 and other PAX proteins. We prepared a representative panel of 10 naturally occurring PAX6 and PAX6(5a) mutants (Fig. 1) using site-directed mutagenesis. The mutation G18W is located in the β-turn motif close to the N-terminal end of the PD, three mutations (R26G, A33P, and S43P) are located within the PAI subdomain, one mutation (G64V) is located in the linker between the PAI and RED subdomains, and three

mutations (I87R, V126D, and R128C) are located in the RED subdomain (Fig. 1B).²⁸ Two nonsense mutations (R317X and S353X) result in premature termination of the C-terminal activation domain of PAX6 (Fig. 1C). The selected mutations cause a broad spectrum of ocular defects (Table 1), and mostly affect amino acid residues that are highly conserved in PDs of other Pax proteins.²⁸ Three missense mutations (R26G, I87R, and R128C) had been characterized in functional and DNA-binding assays^{27,32,33} using the NIH 3T3, P19, B3 lens epithelial, or HeLa cell lines.

The DNA-binding properties of these PAX6 and PAX6(5a) mutants were tested using the “optimal” DNA-binding sites P6CON,³⁴ 5aCON,⁴ and HDCON³⁰ for recombinant PD, PD (5a), and HD proteins, respectively. These “optimal” binding sites were obtained with GST-fusion proteins representing individual DNA-binding subdomains using enrichment of binding sites from pools of random sequences.^{4,30,34} Optimal binding sites for full-length PAX6 and PAX6(5a) remain to be determined. P6CON is a bipartite binding site^{31,34} in which the 5'-portion is strongly recognized by the PAI subdomain, and the 3'-half is recognized weakly by the RED subdomain (Fig. 2). In contrast, 5aCON is recognized by RED subdomain,^{4,5} and HDCON is bound through the HD homodimer.³⁰ Natural Pax6 binding sites are usually recognized by a combination of PAI, RED, and HD for PAX6 or RED and HD for PAX6(5a).³¹ The P6CON binding site is closest to the Pax6-binding site in the guinea pig ζ -crystallin promoter.³⁵ The 5aCON binding site, as a tetramer for PD5a, was shown in mouse γ E- and γ F-crystallin promoters.^{5,36} Binding of Pax6 through both its HD and PD is important for the G1 element in the rat glucagon promoter.³⁷ A sequence of *ATTATGCTAAT* (the HD-binding nucleotides are italic), along with three PD-binding sites, is present in the Pax6-responsive region of the rat c-Maf promoter.³⁸ Thus, P6CON, 5aCON, and HDCON represent well the diversity of natural Pax6 DNA-binding sites.

The PAX6 series of proteins bound to P6CON with apparently different affinities (Fig. 3A). In contrast, the mutants in the RED domain (mutations I87R, V126D, and R128C) compared with mutants affected in the PAI and linker region bound weakly to the 5aCON sites (Fig. 3B). Similarly, the PAX6(5a) series of mutations, except G64V, I87R, and V126D, bound more to 5aCON sites compared with PAX6(5a) (Fig. 3C). In addition, all recombinant PAX6(5a) proteins failed to bind the probe P6CON (data not shown), in agreement with earlier reports.^{4,31} Finally, none of the mutations tested impaired the DNA-binding properties of the mutated Pax6 protein tested with the HDCON sites (Figs. 3D, 3E) although it appeared that a specific group of mutants (G18W, R26G, A33P, and S43P) formed dimers (Figs. 3E, lanes 3 to 6) rather than a monomer generated by PAX6(5a) (lane 2) on the HDCON oligonucleotide. A 50-M excess of identical cold oligonucleotides competed with the specific complexes (data not shown). Western immunoblot experiments showed comparable expression of all proteins (Fig. 3F).

Pleiotropic Effects of Naturally Occurring Missense Mutants in Pax6

To assess functional properties of both PAX6 and PAX6(5a) and their mutants in different cellular environments, we conducted cotransfections in CHO-K1, 293T, and P19 cell lines, as described in the Methods section. These cell lines do not express endogenous Pax6 proteins.³⁹ In addition, we used the lens epithelial cell line N/N1003A, which expresses endogenous Pax6 proteins.^{29,35} To generate diverse reporter plasmids, we used six, four, and four copies of PAX6 PD, PD5a, and HD consensus binding sites cloned 5' of the E4 TATA region,^{4,30,34} as diagrammatically shown in Figure 4A. Initial experiments were performed to generate PAX6 and PAX6(5a) dose-dependent activation curves,³⁰ to identify the optimal range of concentrations of the effector cDNAs. The data for CHO-K1, 293T, and P19 cell lines are shown in Figure 4. Control experiments with the parental vector pKW10 and combinations of pKW10/Pax6 were conducted in parallel, to assure that the cytomegalovirus (CMV) promoter/

enhancer and Pax6-driven promoters did not compete with each other using the concentrations tested. From these data, we decided to study PAX6 at 200 ng or PAX6(5a) at 50 ng amounts per transcriptional activation assay.

The results of cotransfections of PAX6 and PAX6(5a) panels using P6CON, 5aCON, and HDCON reporters in CHO-K1, 293T, P19, and N/N1003A cell lines are shown in Fig. 5. Relative transactivation of these reporters by wild-type PAX6 or PAX6(5a) is shown in Figure 5A. In the absence of endogenous Pax6 proteins, PAX6 activated these reporters in the range between 2.7 and 17.0 in P19, CHO-K1, and 293T cells. Similarly, the highest level of PAX6 (5a) activation was 7.3-fold with 5aCON-reporter and in P19 cells. Next, individual mutants of PAX6 or PAX6(5a) were tested, and the data were expressed as relative ratios between the activation by Pax6 mutants and its corresponding wild-type counterpart (see Fig. 5B). For example, the G18W mutant of PAX6 tested with P6CON in P19 cells gave a change in expression of 0.2; thus, the reporter was still activated by a factor of 3.4. Similarly, the R26G mutant of PAX6(5a) tested with 5aCON in P19 cells gave a change of 10.3-fold. Hence, the reporter was activated by a factor of 75.2-fold. The data are analyzed from the contribution of cellular environment, type of mutations and their physical location, and specific Pax6 DNA-binding mechanism. Because N/N1003A cells differed from other cell lines by endogenous Pax6 expression, their results are presented separately (see below). Generally, the data (Fig. 5) show that the cellular environment of Pax6 plays an important role in gene activation, as identical mutations display different outcomes in different cells. This finding is consistent with in vivo observations that only specific ocular cells and tissues expressing Pax6 are affected in Pax6 heterozygotes.^{10,13-22,26}

Structural and functional analyses between the position of individual mutations and their effect on transcriptional activation resulted in both correlative and sometimes unexpected properties (Fig. 5B), as described in detail later. The G18W mutation in PAX6 yielded reduced activation that was independent of the Pax6 DNA-binding mechanism. In contrast, this mutation in PAX6 (5a) resulted in a moderate activation up to twofold, in no change, or in moderate reduction of activity (down to 0.5-0.7-fold) depending on the cell line (Fig. 5B).

Three mutations in the PAI subdomain, studied in PAX6, yielded either a reduction or no change in activity, except for R26G activating on 5aCON. Each PAX6 G18W, R26G, A33P, and S43P could bind in vitro P6CON (Fig. 3A, lanes 3-6). In contrast, these mutations in PAX6 (5a) had variable properties that were dependent on the Pax6 DNA-binding mechanism. No potentially significant changes (i.e., those changes lying between 0.7-1.3-fold) were observed if P6CON binding sites were used, in agreement with the prediction that the P6CON sequence is not sufficient for PAX6(5a) binding (Fig. 2).¹² All three PAI mutations in PAX6(5a) generated superactivation (i.e., activation between 1.7-13.4-fold higher than the activation by wild type PAX6(5a)) from 5aCON templates.

A linker mutation, G64V, located close to the PAI subdomain, exhibited a pleiotropic impact (Fig. 5B). In PAX6, it resembled other PAI mutations. However, in PAX6(5a) this mutation caused a reduction in the activity (e.g., with 5aCON-driven reporters in P19 and 293T cells) or an increase (e.g., with 5aCON-con driven reporters in CHO-K1 cells).

Three mutations, I87R, V126D, and R128C, located in the RED subdomain, affected PAX6- and PAX6(5a)-mediated transactivation with apparent dependence on the DNA-binding mechanism and cellular context (Fig. 5B). In PAX6, these mutations enhanced transactivation of P6CON-driven reporters in P19 but not in 293T cells, consistent with their ability to bind P6CON (Fig. 3B, lanes 8-10). In CHO-K1 cells, these mutations yielded both reduction (I87R mutation) and hyperactivation (V126D and R128C mutations). These mutants bound in vitro P6CON with higher affinity than PAX6 (Fig. 3B, compare lane 2 with lanes 9 and 10). This

behavior was consistent with earlier data showing that P6CON contains an optimal sequence for PAI recognition, but not for efficient binding of the RED subdomain (Fig. 2).^{4,5,31} Thus, mutations in the RED subdomain, depending on the specific cellular context, can either act as gain- or loss-of-function mutations using the P6CON-binding mechanism. PAX6(5a) activated transcription (by 2.9-fold) from P6CON in CHO-K1 cells, but not in P19 and 293T cells. The data again show that an identical PAX6(5a) mutation can act as a loss-of-function mutation (e.g., V126D in P19 and 293T cells), or gain-of-function mutation (in CHO-K1 cells).

Both nonsense mutations (R317X and S353X) in PAX6 yielded reduced transactivation levels using all three DNA-binding mechanisms (Fig. 5B). This is in agreement with the idea that any C-terminal deletion of PAX6 negatively affects its capacity to activate transcription.^{14, 16,40,41} However; in PAX6(5a), this assumption is not always true. Most notably, S353X either does not have any effect when tested with a P6CON-driven promoter (i.e., in P19 and 293T cells), or it can promote transcription in CHO-K1 cells twofold.

Although CHO-K1, 293T, and P19 cell lines are best suited to study the function of individual Pax6 mutants, it was of interest to examine these mutations in the cellular environment where Pax6 is normally expressed—in this case, in lens epithelial cells N/N1003A.^{29,35} Thus, we conducted a similar set of transfection experiments, and the results are summarized in Figure 5. In contrast to properties of mutants I87R, V126D, and R128C in P19 and CHO-K1 cells, no superactivation was found for the PAX6 series. Twenty of 24 tests performed with eight missense mutations caused reduced activity. However, mutations in PAX6(5a) tested in N/N1003A cells yielded a significant proportion of superactivations (9/24 tests), and only four reactions resulted in reduced activities. Finally, both nonsense mutations, R317X and S353X, tested in N/N1003A lens cells behaved according to patterns found in non-lens cells described earlier.

Discussion

The goal of the present study was to determine the functional properties of 10 representative human PAX6 mutants, and to analyze the structural and functional relationship among them. Understanding of functional properties of Pax6 proteins is important for elucidation of the molecular mechanism of PAX6 as a transcription factor, for a better understanding of the role of Pax6 during development, and for understanding of natural mutations in other PAX genes (e.g., PAX2 and PAX3).⁴²

Structure and Function of Human PAX6 Missense Mutants

The present data show that mutations in Pax6 tested individually show a spectrum of responses: identical mutations in PAX6 and PAX6(5a) could reduce, enhance, or leave transcription unchanged, depending on a specific cellular environment (Fig. 5). These differences did not originate from different stabilities of the expressed proteins, as the mutants were expressed intact (data not shown) and in agreement with earlier studies addressing this possibility.^{16, 19,32,40}

Our study provides surprising answers to predictions based on the crystal structure of Pax6 PD bound to DNA.²⁸ Analysis of the PAI helix-turn-helix and its N-terminal β -turn region revealed critical contacts between glycine 18 and arginine 26 and DNA and suggested that proline substitutions for arginine 33 and serine 43 would be disruptive to the PAI folding. Our functional data combined with DNA-binding properties of Pax6 mutants showed that individual mutations affecting the amino acids at positions 18, 26, 33, and 43 did not abrogate binding of either PAX6 or PAX6(5a) to DNA, and yet, they caused mostly reduced transcriptional activations. The reductions were more profound in 293T cells, but were weak in CHO-K1 cells. We propose that misfolding of PAI caused by substitutions A33P and S43P

may affect the Pax6 conformation and, consequently, its protein-protein interactions with the transcriptional machinery. In contrast, RED contacts with DNA are still uncertain,²⁸ as the optimal binding site for PD(5a) is different from the optimal binding site for PD.^{5,30,31,34} Thus, in the tentative model of RED, arginine 128 makes direct contact with DNA, and isoleucine 87 and valine 126 constitute a hydrophobic core of the RED subdomain.²⁸ The functional variability of mutants affecting residues 87, 126, and 128, using the 5aCON optimal and P6CON suboptimal site, implies that interactions of RED with DNA remain to be established. Mutation R128C actually enhanced binding of the corresponding fulllength PAX6 to P6CON (Fig. 3A, lane 10), although reduced binding of an isolated mutated PD (R128C) was observed earlier.²⁷

Role of Cellular Environment

Earlier functional studies of human Pax6 mutations were limited to studies of a small number of mutations, and typically used a single reporter plasmid.^{16,27,32,33,43} In addition, the cell lines were variably chosen and did not allow direct comparisons between experiments. We used four commonly available cell lines to standardize the assays. The data clearly show that different cellular environments provided by cell lines of different origin affect the properties of most of the mutants tested. Although we do not know the precise molecular mechanism, the present data suggest several possibilities. We show that missense mutations affecting the N-terminal region of the Pax6 PD (i.e., G18W, R26G, A33P, S43P, and G64V) mostly activated transcription in the range of 2- to 13-fold from 5aCON-driven reporter plasmids, though the PD5a was excluded from binding to DNA.^{4,5} It is likely that these missense mutations induce conformational changes in the entire Pax6 proteins and not only in the respective structural subdomain. A direct indication of this possibility is the variation in the mobility of both the PAX6/5aCON (see Fig. 3B) and PAX6(5a)/5aCON complexes (see Fig. 3C) caused by different conformation of the protein and/or bending of the DNA when the Pax6-DNA complex forms. Consequently, Pax6-mutated proteins may differently interact with tissue-restricted proteins of the transcriptional machineries resulting in the observed variabilities. This characteristic of missense mutations in Pax6 is consistent with the in vivo observations that these mutations result in moderate ocular phenotypes compared with the classic aniridia due to the loss of one PAX6 allele. A specific missense mutation may display its detrimental effect in only one or a few sensitive tissues (e.g., G18W and R26G may affect only the lens and cornea).^{15,17}

In conclusion, the present study shows that missense PAX6 mutations should be tested with multiple functional assays and that these mutations are likely to be useful to map protein-protein interactions between Pax6 proteins and other protein components of the transcriptional machinery.

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Figure 1.

Structure of PAX6 and PAX6(5a) and position of human mutations. (A) Schematic representation of PAX6 (amino acid residues 1-422); PAX6(5a) (amino acid residues 1-436); PAX6 PD, HD (yellow); and transcriptional activation domain (P/S/T, green); and subdivision of PD into the PAI (blue) and RED (red) subdomains. Oligopeptide of 14 amino acid residues (light blue) encoded by exon 5a disrupts DNA-binding property of the PD. (B) Schematic representation of secondary structures (α -helices and β -turns) in PD. The DNA-recognition structures are β -turn motifs, β 1 and β 2, recognizing the minor groove of DNA, and two α -helices, α 3 and α 6, recognizing the major groove of DNA. Mutations are shown in circles, and the major characteristics of the human phenotype of specific mutations are displayed. (C) Schematic representation of two truncated PAX6 proteins encoded by R317X and S353X. Mutants of PAX6(5a) have a similar structure (not shown). The references for mutations are: G18W,¹⁷ R26G,¹⁵ A33P, S43P, G64V and V126D,¹⁸ I87R,³⁷ R128C,²⁷ R317X,¹⁸ and S353X.¹⁴ For additional information, see Table 1. AN, Aniridia; pAN, partial aniridia; CAT, cataract; FOV, foveal hypoplasia; and PET, Peters' anomaly.

Figure 2.

Three distinct DNA-binding mechanisms of Pax6 proteins. The models were deduced using recombinant PD, PD(5a), and HD Pax6 proteins.^{4,5,30,34} The PD-binding site is a bipartite site. Its 5'-half dominantly and strongly binds the PAX6 PAI subdomain. In contrast, it does not bind the alternatively spliced PD(5a) PAI subdomain.^{4,31} Although the 3' half makes contacts with DNA in the crystal structure of the Pax6 PD with P6CON,²⁸ it cannot bind the isolated PD(5a)^{4,31} and is considered to be a weak binding sequence. XXX: Areas of strong (i.e., optimal) binding; vertical bars: area of weaker binding.

Figure 3.

DNA-binding properties of PAX6 and PAX6(5a) proteins. EMSAs with wild-type and (A) mutated PAX6 series with P6CON, (B) mutated PAX6 proteins with 5aCON, (C) mutated PAX6(5a) series with 5aCON, (D) mutated PAX6 proteins with HDCON, and (E) mutated PAX6(5a) series with HDCON. Solid arrow: Specific complexes in (A) to (E) formed by full-length proteins (lanes 2-10). Open arrows: truncated complexes (lanes 11 and 12). (*) Presumptive dimers (see Refs. 4,5,31) formed by truncated Pax6 proteins (lanes 11 and 12). (E, open, wide arrow) Possible Pax6 dimers (lanes 3-6). (F) Western blot of PAX6 and PAX6(5a) and their missense mutants, to demonstrate comparable level of expression of proteins used in EMSAs.

Figure 4.

Pax6 dosage-dependent activation of transcription in three cell lines. **(A)** Schematic diagram of P6CON-, 5aCON-, and HDCON-driven promoters combined with the E4 TATA element. **(B)** Concentration dependence of the transactivation function of PAX6 in the P19, CHO-K1, and 293T cell lines. **(C)** Concentration dependence of the transactivation function of PAX6 (5a) in the P19, CHO-K1, and 293T cell lines. The results were calculated as ratios of light units obtained in the presence of given amount of PAX6 or PAX6(5a) cDNA divided by the value obtained in the presence of the corresponding amount of empty vector, pKW10.

Figure 5.

A summary of relative transactivation of 10 PAX6 and PAX6(5a) mutants compared with their wild-type counterparts. (A) Relative activation by PAX6 and PAX6(5a) compared with the absence of Pax6. (B) Relative activation of PAX6 or PAX6(5a) mutants compared with their respective wild-type counterparts. Changes less than 0.5-fold are in *purple*, between 0.5- and 0.8-fold in *light purple*, between 0.8- and 1.2-fold in *light orange*, between 1.2- and 1.5-fold in *orange*, and more than 1.5-fold in *dark orange*. The location of the mutation is shown in Figure 1. The position of mutation is indicated by the corresponding subdomain: β-linker; the PAI subdomain, PAI; the linker between PAI and RED subdomain, L; the RED subdomain, RED; and the activation domain, AD. The experiments were performed either with 200 ng of PAX6 or pKW10; or 50 ng of PAX6(5a) or pKW10 expression plasmid, respectively. The

number of experiments used for the calculation was six or nine. The relative activation by wild-type PAX6 and PAX6(5a) are shown for comparison.

Table 1
Phenotypes and Functional Data of the Representative Human Mutants in *PAX6*

Mutation*	Phenotypes	Functional Data
Missense		
G18W	Congenital cataracts, Peters' anomaly, secondary glaucoma ¹⁷	
R26G	Peters' anomaly ¹⁵	Tested in P19 cells, ²⁷ NIH 3T3 and HLE B3 lens cells ^{32,33}
A33P	Congenital cataracts, partial aniridia ¹⁸	
S43P	Aniridia, nystagmus, cataracts, microcomeae, peripheral corneal vascularization ¹⁸	
G64V	Congenital cataracts, foveal hypoplasia, nystagmus ¹⁸	
I87R	Aniridia ³⁵	Tested in NIH 3T3 and HLE B3 lens cells ^{32,33}
V126D	"Atypical aniridia," optic nerve hypoplasia, macular hypoplasia, mild limbal corneal dystrophy ¹⁸	
R128C	Foveal hypoplasia ²⁷	Tested in P19 cells ²⁷
Nonsense		
R317X	Aniridia ¹³	
S353X	Congenital and bilateral cataracts ¹⁴	Tested in NIH 3T3 and HLEB3 lens cells ¹⁶

* Eight missense mutations were selected from 47 missense mutations, and 2 nonsense mutations were selected from 34 mutations summarized in <http://pax6.hgu.mrc.ac.uk/>, as of July 4, 2003.