Truncation Mutations in the Transactivation Region of PAX6 Result in Dominant-Negative Mutants*

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PAX6 is a transcription factor with two DNA-binding domains (paired box and homeobox) and a proline-serine-threonine (PST)-rich transactivation domain. PAX6 regulates eye development in animals ranging from jellyfish to Drosophila to humans. Heterozygous mutations in the human PAX6 gene result in various phenotypes, including aniridia, Peter's anomaly, autosomal dominant keratitis, and familial foveal dysplasia. It is believed that the mutated allele of PAX6 produces an inactive protein and aniridia is caused due to genetic haploinsufficiency. However, several truncation mutations have been found to occur in the C-terminal half of PAX6 in patients with Aniridia resulting in mutant proteins that retain the DNA-binding domains but have lost most of the transactivation domain. It is not clear whether such mutants really behave as loss-of-function mutants as predicted by haploinsufficiency. Contrary to this theory, our data showed that these mutants are dominant-negative in transient transfection assays when they are coexpressed with wild-type PAX6. We found that the dominant-negative effects result from the enhanced DNA binding ability of these mutants. Kinetic studies of binding and dissociation revealed that various truncation mutants have 3-5-fold higher affinity to various DNA-binding sites when compared with the wild-type PAX6. These results provide a new insight into the role of mutant PAX6 in causing aniridia.

PAX6 is an evolutionarily conserved gene that regulates the development of the eye in animals ranging from jellyfish to Drosophila to humans (for review, see Ref. 1). The induction of ectopic compound eyes by overexpressing mouse and squid PAX6 (2, 3) clearly indicates that not only is the structure of PAX6 conserved but also its biochemical properties are conserved. Recent reports have shown that PAX6 is also involved in pancreas development (4, 5).

Like other members of the PAX family, PAX6 functions as a transcriptional activator. Structural analysis of PAX6 has identified two DNA-binding domains (a paired domain at the N terminus and a paired like homeodomain in the middle), a glycine-rich hinge region that links the two DNA-binding domains, and a proline-serine-threonine-rich (PST) transactivation domain at the C terminus. Our recent studies designed to characterize the transactivation domain revealed that the four exons, which constitute the PST domain, synergistically stimulate transcriptional activation and that the transactivation potential is not localized but distributed throughout the PST domain (6). The transcriptional activity of the PAX family recognizes their target genes via the DNA binding function of the paired domain (7, 8). Several PAX6 paired-domain binding sequences have already been identified (reviewed in Ref. 1). However, studies of Czerny and Busslinger (9) identifying the P3 site as the optional binding site for cooperative binding and transactivation by the PAX6 homeodomain, conservation of P3 in eye-specific promoters (10), and the presence and requirement of the P3 site in the rhodopsin expression in Drosophila (11) indicated that several genes could also be regulated through the homeodomain of PAX6.

Mutations in PAX6 genes are responsible for several naturally occurring mutant phenotypes including aniridia in humans (12-18), small-eye (Sey) in rodents (19-21), and eyeless in Drosophila (22). The human and murine PAX6 proteins have an identical amino acid sequence (23, 54). Aniridia and small-eye are similar eye phenotypes (19), with both being characterized by iris hypoplasia and cataracts.

Mutations in PAX6 result in semidominant phenotypes, i.e. heterozygous mutations cause aniridia in humans and small-eye in rodents, while homozygous mutations lead to severe brain abnormalities, microencephaly, and early postnatal death with no eyes and no nose in rodents (20, 24) and humans (25). In addition, mice with heterozygous mutations in PAX6 have lower levels of pancreatic hormones (4). A haploinsufficiency mechanism has been postulated for the heterozygous phenotype. According to this theory, the function of the protein product of the mutant allele is lost and the normal PAX6 protein produced by the wild-type allele does not reach the threshold level necessary for normal eye development (1).

The mutation spectrum of PAX6 observed in aniridia and small-eye includes large deletions as well as intragenic mutations (1, 26, 27, 41). Most intragenic mutations result in truncations of the PAX6 protein; only four of the detected mutants are due to missense mutations, one that affects the nuclear translocation signal (15, 17) and three that affect the paired DNA-binding domain (28-30). The loss of function in the mutant PAX6 proteins that have lost the DNA binding can be clearly explained. What is not clear is the role of truncated PAX6 proteins that arise due to certain mutations that occur at the C-terminal region of PAX6 and result in mutants that (i) lack all or part of the PST domain but retain both DNA-binding domains or (ii) lack all of the PST domain and part of the homeodomain but retain the paired-box DNA-binding domain (1, 26, 27).

In the study reported here, we demonstrate that these mutants are not simple loss-of-function mutants or unstable mutants. On the contrary, they have gained a stronger DNA-binding
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Materials and Methods

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 (31), was maintained in Eagle's minimal essential medium supplemented with 20% fetal calf serum.

Plasmid Constructs—pRC-CMV-PAX6 expression plasmids were constructed by using a polymerase chain reaction cloning strategy. In brief, specific regions of PAX6 were amplified by polymerase chain reaction using the DNA clone pH12 (21) as a template. Primers used to amplify specific regions of PAX6 are listed in Table I. All the C-terminal deletion constructs used a common 5′ primer containing a HindIII restriction site and a Kozak consensus sequence for translational initiation (PAX6(1–422)). The 3′ primers differed in sequence, but all contained an SphI restriction site and a stop codon. Endonuclease digestion with SphI and XhoI generated compatible cohesive ends. The HindIII-SphI-digested polymerase chain reaction products were ligated into the HindIII-XhoI restriction sites in the polylinker region of the expression vector pRC-CMV (Invitrogen). The construction of R26G and I87R mutants from transfected NIH 3T3 cells according to the method of Schreier et al. (32) as modified by Singh and Aggarwal (33). The cells were harvested from each 60-mm culture dish and lysed in 0.2 ml of lysis buffer (10 mM HEPES, pH 7.9, 10 mM KC1, 0.1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 mg/ml leupeptin, 2.0 mg/ml aprotonin, and 0.5 mg/ml benzamidine). The nuclear pellet was resuspended in 15 μl of extraction buffer (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2.0 mg/ml leupeptin, 2.0 mg/ml aprotonin, and 0.5 mg/ml benzamidine). The protein concentration of each nuclear extract was measured by the Bradford assay and adjusted to equal protein concentration after dilution with the nuclear extraction buffer.

Electrophoretic Mobility Shift Assays—Electrophoretic mobility shift assays were performed in a 20-μl binding reaction incubated at room temperature for 30 min in a binding buffer (25 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM dithiothreitol, 1% Nonidet P-40, 50% glycerol, and 150 mM NaCl) (33). Each binding reaction contained 0.25 to 1 μg of nuclear extracts, 1–3 μg of poly(dI-dC), and 16–100 fmol of probes labeled with 32P by T4 polynucleotide kinase. Protein-DNA complexes were separated from free probes on a 4.5% or 6% native polyacrylamide gel using a buffer containing 50 mM Tris, 200 mM glycine, pH 8.5, and 1 mM EDTA. The gel was dried and then analyzed by a PhosphorImager (Molecular Dynamics) using Image Quant 3.3 software (Molecular Dynamics, Sunnyvale, CA). For supershift assays, the binding reaction mixture was incubated with the polyclonal antibody against PAX6 at room temperature for 30 min before loading onto the gel.

Western Blotting Assay—Crude nuclear extracts prepared from transfected NIH 3T3 or HLEB3 cells were resolved by 10% SDS-PAGE, 3 electrotransferred to a nitrocellulose membrane, and then analyzed for PAX6 expression by hybridization with polyclonal antibodies raised against PAX6(1–422) or against the paired domain or linker region of PAX6 (34). The protein band was detected by enhanced chemiluminescence using a horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (ECL, Amersham). The bands on the Western blots were quantitated using Personal Densitometer Scan v1.30 and Image Quant v3.3 software (Molecular Dynamics).

Results

Dominant-Negative Effects of Mutant PAX6 with Truncated PST Domains—Haploinsufficiency diseases such as aniridia and small-eye usually result from heterozygous mutations in which a mutant null allele coexists with a wild-type allele. In some cases, competition between the protein products of the mutant allele and the wild-type allele may result in phenotypic variability. PAX6 strongly activates transcription of the CD19-luc reporter, whereas most mutant PAX6 proteins with truncated PST domains have less than 30% of the wild-type activity (Figs. 2A and 6). Since such mutant PAX6 proteins retain the

The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; O/E BP, cAMP response element-binding protein; luc, luciferase; aa, amino acid(s).
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**FIG. 1.** Schematic diagram of wild-type and mutant PAX6 expression constructs. PAX6 cDNA and its mutant derivatives were inserted into the parental expression vector pRc-CMV. Schematic diagram of the full-length human PAX6(1–422) protein is shown at the top. The numbers above the diagram refer to amino acid number. The numbers inside the parentheses in the name of each mutant construct correspond to the N-terminal and C-terminal amino acid residues. **PD**, paired domain; **G**, glycine-rich domain; **HD**, homeodomain; **PST**, proline/serine/threonine-rich domain.

Paired domain, they may compete for target binding sites with the wild-type PAX6. To test this possibility in vivo, we cotransfected equal amounts of mutant and wild-type PAX6 expression plasmids (Fig. 1) with a CD19-luc reporter into NIH 3T3 cells, and then assessed the combined transactivation potential of mutant and wild-type PAX6 (Fig. 2, A and B). Interestingly, all mutant PAX6 proteins with truncations in the PST domain repressed the wild-type PAX6 activity (Fig. 2, A and B). However, the missense mutants PAX6(187R) and PAX6(R26G), which lose their DNA binding ability through the paired domain (30), did not repress the activity of wild-type PAX6. The repression by truncation mutants was much stronger than expected from a simple competition for DNA-binding sites of the wild-type and mutant PAX6 proteins and suggested that mutant PAX6 had dominant-negative effects.

To analyze the mechanism of the dominant-negative effects by mutant PAX6, we assessed the DNA binding ability of wild-type and mutant PAX6 in electrophoretic mobility shift assay (EMSA). We analyzed the DNA binding properties of the nuclear extracts prepared from NIH 3T3 cells co-transfected with mutant and wild-type PAX6 expression vectors. The wild-type PAX6 showed binding to the CD19–2(A-ins) probe (Fig. 2C, lane 2). In the presence of mutant PAX6, however, the wild-type PAX6 showed very weak DNA binding, whereas mutant PAX6 showed strong DNA binding (Fig. 2C, lanes 3–6). No intermediate complex was detected between the wild-type and mutant PAX6-DNA complexes, suggesting that both mutant and wild-type PAX6 bound DNA as monomers. Assuming that both the mutant and wild-type PAX6 proteins accumulated in nuclei at comparable levels, the simplest model for the dominant repression effect of the mutants would be that depletion of the PST domain resulted in increased DNA binding ability of mutant PAX6. The same nuclear extracts used in the EMSA were assessed for expression of wild-type and mutant PAX6 proteins by Western blotting using an immune serum raised against *Escherichia coli*-produced recombinant PAX6 (16–422). As expected, the wild-type PAX6 protein was detected at constant levels in extracts transfected with wild-type PAX6 alone or co-transfected with wild-type and mutant PAX6 (data not shown), indicating that the reduction in DNA binding of wild-type PAX6 was due to the competition for DNA-binding sites by the mutant PAX6 rather than a reduction in protein level. Although the antibody used cannot quantitatively detect each mutant PAX6 protein because it was raised against the PAX6 with an intact PST domain, the mutant proteins had accumulated in sufficient amounts to be detected by the polyclonal antibody and EMSA. It is also possible that the mutant proteins were expressed in much higher quantity than the wild type, and therefore showed higher binding. However, later experiments shown in Figs. 5, A–C, 7, and 8 revealed that this was not the reason for lower PAX6 binding.

**DNA Binding Specificity of Wild-type and Mutant PAX6 Proteins**—To examine the specificity of DNA binding of mutant and wild-type PAX6 in the nuclear extracts, we performed oligonucleotide competition and antibody supershift analyses in EMSA. Nuclear extracts were prepared from NIH 3T3 cells transfected with either a single plasmid of the wild-type PAX6, mutant PAX6(1–344), mutant PAX6(4V/G), or an equimolar mixture of wild-type PAX6 and mutant PAX6(1–344) plasmids. Since missense mutations have not been detected in aniridia patients, mutant PAX6(4V/G) was generated to find out the possible effect of missense mutations. This mutant did not show any significant difference in the transactivation potential nor on DNA binding (6). Protein expression of transfected mutant and wild-type PAX6 was detected by the polyclonal antibody against the paired domain (data not shown). The protein-DNA complexes formed by the binding of each nuclear extract to the CD19–2(A-ins) probe (Fig. 3A, lanes 3, 7, 11, and 15) were efficiently ablated by adding either a 100-fold excess of unlabeled CD19–2(A-ins) probe (Fig. 3A, lanes 4, 8, 12, and 16) or polyclonal antibody against PAX6 (aa 16–422) (Fig. 3A, lanes 5, 9, 13, and 17), but not by adding the nonspecific antibody (Fig. 3A, lanes 6, 10, 14, and 18). Addition of the anti-PAX6 antibody also resulted in supershifting of the protein-DNA complexes (Fig. 3A, lanes 5, 9, 13, and 17). Interestingly, the DNA-PAX6(1–344) complex was ablated less efficiently by the specific oligonucleotide and antibody than was the wild-type PAX6-DNA complex (Fig. 3A, lanes 16 and 17), consistent with our finding that the DNA binding ability of the mutant PAX6(1–344) was stronger than that of the wild-type PAX6. Thus, these experiments demonstrate that the CD19–2(A-ins) probe was recognized specifically by the wild-type and mutant PAX6 proteins expressed in transfected NIH 3T3 cells.

To confirm that the repression of transcription was specific to PAX6 and that it did not affect transcription mediated through some other transcription factor, we used a transactivation system in which the effector was C/EBP with a respective chloramphenicol acetyltransferase reporter (35). At the concentration of effector used in the experiment 3–4-fold transactivation was observed in the control sets and no difference was seen in the level of activation when PAX6 or the mutants were coexpressed (Fig. 3B) indicating that the repression effect was limited to PAX6.

**Modulation of Transcriptional Activation by Changes in the PAX6 Mutant to Wild-type Ratio**—To examine the importance of the ratio of mutant to wild-type PAX6 proteins for transcriptional activation, we co-transfected a constant amount of wild-type PAX6 expression plasmid and increasing amounts of mutant proteins PAX6(1–306), PAX6(1–317), and PAX6(1–344) expression plasmid with the CD19-luc reporter plasmid into NIH 3T3 cells and measured the combined transcriptional ac-
Fig. 2. Dominant-negative effects of mutant PAX6 with truncated PST domains. A, the repression of the transactivation activity of PAX6 by the truncation mutants. 0.5 μg of plasmid for wild-type or mutant proteins were used in each transfection. Total concentration of plasmid was maintained at 1.0 μg using empty expression vector. Mutant proteins were transfected alone or in the presence of PAX6. The relative luciferase activities of the reporter construct are shown as mean ± S.E. of three separate transfection experiments. Mutant PAX6 (I87R) and PAX6 (R26G) that lose the ability to bind DNA through the paired domain were used as controls. B, the repression of the transactivation activity of PAX6 by the truncation mutants. Concentration of expression plasmids used for transfection was the same as described in A. Mutant proteins were transfected in the presence of PAX6. The relative luciferase activities of the reporter construct are shown as mean ± S.E. of three separate transfection experiments. Transfections were performed in triplicate, from which two sets were used for luciferase assays and one set used for preparing nuclear extracts. C, competition for target DNA-binding sites of mutant and wild-type PAX6. EMSA analysis of CD19–2(A-ins) binding to wild-type and mutant PAX6 using nuclear extracts as described in the legend to B. Nuclear extract of HEB3 cells that contained endogenous PAX6 protein was used as a positive control for wild-type PAX6 binding. All lanes contained equal amounts of nuclear extract. Lanes 1–6 correspond to lanes 1–6 in B.
that neither PAX6 nor truncation mutants interfered with the transactivation activity, which was assigned a value of 100 (Fig. 4A, lane 2). In contrast, the mutant PAX6 protein (aa 1–344) alone produced only 20% of the wild-type activity (Fig. 4A, lane 7). Co-transfection of mutant and PAX6 wild-type plasmids resulted in attenuation of the wild-type PAX6 activity from 100 to 25% by increasing the ratio of mutant to wild-type PAX6 plasmid until an equimolar plasmid ratio was reached (Fig. 4A, lanes 3–6). Similar results were obtained using mutant PAX6 (aa 1–317) and PAX6 (aa 1–306) (data not shown). Thus, the repression phenomenon of the mutant PAX6, illustrated here in a dose-dependent manner, was consistent with our previous observations.

To explore the dominant-negative mechanism of the mutant PAX6, it was crucial to measure the relative ratio of mutant to wild-type PAX6 protein. These experiments were performed using three mutants and all three gave similar results; however, for clarity, we show only the data using mutant PAX6(aa 1–344). First the protein expression of mutant and wild-type PAX6 was verified (Fig. 4B). We took advantage of a polyclonal antibody directed against the paired domain of PAX6 (aa 1–127) (34). This polyclonal antibody allowed us to assess the ratio of mutant to wild-type PAX6 proteins in the same nuclear extract. To closely correlate the protein expression level and DNA binding ability with the transactivation potential of the mutant and wild-type PAX6 proteins, the co-transfection experiment was performed in triplicate; two experiments were used for luciferase assays, and one was used for preparing nuclear extracts. An increasing level of mutant PAX6 protein (aa 1–344) (Fig. 4B, lanes 3–7, corresponding to Fig. 4A, lanes 3–7) was detected in parallel co-transfection assays. Quantitation of each band in the autoradiogram corresponding to the wild-type and mutant PAX6 proteins was performed by densitometry scanning. The densitometry units of the wild-type PAX6 protein in each lane were assigned a value of 1. The densitometry score of mutant PAX6(1–344) was divided by that of the wild-type PAX6 to yield ratios of mutant to wild-type PAX6 protein ranging from 0.34 to 1.39, with an equimolar ratio detected in lane 6. Interestingly, the equimolar mixture of mutant and wild-type PAX6 proteins resulted in a 70% reduction in the transactivation potential of the wild-type PAX6 (Fig. 4A, lane 6). A low 0.44 ratio of mutant to wild-type PAX6 protein (Fig. 4B, lane 4) resulted in a 50% reduction in wild-type PAX6 activity (Fig. 4A, lane 4). Thus, the dominant-negative effect did not reflect a predominance of the mutant protein level but probably an increase in the DNA binding ability of the mutant PAX6. These data strongly support the notion that mutant PAX6(1–344) acts as a repressor of the wild-type PAX6 protein and that the ratio of mutant to wild-type PAX6 protein is instrumental in modulating the transcriptional activation potential of the wild-type PAX6.

To compare directly the DNA binding ability of the mutant and wild-type PAX6 proteins, we performed gel-shift analyses using the same nuclear extracts assessed by Western blotting analysis (Fig. 4C). In the absence of mutant PAX6, the wild-type PAX6 protein bound strongly to the CD19–2(A-ins) probe (Fig. 4C, lane 2). However, the DNA binding activity of the wild-type PAX6 protein was gradually attenuated by a gradual increase in the level of mutant PAX6 protein (aa 1–344) (Fig. 4C, lanes 3–7). Quantitation of the shifted bands in the autoradiogram corresponding to the mutant or wild-type PAX6 protein-DNA complexes was performed by densitometric scan-

**Fig. 3. DNA binding specificity of wild-type and mutant PAX6.**

A. EMSA analysis of CD19–2(A-ins) binding to wild-type PAX6 alone, PAX6(1–344) alone, PAX6(4V/G) alone, or a mixture of wild-type and mutant PAX6(1–344). Nuclear extracts were prepared from NIH 3T3 cells transfected with mutant or wild-type PAX6 expression plasmids under the same experimental conditions as used for luciferase assays. Wild-type and mutant PAX6 proteins in the nuclear extracts were detected with a polyclonal antibody against the paired domain of PAX6(aa 1–127) and quantitated by densitometry scanning. Free probe (lane 1), nuclear extracts from untransfected cells (lane 2), wild-type PAX6 (lanes 3–6), mutant PAX6 (lanes 7–14), or wild-type and mutant PAX6 in combination (lanes 15–18). Super Shift, antibody supershift. 100X Oligo, 100-fold molar excess of unlabeled CD19–2(A-ins) probe; Anti-PAX6, polyclonal antibody against PAX6(aa 16–422); N.S.-Ab, anti-TFID used as nonspecific antibody. B, PAX6 or PAX6 truncation mutants do not inhibit the C/EBP-mediated transactivation of a chloramphenicol acetyltransferase reporter gene. The histogram shows the chloramphenicol acetyltransferase activity of a reporter construct containing C/EBP response element in the presence (lanes 2–6) or absence (lane 1) of C/EBP expression vector. Lanes 3–6 also contained PAX6 or one of the PAX6 truncation mutants as marked. Data clearly showed that neither PAX6 nor truncation mutants interfered with the transactivation activity of C/EBP.
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Fig. 4. Modulation of transcriptional activation by truncation mutant (PAX6 aa1-344) to wild-type PAX6 ratio. A, mutant PAX6 (1–344) and wild-type PAX6 were coexpressed in NIH 3T3 cells by transient transfection with a constant amount of wild-type PAX6 expression plasmid, the DNA binding of the wild-type PAX6 protein was gradually reduced. This reduction was inversely correlated to the gradual increase in DNA binding of the mutant PAX6 protein (aa 1–344). When the binding activity was normalized to that of the wild-type PAX6 protein-DNA complex, the DNA binding activity of the mutant PAX6 (aa 1–344) increased from 0.28- to 4.9-fold of the wild-type PAX6 activity. An equimolar mixture of mutant to wild-type PAX6 proteins (Fig. 4B, lane 6) showed that the DNA binding activity of the mutant PAX6(1–344) protein was 2.7-fold greater than that of the wild-type PAX6 protein (Fig. 4C, lane 6).

Higher DNA Binding Abilities of Truncated PAX6—To further characterize the differences in DNA binding between wild-type and truncated (mutant) proteins we performed kinetic studies that included saturation analysis using increasing concentrations of the DNA-binding site, or increasing concentration of proteins, and dissociation analysis where increasing concentrations of unlabeled DNA was used to compare the dissociation between wild-type and mutant proteins. For these studies NIH 3T3 cells were transfected with various expression constructs, and nuclear extracts were prepared. Western blot analysis (data not shown) using polyclonal antibodies raised against the paired domain revealed that the recombinant proteins were intact. To normalize the level of different proteins in different sets of nuclear extracts, serial dilutions of proteins were loaded on the gel, and the bands obtained by Western blot were quantitated using a densitometer to establish the linearity and relative quantity. After adjustment of the quantity, the Western blots were repeated occasionally to verify the relative quantities of wild-type and mutant proteins (Fig. 5A, upper left panel).

Saturation analysis was performed as described by Underhill et al. (36). Nuclear extracts containing equimolar amounts of mutant or wild-type PAX6 were tested for their binding activity to increasing amounts of the CD19–2(A-ins) probe (Fig. 5A). Mutant PAX6 (aa 1–344) showed consistently stronger binding activities than wild-type PAX6 (Fig. 5A, lanes 1–8). The bands were quantitated using a PhosphorImager as described under "Materials and Methods" and plotted as bound versus total probe. Both proteins achieved maximal binding when the probe concentration was 5 pmol in the reaction, and binding did not increase further when the probe concentration was increased to 8 pmol (Fig. 5B). However, there was at least a 4-fold higher binding for the mutant protein. These data confirmed our earlier results that the mean ± S.E. of duplicate transfections. B, nuclear extracts were prepared as described in A. Proteins were resolved on 10% SDS-PAGE. Each lane contained equal amounts of nuclear extracts. Lanes 1–7 correspond to lanes 1–7 in A. Wild-type PAX6 was detected at constant levels, whereas mutant PAX6 was detected at increasing levels. Quantitation of each band on the autoradiogram was performed with a densitometer. The volume reports from the quantitation were listed at the bottom of each lane. The ratio of mutant to wild-type PAX6 protein was calculated by assigning the amount of wild-type PAX6 a value of 1. Equimolar amount of wild-type PAX6 and mutant PAX6(1–344) was detected in lane 6. Each lane contained equal amounts of nuclear extract. Quantitation of the DNA-protein complex was performed by scanning the shifted band on the autoradiogram with a densitometer. The volume reports of each band are listed at the bottom of each lane. The ratio of DNA binding of mutant to wild-type PAX6 was calculated by assigning the wild-type activity a value of 1. In lane 6, where the mutant and wild-type PAX6 proteins were present in equimolar amounts, the DNA binding of the mutant is 2.7-fold higher than that of the wild-type PAX6.
mutant protein has higher affinity for the binding site used in the assay. To further elucidate the difference in binding, the amount of the mutant protein was kept constant while the amount of wild-type protein was increased up to 5-fold (Fig. 5A, lanes 9–11). As expected, close to equal binding for the mutant and wild-type proteins was achieved when the ratio was increased to 1:5 in favor of the wild-type protein. Results of a similar experiment using the mutants PAX6(1–317) and PAX6(1–353) (Fig. 5C and data not shown) were similar. The binding of the mutant proteins was almost 4-fold higher than that of the wild type.

In an effort to further compare the difference in affinity between wild-type and mutant proteins, we used increasing concentrations of wild-type and truncated (mutant) proteins individually with a constant amount of the CD19–2(A-ins) probe. The results of these experiments are displayed in the graphic shown in Fig. 6. The fold difference in $K_D$ value between wild-type and truncated (mutant) PAX6 proteins, since PAX6 binds DNA as monomer through its paired domain, we calculated the fold difference in $K_D$ values using the following formula: $D$ is DNA, $P$ is wild-type protein, and $P_m$ is mutant protein: $K_{Pm}/K_{PM} = ([P][D]/[PD]) \times ([P_m][D]/[P_mD])$. At any point in the log phase of reaction where amount of probe and protein used were same: $K_{Pm}/K_{PM} = ([P][D]/[P_mD])$. Increasing concentrations of wild-type and mutant proteins were individually incubated at the indicated concentrations with the $^{32}$P-labeled probe as described under “Materials and Methods.” The protein-DNA complexes were separated from free DNA by gel electrophoresis and the amount of free DNA and protein-DNA complexes were quantitated using a PhosphorImager and Image Quant program as described under “Materials and Methods.” The data shown is mean ± S.E. of three separate experiments.

The mutant protein has higher affinity for the binding site used in the assay. To further elucidate the difference in binding, the amount of the mutant protein was kept constant while the amount of wild-type protein was increased up to 5-fold (Fig. 5A, lanes 9–11). As expected, close to equal binding for the mutant and wild-type proteins was achieved when the ratio was increased to 1:5 in favor of the wild-type protein. Results of a similar experiment using the mutants PAX6(1–317) and PAX6(1–353) (Fig. 5C and data not shown) were similar. The binding of the mutant proteins was almost 4-fold higher than that of the wild type.

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The mutant and wild-type PAX6 were compared for their DNA binding affinities to a murine genomic binding site, which bound probe was plotted against the total probe. The data shown is mean ± S.E. of three separate experiments.
is located in the upstream regulatory region of the murine N-CAM gene (neural cell adhesion molecule) (38). As seen with CD19–2(A-ins), we found that mutant PAX6(1–344) and PAX6(1–317) bound the neural cell adhesion molecule site also with a stronger affinity than the wild-type PAX6 (data not shown), suggesting that mutant PAX6 was also able to dominantly bind at natural target binding sites.

Considering our results from these various experiments, we conclude that (i) truncations in the PST domain of PAX6 result in mutant PAX6 with decreased transactivation potential but increased affinity for DNA binding and (ii) these mutant PAX6 proteins can function as dominant repressors of the transactivation activity of wild-type PAX6 by competing for target DNA-binding sites.

*Dominant Repression Function with Homeodomain-binding Sites*—We discussed earlier that certain genes have P3 homeodomain-binding sites, therefore we tested the effect of these mutants on transcriptional activation through homeodomain-binding sites using a different reporter plasmid, P3-luc. P3-luc has one copy of the P3 homeodomain-binding site replacing the CD19–2(A-ins) sites in the upstream regulatory region of the CD19-luc reporter. Transcriptional activation of the P3-luc reporter by mutant and wild-type PAX6 was previously examined in transiently transfected NIH 3T3 cells, and the transcriptional activation pattern was similar to that of the CD19-luc reporter (6). Interestingly, all the truncation mutants used in the study repressed the wild-type PAX6 activity (Fig. 8). PAX6(aa 1–395) inhibited activity only 50%, but other mutants inhibited more than 70% of the activity. Experiments similar to that described in Fig. 5 were performed using the P3-luc reporter to study the dose response with the homeodomain-binding site. We again found that, an increasing concentration of mutant expression plasmids inhibited the activity of wild-type PAX6 in a dose-dependent manner (data not shown). It is possible that alterations in the structure of the PST domain may have increased the DNA binding affinity of the homeodomain also, giving those mutants that lost transcriptional activity a dominant-repression function. However, the DNA binding activity of mutant proteins through the homeodomain has not been tested.

*Modulation of Transcriptional Activation by Over Dosage of Wild-type PAX6*—The aniridia and small-eye phenotypes result from haploinsufficiency but are characterized by variable expressivity. Now, we have demonstrated here that mutant PAX6 with truncated PST domains lose their transactivation ability but gain a stronger DNA binding ability and that these mutants can dominantly repress the transcriptional activation potential of wild-type PAX6 at an equimolar ratio of mutant to wild-type PAX6. Theoretically, this may result in a more severe
phenotype. On the other hand, in circumstances where the wild-type PAX6 and the mutant PAX6 alleles are inherited from different parents, there is a possibility that the abundance of protein product of the wild-type allele will predominate over that of the mutant allele. This may override the dominant repression by mutant PAX6 as shown previously and result in a less severe phenotype. A transgenic mouse model of multiple copies of the PAX6 gene showed an overdosage phenotype of the eye is distinct from the small-eye phenotype (39), indicating that overdosage of the PAX6 gene also causes an abnormal phenotype in vivo.

We therefore tested the effect of unbalanced gene dosage of mutant to wild-type PAX6 with wild-type PAX6 in excess. Increasing amounts of the wild-type PAX6 plasmid and constant amounts of mutant plasmid were co-transfected with the CD19-luc reporter into NIH 3T3 cells and the transcriptional activation of the luciferase reporter was examined (Fig. 9A). As expected, the transcriptional activation level gradually increased as the ratio of wild-type to mutant PAX6 increased. Interestingly, the maximal level of transcriptional activation was reached at a submaximal (optimal) plasmid dosage, after which the level of transcriptional activation gradually declined as the amount of the wild-type PAX6 plasmid exceeded the optimal dosage. The basis for the decreasing transactivation level with overdosage of wild-type PAX6 plasmid is not clear, but it may be related to a self-squelching mechanism (9, 40) since the activity of the SV40 promoter, which drives the expression of the LacZ gene, was not affected (data not shown).

We verified the protein expression of the transfected plasmids by Western analysis of the nuclear extracts prepared from a triplicate transfection experiment (one experiment was used for preparing nuclear extracts and the other two for luciferase assays). As expected, the mutant PAX6(1–344) was detected at constant levels, whereas the wild-type PAX6 protein was detected at gradually increasing levels corresponding to the increase in plasmid dosage (Fig. 9B, lanes 1–6). Our data demonstrated that the transcriptional activation level of a target gene could be modulated by the relative ratio of mutant to wild-type PAX6 proteins and that excessive amounts of wild-type PAX6 protein caused a decrease in transcriptional activation.

Repression of Endogenous PAX6 Activity by Transfected Mutant PAX6—HEB3 cells derived from human lens epithelia (31) expressed high levels of endogenous wild-type PAX6 protein (data not shown). We therefore used HEB3 cells to test whether the mutant PAX6 could repress the endogenous wild-type PAX6 activity upon transfection. When the CD19-luc reporter plasmid was co-transfected with increasing amounts of mutant PAX6(1–306) plasmids into HEB3 cells and examined for the repression of endogenous PAX6 activity by transfected mutant PAX6(1–306). The luciferase activities are shown as mean ± S.E. of duplicate luciferase assays.

![Fig. 9. Modulation of transcriptional activation by overdosage of wild-type PAX6. A, increasing amounts of wild-type PAX6 expression plasmid and constant amount of mutant PAX6(1–344) plasmid were co-transfected into NIH 3T3 cells with the CD19-luc reporter and tested for the effect of overdosage of wild-type PAX6 on the expression of the reporter gene. Transfections were performed in triplicate, of which two sets were used for duplicate luciferase assays and one set for preparing nuclear extracts. The luciferase activities are shown as mean ± S.E. of duplicate transfection assays. B, protein expression of wild-type PAX6 and mutant PAX6(1–344) in transfected NIH 3T3 cells. Western analysis of nuclear extracts prepared from transfected NIH 3T3 cells was as described under “Materials and Methods.” Wild-type and mutant PAX6 proteins were detected by enhanced chemiluminescence using a polyclonal antibody against the paired domain of PAX6. Proteins were resolved on 10% SDS-PAGE. All lanes contained equal amounts of nuclear extract. Lanes 1–6 correspond to lanes 1–6 in A.](image-url)

![Fig. 10. Repression of endogenous wild-type PAX6 activity by transfected mutant PAX6(1–306) in HEB3 cells. Increasing amounts of mutant PAX6(1–306) plasmids were co-transfected with the CD19-luc reporter into HEB3 cells and examined for the repression of endogenous PAX6 activity by transfected mutant PAX6(1–306). The luciferase activities are shown as mean ± S.E. of duplicate luciferase assays.](image-url)
other two for luciferase assays. We analyzed the nuclear extracts for protein expression of the transfected mutant PAX6 and the endogenous PAX6, using a polyclonal antibody directed against the paired domain of PAX6. The endogenous PAX6 protein was detected at constant levels, while the exogenous mutant PAX6 protein was detected at increasing levels (data not shown). The repression of the endogenous wild-type PAX6 activity by transfected mutant PAX6 in the HE3B cells was not as dramatic as that in NIH 3T3 cells co-transfected with mutant and wild-type PAX6, probably because PAX6 was already present in high amounts before the mutant protein was expressed. Next, we tested the repression effect of another mutant PAX6 (1-344) in HE3B cells, using a different reporter plasmid, P3-luc. Interestingly, the transcriptional level of the P3-luc reporter stimulated by the endogenous PAX6 in HE3B cells was also reduced significantly as the transfected mutant plasmid dosage was gradually increased (data not shown). Transfection of wild-type PAX6 plasmid into the HE3B cells did not show any significant difference in transactivation activity (data not shown). Thus, it appears that mutant PAX6 with truncated PST domains was able to repress the wild-type PAX6 activity not only at target genes with paired domain-binding sites but also at target genes with homedomain-binding sites.

**DISCUSSION**

Aniridia is a congenital, bilateral panocular disorder that occurs at a rate of between 1 in 64,000 and 1 in 96,000 in the human population. The characteristic clinical manifestations of heterozygous aniridia are a complete or partial absence of the iris and iris hypoplasia. The ophthalmic complications associated with aniridia include poor vision, iris coloboma, glaucoma, cataracts, ectopia lentis, corneal opacification, optic nerve hypoplasia, and nystagmus (42).

The mutation spectra of aniridia and small-eye have revealed premature termination of the PAX6 protein in most cases. Some truncations occur at the beginning of the PAX6 protein, resulting in short peptides that are unlikely to have any function (17). This type of loss-of-function mutation is consistent with a haploinsufficiency mechanism for producing the aniridia phenotype, in which the normal PAX6 protein produced by a wild-type allele does not reach the threshold level necessary for normal eye development. Interestingly, two missense mutations, R26G (28) and I87R (30) in the paired box produced by a wild-type allele does not reach the threshold level necessary for normal eye development. These mutant proteins retain certain functions such as the DNA binding function and some of them retain a partial PST domain have residual transactivation activity (6, 9). So far, 13 mutations in PAX6 have been reported to result in truncations in the PST region (summarized on website: www.hgmp.mrc.ac.uk/Softdata/PAX6/home.htm). Four of them are simple nonsense mutations that truncate PAX6 at codons 240, 267, 317, and 353. The others are splicing mutations that result in truncation of the PST domain and its fusion to a nonsense peptide. Our biochemical findings demonstrated that the DNA binding functions of such mutants were not only retained but also enhanced, and that this enhanced DNA binding ability resulted in mutants with dominant-negative effects that competed for target DNA-binding sites with the wild-type PAX6 protein. Because PAX6 undergoes DNA-induced conformational changes (43), the smaller mutant protein may be able to fold differently and thus have an advantage over the wild-type PAX6 during binding.

It is clear that dominant-negative mutants are by themselves not fully functional; however, they can antagonize the activity of the wild-type protein in the heterozygous state. Two classes of dominant-negative mutations that produce such mutants have been described with respect to transcriptional regulatory proteins (44). The major group comprises proteins that can form dimers or multimers and whose activity depends on oligomerization. Thus, the presence in a multimer of a mutant subunit with an intact oligomerization sequence but altered DNA-binding domains will abrogate the DNA binding function of the entire multimer. The second class of dominant-negative mutants comprises monomeric proteins. The dominant-negative effects of these mutants result from competition for DNA-binding sites between mutant (repressor) and wild-type (activator) proteins. The PAX6 dominant-negative mutants fall into the second category. An example of the second class is the interferon activator IRF1 and its antagonist IRF2; IRF2 has an enhanced DNA binding activity and displaces IRF1 from the interferon promoter (45). The conversion of activators to repressors by removing the transactivation domains has been observed in other transcription factors (46).

PAX6 contains two DNA-binding domains and is able to activate transcription of target genes bearing binding sites for either domain. The dissociation of a paired-domain phenotype from a homeodomain phenotype has been observed in association with mutations in Caenorhabditis elegans pax6, in which mutations in the paired domain resulted in defects in head development (47) but mutations in the homeodomain resulted in a peripheral sense organ defect (48). The homeodomain phenotype has been linked to a paired-less isof orm of the C. elegans pax6 that lacked a paired domain (48). Interestingly, a paired-less isof orm of pax6 was also found in quail (34). It remains to be tested whether a paired-less isof orm of PAX6 is conserved in all vertebrate animals.

Several molecular mechanisms for dominant inheritance have been proposed, including haploinsufficiency, increased gene dosage, ectopic or temporally altered mRNA expression, increases in protein levels, interaction with toxic proteins, and dominant-negative mutations (49). PAX6 dosage appears to be critical for normal eye development. Indeed, subtle differences in phenotype may result from small variations in PAX6 levels. As we have shown here, the expression level of a PAX6 target gene can be modulated by the mutant to wild-type PAX6 ratio. However, in addition to the genotypic variations of mutant alleles, other factors that control phenotypic expressivity must also be considered. For instance, in a large family with familial aniridia mutation VMR-1, all affected members had the same mutation (an 11-base pair duplication that results in truncations within the paired domain) (17) but showed a widely variable phenotype ranging from complete loss of the iris to essentially an eye of normal appearance (50). This suggests that multiple PAX6 targets and restricted control of PAX6 expression are also critical aspects of PAX6 function. In fact, the 5' promoter region of PAX6 is composed of multiple positive and negative cis-regulatory elements (51), and PAX6 overexpression is associated with eye abnormalities that are distinct from the small-eye phenotype (39). A suggested autoregulation (52) can further add to complications involving restricted expression of PAX6. Recent studies using Drosophila have raised the possibility of other master regulators of eye development that may cooperatively function with PAX6 but at the same time...
time may have their expression under the control of PAX6 (for review, see Ref. 53).

Our study clearly demonstrated that truncation mutants of PAX6 function as dominant-negative in transient transfection assays. We also found that this dominant-negative effect was the result of enhanced DNA binding ability of these mutants. Since critical target genes of PAX6 are not known and other regulatory partners are not identified, it is difficult to assess the effect of these mutants. It is clear, however, that the cause of variability in the expression of aniridia is more complicated and may involve a multiplicity of target genes.

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