Missense Mutation in the Alternative Splice Region of the PAX6 Gene in Eye Anomalies

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Summary

The PAX6 gene is involved in ocular morphogenesis, and PAX6 mutations have been detected in various types of ocular anomalies, including aniridia, Peters anomaly, corneal dystrophy, congenital cataract, and foveal hypoplasia. The gene encodes a transcriptional regulator that recognizes target genes through its paired-type DNA-binding domain. The paired domain is composed of two distinct DNA-binding subdomains, the N-terminal subdomain (NTS) and the C-terminal subdomain (CTS), which bind respective consensus DNA sequences. The human PAX6 gene produces two alternative splice isoforms that have the distinct structure of the paired domain. The insertion, into the NTS, of 14 additional amino acids encoded by exon 5a abolishes the DNA-binding activity of the NTS and unmasks the DNA-binding ability of the CTS. Thus, exon 5a appears to function as a molecular switch that specifies target genes. We ascertained a novel missense mutation in four pedigrees with Peters anomaly, congenital cataract, Axenfeldt anomaly, and/or foveal hypoplasia, which, to our knowledge, is the first mutation identified in the splice-variant region. A T→A transition at the 20th nucleotide position of exon 5a results in a Val→Asp (GTC→GAC) substitution at the 7th codon of the alternative splice region. Functional analyses demonstrated that the V54D mutation slightly increased NTS binding and decreased CTS transactivation activity to almost half.

Introduction

The PAX6 gene, which encodes a transcription regulator that recognizes target genes through DNA binding of its paired domain, is involved in ocular morphogenesis (Treisman et al. 1991; Czerny et al. 1993; Gehring 1996). The gene was isolated, by positional cloning, as a candidate gene for aniridia (Ton et al. 1991), and numerous mutations have been detected in patients with aniridia. These mutations are summarized in The Human PAX6 Mutation Database. Since most of these mutations result in premature translational termination on one of the alleles, haploinsufficiency of the gene has been suggested to cause the aniridia phenotype (Fisher and Scambler 1994; Martha et al. 1994). In contrast, few missense mutations have been detected to date. Six mutations occur in the paired domain, one in the homeodomain, one in the linkage portion between the paired domain and the homeodomain, and one in the proline-serine-threonine transactivation domain. Each of these mutations generates a distinctive phenotype including aniridia; anterior-segment dysgenesis, which includes Peters anomaly and corneal dystrophy; and foveal hypoplasia (Hanson et al. 1994; Martha et al. 1994; Mirzayans et al. 1995; Azuma et al. 1996, 1998a, 1998b; Tang et al. 1997). The expression pattern of the PAX6 gene also indicates multiple functions of the gene. The gene is expressed in the developing CNS, as well as in ocular tissues—including tissues of the corneal epithelium, lens, and retina—that are derived from the ectoderm and neuroectoderm (Walther and Gruss 1991). Thus, PAX6 mutations, especially missense mutations, may cause numerous types of ocular anomalies, and the phenotype-genotype correlation must be carefully examined to elucidate the function of PAX6.

The PAX6 paired domain comprises two structurally distinct subdomains, the N-terminal subdomain (NTS) and the C-terminal subdomain (CTS), which bind respective consensus sequences (Epstein et al. 1994a, 1994b). The NTS region is well conserved among members of the PAX family of genes, whereas the CTS region is variable. Initial studies have shown that the NTS plays

a primary role in DNA binding (Xu et al. 1995). So far, most missense mutations detected in the PAX family of genes occur in the NTS, where DNA-binding ability may be altered (Balling et al. 1988; Baldwin et al. 1992; Tassabehji et al. 1992, 1993; Vogan et al. 1993). However, recent evidence strongly suggests that the CTS also has a distinct DNA-binding ability (Epstein et al. 1994*a*, 1994*b*). Three missense mutations recently identified in the CTS also suggest an essential role for the CTS in the formation of the eye (Chisholm et al. 1995; Azuma et al. 1996; Tang et al. 1997).

The PAX6 gene produces two isoforms by alternative splicing with or without exon 5a, and a variant form of exon 5a has 14 additional amino acid residues inserted into the NTS of the paired domain. Interestingly, the insertion not only abolishes NTS function but also enhances transactivation activity via the CTS (Epstein et al. 1994*b*; Yamaguchi et al. 1997). In contrast, in a case of isolated foveal hypoplasia, a missense mutation detected in the CTS (Azuma et al. 1996) abolished CTS function and caused hyperactivation of NTS transcription activity. On the basis of these lines of evidence, it has been proposed that the two subdomains negatively regulate each other (Yamaguchi et al. 1997). Exon 5a thus appears to function as a molecular switch that selects and specifies target genes (Epstein et al. 1994*b*; Yamaguchi et al. 1997). In the present study, we describe both a novel missense mutation in the splice-variant region and the DNA-binding activity of the mutated form.

Material and Methods

DNA Samples

The present study was conducted in accordance with the World Medical Association Declaration of Helsinki. Our examination of human subjects was conducted according to the program approved by the National Children's Hospital Experimental Review Board and was deemed exempt from human-subject regulations. We analyzed PAX6 mutations in 55 Japanese pedigrees with ocular anterior-segment anomalies, including Peters anomaly and Axenfeldt anomaly; 55 pedigrees with aniridia; 70 pedigrees with congenital cataract; 25 pedigrees with isolated foveal hypoplasia; and 140 pedigrees with optic-nerve anomaly. After informed consent was obtained from all patients, samples of blood from peripheral veins were collected into lithium-heparin tubes. Unaffected individuals who were immediate family members were also examined. Genomic DNA from isolated leukocytes was prepared by means of a standard phenol/chloroform procedure. DNA samples from normal controls were prepared as described elsewhere (Tadokoro et al. 1993).

PCR-SSCP Assay and Sequencing

PCR primers used for the amplification of 14 exons of PAX6 were synthesized according to the sequence reported by Glaser et al. (1992), by use of a DNA/RNA synthesizer (model 392; Applied Biosystems). The PCR conditions that we used (Tadokoro et al. 1993) were adjusted so that (1) the annealing temperature was 55°C for exons 5a and 13 and 60°C for the remainder of the exons, and (2) the Mg^{++} concentrations were 1.5 mM. SSCP analyses were done after the samples were radiolabeled with $\alpha[^{32}P]$-dATP, in the PCR reaction, by use of conventional-size 5% polyacrylamide gels under at least three different conditions for concentrations of glycerol and running buffer and running temperature. Nucleotide sequences were determined, either immediately or directly after cloning on pUC18, by use of a Sequenase Version 2 kit (Amersham) with PCR primers or universal primers in pUC18. Each sequence variation was confirmed in at least six independent clones.

PAX6 Expression Plasmids

Under the control of the cytomegalovirus enhancer and the chicken β-actin promoter, bacterial expression plasmids (pGEX-PrD WT and pGEX-PrD5a WT) have been used to produce a portion of the PAX6 paired domain, with or without exon 5a, and mammalian expression plasmids (pCMV-PAX6 WT and pCMV-PAX6-5a WT) have been used to produce the entire PAX6 coding region, with or without exon 5a (Yamaguchi et al. 1997). The V54D mutation (GTC→GAC) was introduced into a cDNA construct with exon 5a by use of PCR that incorporated a reverse primer, 5'-TTTCTC-GAGTTACTGTAATCTTGGCCAGTATTG-3', and a forward mutagenic primer, 5'-TTCTGCAGACCCATG-CAGATGCAAAA_GAC_CAAGTGCTG-3' (the mutated codon is underlined). The wild-type sequences pGEX-PrD5a WT and pCMV-PAX6-5a WT were replaced with the *Pst*I-*Nhe*I fragment derived from the PCR products, generating pGEX-PrD5a V54D and pCMV-PAX6-5a V54D. Mutated sequences were verified by sequencing in the final constructs.

Gel-Shift Assays

In *Escherichia coli* cells of strain DH5α, a portion of the PAX6 paired domain was produced as a fused form with glutathione S-transferase (GST), by use of pGEX-PrD WT and pGEX-PrD5a WT, and it was purified with a glutathione Sepharose column, according to the manufacturer's instructions (Amersham Pharmacia). Oligonucleotides containing the consensus binding sequences P6CON (5'-GGAATTCAGGAAAA-ATTTTCACGCTTGAGTTCACAGCTCGAGT-3') and 5aCON (5'-AAATCTGAACATGCTCAGTGAAT-
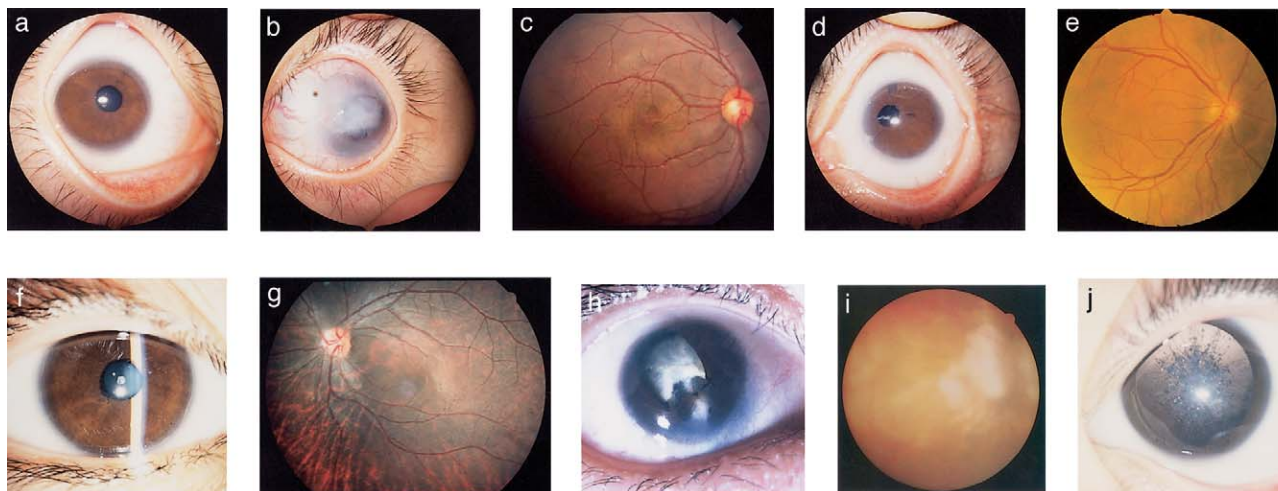
Figure 1   Photographs of the anterior segments (*a* and *b*) and the fundus of the right eye (*c*) of patient 1. In the left eye, the cornea is opacified, and the cornea and iris adhere at the central portion (*b*). In the right eye of patient 1, the corneoscleral limbus is obscure (*a*), and the foveal reflex is absent, with a residue of slight luteal pigment, and the blood vessels of the retina are not far from the presumed foveal region (*c*). Patient 1's mother had microphthalmos, cloudy corneoscleral limbus, aphakia after cataract surgery (*d*), and foveal hypoplasia (*e*). Photographs of the anterior segments (*f*) and the fundus (*g*) of patient 2 show an obscure corneoscleral limbus and iridocorneal adhesion (posterior embryotoxon) in the periphery. In the fundus of patient 2, the foveal reflex is absent, with a residue of slight luteal pigment, and the blood vessels of the retina are not far from the presumed foveal region. Patient 3 shows microcornea and Peters anomaly (*h*) and retinal degeneration (*i*). The right eye of patient 4 shows microcornea and congenital cataract (*j*).

GTTCATTGACTCTCGAGGTC-3′) were synthesized (Epstein et al. 1994*a*, 1994*b*). Gel-shift assays were performed as described elsewhere (Yamaguchi et al. 1997). In brief, an indicated amount of GST-PrD protein was incubated with the oligonucleotide in 10 ml reaction buffer containing 50 mM Tris (pH 7.9), 20% glycerol, 50 mM KCl, 0.1 mM EDTA, 5 mg BSA, and 1 mg poly dIdC for 30 min at 37°C and was then subjected to electrophoresis through a 4% native polyacrylamide gel, with 0.5 × Tris-borate EDTA used as running buffer.

Transfection and Luciferase Assays

Mouse embryonal carcinoma P19 cells were maintained in MEM medium (Gibco) supplemented with 10% fetal bovine serum at 37°C and a humidified atmosphere of 5% CO_2. Reporter plasmids P6CON-luc and 5aCON-luc, in which either six copies of P6CON or two copies of 5aCON were situated upstream of the adenovirus E4 minimal promoter, directed expression of the luciferase gene, as described elsewhere (Yamaguchi et al. 1997). A total of 5×10^4 P19 cells were plated on 60-mm dishes, cultured for 20 h, and transfected with a total of 10 μg DNA (5 μg of either P6CON-luc or 5aCON-luc, a various amount of pCMV-PAX6 or its derivatives, and an empty vector, pCAGGS) by use of a standard calcium phosphate method (Ausubel et al. 1995). Cells were harvested 42 h post transfection, and

luciferase activities were measured with cell lysates. The luciferase activity was expressed as fold activation, with the mean of three or five independent experiments, after normalization with protein concentrations.

Results

Clinical Reports of Patients with the PAX6 Missense Mutation

Patient 1, a 3-year-old girl, showed nystagmus, exotropia, corneal opacity with iridocorneal adhesion (Peters anomaly) in the left eye, and corneal opacity with sclerocornea and posterior embryotoxon in the right eye. The fundus of the left eye could not be seen because of a cloudy cornea, but the right eye showed foveal hypoplasia (fig. 1*a–c*). At 8 years of age, the patient's visual acuity was 0.6 in the right eye, and her left eye could detect hand movement only. Electroencephalography results were normal bilaterally, but echography findings showed microphthalmos of the left eye (eyeball size, 21 mm). Her 36-year-old mother, who had undergone surgery for bilateral congenital cataracts at the age of 14 years, had nystagmus, bilateral microphthalmos (eyeball size, 22 mm), and foveal hypoplasia (fig. 1*d* and *e*). The patient's 37-year-old father had no ocular abnormalities.

Patient 2, a 16-year-old girl, showed nystagmus, slight microcornea (corneal diameter, 10 mm), peripheral iridocorneal adhesion (Axenfeldt anomaly), and foveal hy-
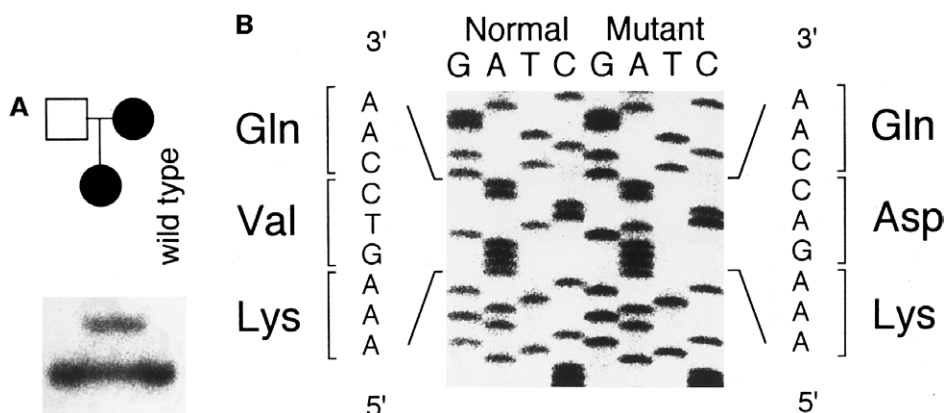
Figure 2 *A,* Band shift of SSCP, indicating that a heterozygous mutation was detected in the exon 5a region of patient 1 and her mother but that it was not seen in their unaffected family members or in >100 normal controls. *B,* Sequencing of the normal and mutant alleles, which identified, at the 20th nucleotide of exon 5a, a heterozygous T→A transversion resulting in a Val→Asp substitution in the protein. The same mutation was also identified in patients 2–4.

poplasia in both eyes (fig. 1*f* and *g*). Her visual acuity was 0.1 bilaterally. Her electroencephalography results were normal.

Patient 3, a 43-year-old woman, had undergone iridectomy in both eyes after congenital corneal opacity and cataract were diagnosed in childhood. She had bilateral microcornea (corneal diameter, 8.5 mm), central corneal opacity with iridocorneal adhesion (Peters anomaly), cataract, posterior staphyloma with high myopia, and nystagmus (fig. 1*h* and *i*). Her vision was 0.04 in the right eye and 0.01 in the left eye, and her electroencephalography results were subnormal bilaterally. Computed-tomography and radiology findings showed adhesion of cervical bones 1 and 2 plus a vascular anomaly in the brain stem.

Patient 4, a 3-mo-old female infant, had nystagmus and bilateral congenital cataract (fig. 1*j*) and underwent cataract surgery at 4 mo. Ophthalmoscopy and electroencephalography results were normal bilaterally; however, echography results showed microphthalmos (eyeball size, 20 mm). At 6 years of age, her visual acuity with contact lenses was 0.6 bilaterally.

Computed-tomography results showed brain abnormality in patient 3 only. All affected patients otherwise had normal growth and intelligence, and their karyotypes were normal.

Missense Mutation in the PAX6 Alternatively Spliced Region

Using genomic DNA, we amplified 14 exons by PCR and subjected the PCR products to SSCP analyses. An abnormal pattern indicating a heterozygous mutation was detected in the exon 5a region in patients 1–4 and in the mother of patient 1 but was not detected in the

unaffected family members or in >100 normal controls (fig. 2*A*). Sequencing analysis demonstrated that, in each of the affected patients, one allele had, at the 20th nucleotide position of exon 5a, a T→A transversion that resulted in a Val→Asp (GTC→GAC) substitution at the 7th codon of the 14-amino-acid stretch inserted into the NTS region by alternative splicing (fig. 2*B*). No other changes in nucleotide sequences were detected in PCR products from patients' genomic DNA. The mutation in patient 4 is sporadic, since neither of her parents had the mutation. In contrast, the origin of the mutation in other families could not be determined, since the unaffected family members of patients 2 and 3 were not available for examination. All four pedigrees are Japanese and originated in and live in a particular geographic area in and near Tokyo. However, it remains unknown whether the mutations found in patients 1, 2, and 3 are derived from a common ancestral origin.

Effect of the V54D Mutation on PAX6-5a DNA-Binding Activity

To analyze how the detected mutation affects the regulatory role of the PAX gene, we first examined the DNA-binding activity of the V54D mutant, by use of a gel-shift assay. Short peptides containing either the wild-type or the V54D-mutated paired domain, which were expressed in *E. coli* as a GST-fused form, were purified and then were incubated with $\alpha[^{32}P]$-labeled P6CON or 5aCON probe. In agreement with the results of previous studies, the paired domain containing 14 additional amino acid residues encoded by exon 5a (GST-PrD5a WT) barely bound to P6CON (fig. 3*A*, lanes 11–15). However, the similar peptide containing the mutation (GST-PrD5a V54D) slightly enhanced the binding to
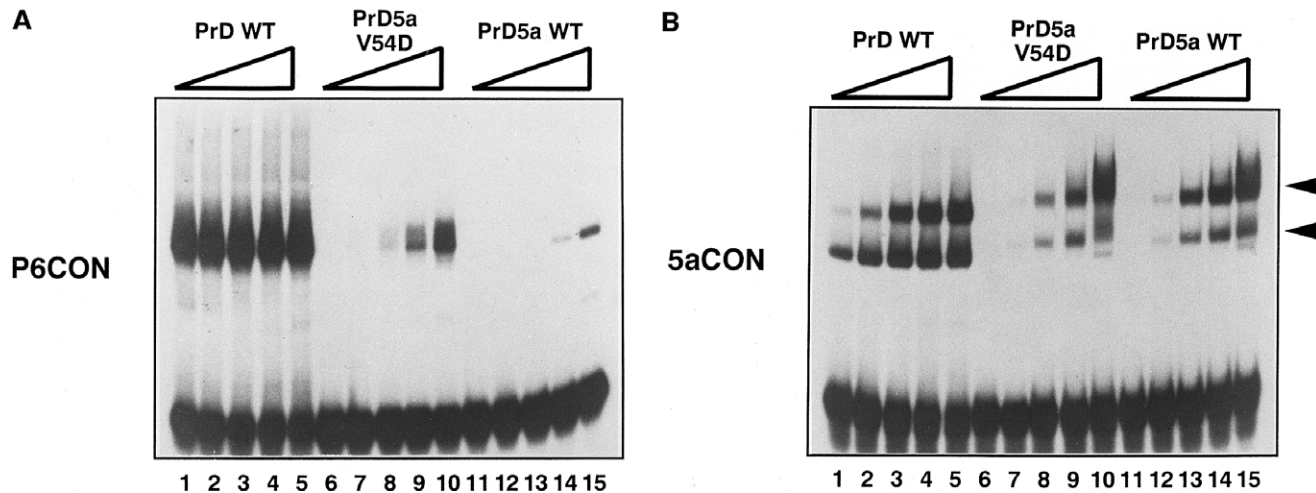
Figure 3   DNA-binding activity of the PAX6-5a V54D mutant. The PAX6 paired-domain wild type (PrD WT; lanes 1–5, at 3, 10, 30, 100, and 300 ng genomic DNA), the paired domain containing exon 5a (PrD5a WT; lanes 11–15), and the paired domain containing exon 5a with the V54D mutation (PrD5a V54D; lanes 6–10) were incubated with either an $\alpha[^{32}P]$-labeled P6CON probe (*A*) or 5aCON (*B*) probe. PrD5a WT shows barely detectable levels of P6CON binding, but the V54D mutation increases the level of P6CON binding. 5aCON binding is not affected by the mutation.

P6CON (fig. 3*A*, lanes 6–10), indicating that the V54D mutation is somehow likely to neutralize the negative effect of insertion of exon 5a. On the other hand, both peptides bound to 5aCON at almost similar levels, indicating that the mutation has no effect on the 5aCON binding (fig. 3*B*, lanes 6–15). Thus, in this binding assay, the V54D mutation seems to affect only the NTS structure.

Effect of the V54D Mutation on PAX6-5a Transcriptional Activation

We next examined the transactivation activity of the V54D mutation by use of a luciferase assay of transfected cells. Two reporter plasmids, P6CON-luc and 5aCON-luc, contained six copies of P6CON and two copies of 5aCON, respectively, which were located upstream of the adenovirus E4 minimal promoter, to direct expression of the luciferase gene (Yamaguchi et al. 1997). Increasing amounts (1–300 ng) of the expression plasmid for either PAX6 WT or the V54D mutant were cotransfected, with a constant amount (5 μg) of either P6CON-luc or 5aCON-luc, into P19 cells, and then the luciferase activities were measured. As shown in figure 4*A,* the V54D mutant, as well as PAX6-5a WT, failed to stimulate transcription from P6CON, whereas PAX6 without exon 5a stimulated transcription ∼20-fold. This result seems to be inconsistent with that seen on gel-shift assay. However, since P6CON binding by the V54D mutant is ≥100-fold weaker than that by PAX6 WT (fig. 4*A*), transactivation via P6CON by PAX6-5a V54D may be too weak to be detected by this assay. On the other

hand, transactivation via 5aCON was primarily observed with PAX6 with exon 5a, as demonstrated previously, and the V54D mutation reduced the transactivation activity to almost half (fig. 4*B*). Since, as demonstrated above, the V54D mutation slightly regained NTS binding ability, this observation is consistent with the hypothesis that the transactivation activity via 5aCON is negatively correlated with the DNA-binding activity of the NTS.

Discussion

We have identified a novel missense mutation, a Val→Asp substitution at the 7th codon of the PAX6 alternative splice region, in the pedigrees of four patients with various eye anomalies. It is well known that PAX6 mutations cause various phenotypes; even in a pedigree with aniridia, individuals with the same mutation showed phenotypes ranging from total absence of the iris to the presence of a normal-shaped iris. With the exception of the pedigree of patient 1, the patients' pedigrees did not have apparent family history of eye disease; however, members with extremely mild or nearly normal phenotypes may have been overlooked.

Splice variants (with or without exon 5a) of PAX6 show distinct DNA-binding and transactivation properties. This has led to the idea that the 14 amino acids encoded by exon 5a function as a molecular switch that determines the target genes and their activation (Epstein et al. 1994*b;* Yamaguchi et al. 1997). Functional analyses have shown that the V54D mutation
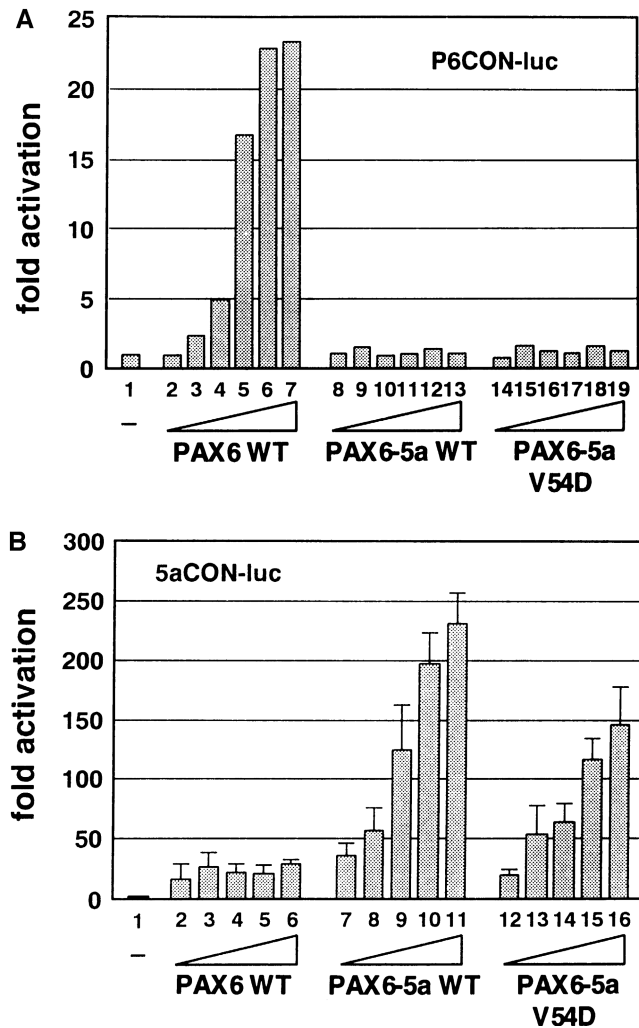
Figure 4 Transactivation potential of the PAX6-5a V54D mutant. *A,* Expression plasmid expressing PAX6 WT (lanes 2–7, at 1, 3, 10, 30, 100, and 300 ng genomic DNA), PAX6-5a WT (lanes 8–13), and PAX6-5a V54D (lanes 14–19) was cotransfected, with 5 μg of either P6CON-luciferase reporter plasmid, into mouse embryonal carcinoma P19 cells. Both PAX6-5a V54D and PAX6-5a WT failed to stimulate transcription from P6CON, whereas PAX6 WT stimulated transcription ~20-fold. *B,* Transfection performed as in panel *A,* with the exception that 5aCON-luciferase plasmid was used as the reporter. Pax6-5a WT possesses higher stimulatory activity than does PAX6 WT. V54D mutation reduces the transactivation potential approximately twofold.

slightly increases the affinity of PAX6-5a for P6CON, without affecting its ability to bind 5aCON (fig. 3*A*).The NTS of the paired domain is responsible for P6CON binding, and insertion of exon 5a into the NTS abolishes the DNA-binding activity of the NTS (Epstein et al. 1994*b;* Yamaguchi et al. 1997). Since the amino acids Val and Asp are significantly different in terms of their electronic charge, the mutation may affect the NTS structure and, thereby, partially restore

the P6CON binding. In contrast to the result of a gel-shift assay, however, the mutation could not recover the P6CON-mediated transactivation. Instead, the mutation mildly reduced transactivation from the 5aCON.

Several lines of evidence suggest that NTS and CTS, the two DNA-binding subdomains of the PAX6 paired domain, can independently bind to their cognate sites (Epstein et al. 1994*b*). Our previous analyses, however, have shown that the two subdomains negatively regulate the transactivation potentials of each other in vivo (Yamaguchi et al. 1997). For instance, the insertion of 14 additional amino acids encoded by exon 5a abolishes the NTS function and markedly enhances transactivation via the CTS and 5aCON. This finding can be interpreted as follows: when the CTS binds 5aCON, the NTS also interacts with the flanking sequences nonspecifically; this interaction constrains the structure of the NTS, to provide a reduced level of transactivation. In all cases examined to date, the transactivation potentials of one subdomain are inversely correlated with the DNA binding activity of the other subdomain. In the present study, the V54D mutation mildly increased P6CON-binding activity and decreased transactivation via 5aCON, a result that also fits with this model.

Normal eye development is highly susceptible to the level of PAX6 expression: haploinsufficiency causes aniridia, and overexpression also causes developmental defects of the eye (Fisher and Scambler 1994; Schedl et al. 1996). A recent biochemical assay has indicated that truncation mutations in the proline-serine-threonine transactivation region of PAX6 have threefold- to five-fold-higher affinity for DNA-binding sites than does the wild-type PAX6; the assay also indicated that the mutations are dominant-negative mutations, rather than loss-of-function mutations (Singh et al. 1998). Therefore, it is not surprising to expect that the twofold variation is responsible for the observed eye anomalies. Another possibility is that the binding and transcription assays do not accurately show the PAX6 protein defect that is due to the mutation. We might have failed to detect the full consequence of the mutation occurring in vivo. With the use of different cis-elements or different cells (e.g., in the presence of putative cell type–specific cofactors for PAX6), the mutation may cause a more potent effect on its function.

A missense mutation in the NTS was identified in individuals with Peters anomaly who had a phenotype limited to the anterior segments. Another mutation in the CTS was detected in a case of foveal hypoplasia, in which only the neural retina is affected. Aniridia involves the entire eye (Glaser et al. 1992; Hanson et al. 1994; Martha et al. 1994; Azuma et al. 1996). Almost all mutations detected in aniridia are hemizygous or lead to a

premature termination; thus, both the NTS and the CTS must be involved. We previously had proposed that the NTS affects the anterior segments and that the CTS affects the posterior areas (Azuma et al. 1996). However, the patients described in the present study have defects in the anterior segment, in addition to foveal hypoplasia. Thus, the regulation of eye formation by the PAX6 gene may be more complex or may involve unknown mechanisms; for instance, the inactive mutants may act in a dominant-negative manner by competing with some coactivators.

According to the expression pattern of PAX6, the gene is repeatedly expressed in the development of various eye tissues (Walther and Gruss 1991). Among patients in the present study, the phenotype had a range of expression, manifesting from anterior to posterior segments of the eye; this finding indicates that there are multiple functions of the alternative splice. The alternative splice may help to control the use and activity of the NTS and the CTS at different stages and in different tissues, according to circumstances and in association with some cofactors. Intriguingly, such a splice variant is found only in vertebrates (Walther and Gruss 1991; Epstein et al. 1994*b*). Thus, acquisition of the splice variant during evolution may contribute to the formation of a highly complex eye structure to obtain better vision.

Acknowledgments

Electronic-Database Information

The URL for data in this article is as follows:

The Human PAX6 Mutation Database, http://www.hgu.mrc .ac.uk/Softdata/PAX6#Summary

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