Published online March 12, 2004

1696–1709 *Nucleic Acids Research, 2004, Vol. 32, No. 5*
DOI: 10.1093/nar/gkh334

Functional interactions between alternatively spliced forms of Pax6 in crystallin gene regulation and in haploinsufficiency

Bharesh K. Chauhan, Ying Yang, Květa Cveklová and Aleš Cvekl*

Department of Ophthalmology and Visual Sciences and Department of Molecular Genetics, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA

Received February 4, 2004; Revised and Accepted February 19, 2004

ABSTRACT

***Pax6* is essential for development of the eye, olfactory system, brain and pancreas. Haploinsufficiency of Pax6 causes abnormal eye development. Two forms of Pax6 protein, PAX6 and PAX6(5a), differ in a 14 amino acid insertion encoded by an alternatively spliced exon 5a in the N-terminal DNA-binding paired domain (PD), and they are simultaneously expressed. Here, we show that PAX6 and PAX6(5a) together synergistically activate transcription from promoters recognized by Pax6 PD and PD5a, but not by their homeodomain. This synergism promotes activation of transcription by c-Maf and MafA on the αB-crystallin promoter, and is required for transcriptional co-activation by RARβ/RXRβ and PAX6/PAX6(5a) on the γF-crystallin promoter. To determine the role of this synergism in haploinsufficiency, we tested four human missense (G18W, R26G, G64V and R128C) and one nonsense (R317X) mutants, with reporters driven by Pax6 PD consensus binding sites and the αB-crystallin promoter. The simultaneous activity of Pax6 proteins [PAX6, mutated PAX6, PAX6(5a) and mutated PAX6(5a)] modeling haploinsufficiency yielded results not predicted by properties of individual PAX6 or PAX6(5a). Taken together, these results indicate that complex ocular phenotypes due to Pax6 haploinsufficiency originate, at least partially, from functional interactions between alternatively spliced PAX6 and PAX6(5a) variants and other factors, e.g. MafA/c-Maf.**

INTRODUCTION

The *Pax* genes (*Pax1–Pax9*) share a 128 amino acid DNA-binding domain called the paired domain (PD), and represent a family of evolutionary conserved genes. They play critical roles acting as specific DNA-binding transcription factors in pattern formation, cellular proliferation, differentiation, migration and apoptosis in mammalian development, and are required for tissue maintenance in adults (1–3). Pax proteins mostly act as transcriptional activators. Mutations in *Pax* genes produce a number of human and mouse developmental disorders, and studies of these mutants have provided critical information about the formation of tissues and organs. The most widely studied member of the Pax family, Pax6, is a multifunctional protein playing essential yet diverse roles in the organogenesis of the brain, visual and olfactory systems, as well as peptide hormone gene expression in the pancreas (3–7).

Two independent DNA-binding globular helix–turn–helix subdomains, PAI and RED, form the 'pai-red' domain, PD (8). The crystal structure of Pax6 PD in complex with DNA provides a model to understand the function of individual structural motifs comprising the PD (9). Alternative splicing within the PD was observed with Pax3, Pax6, Pax7 and Pax8 (10). Alternatively spliced Pax proteins possess different biochemical properties (10–13). *Pax6* gene encodes predominantly two forms of Pax6 protein, Pax6 and Pax6(5a) (10,11,14–16). Pax6 contains a canonical PD comprised of 128 amino acid residues. In contrast, Pax6(5a) contains a 14 amino acid insertion within the PAI subdomain (see Fig. 1A). Much of the earlier molecular studies focused on Pax6 since it contained the canonical PD, and appeared to be more abundant than Pax6(5a) (14–16). Subsequent studies of ocular tissues and cells have found that transcripts encoding Pax6 and Pax6(5a) are equally abundant in the human adult lens epithelium, cornea and monkey retina (17) and in bovine iris (18), raising the possibility that Pax6 and Pax6(5a) transcripts are regulated in a tissue- and temporal-specific manner. A gene targeting study of exon 5a revealed specific roles of Pax6(5a) in ocular and pancreatic development, although the phenotypes were not analyzed at the molecular level (19).

Pax3, 4, 6 and 7 also contain an internal, paired-type homeodomain (HD). This HD can bind DNA in a form of homodimers using a symmetric binding site with two inverted ATTA motifs separated by three nucleotides (20). The Pax HD not only plays a role in DNA recognition but also provides surface for interactions with specific transcription factors including TFIID, retinoblastoma protein, and a number of HD-containing proteins co-expressed with Pax6 (21–23).

*To whom correspondence should be addressed. Tel: +1 718 430 3217; Fax: +1 718 430 8778; Email: cvekl@aecom.yu.edu

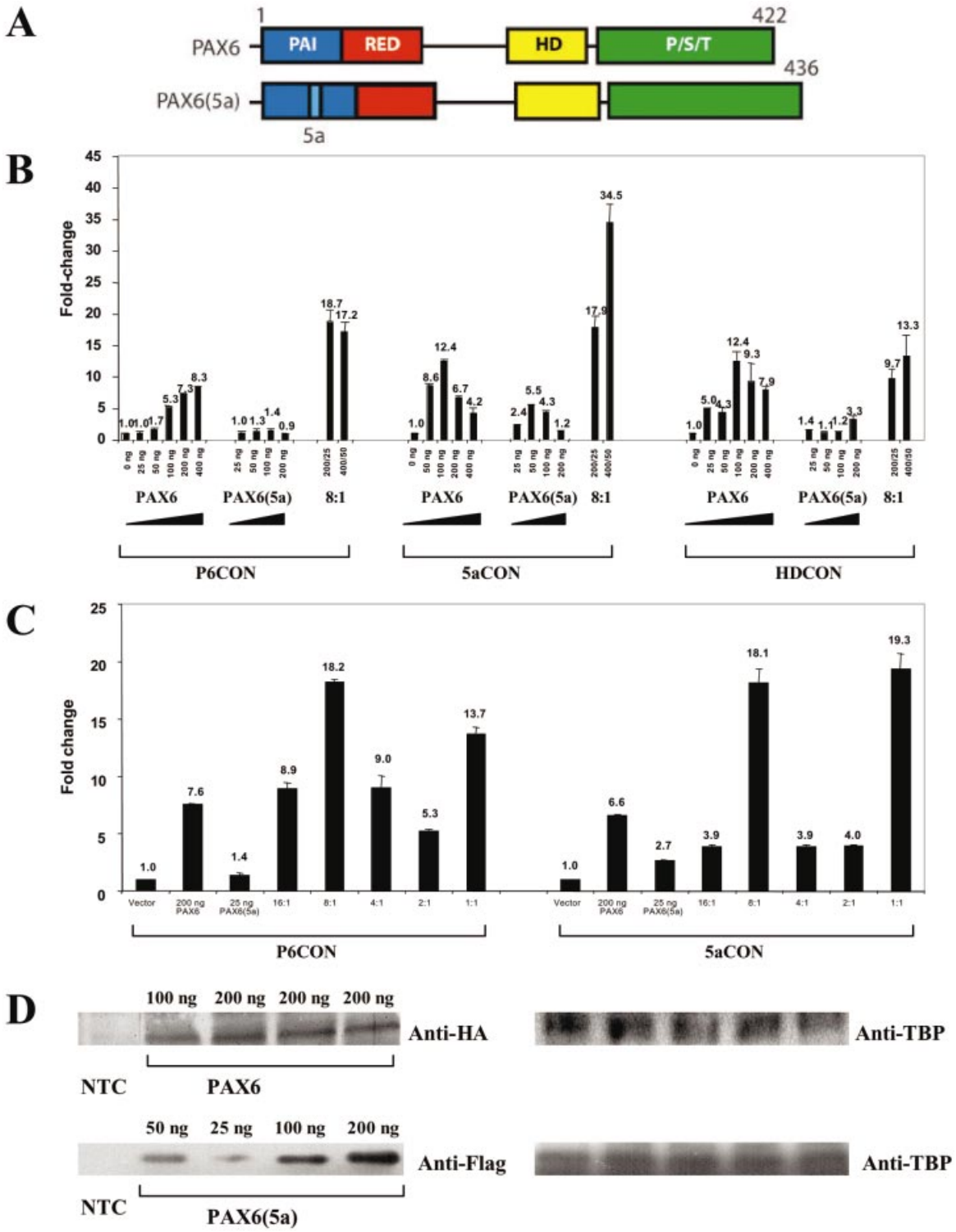

Figure 1. Transcriptional synergism between PAX6 and PAX6(5a) using synthetic promoters. (**A**) Schematic representation of PAX6 PD and HD and subdivision of PD into PAI and RED subdomains. An oligopeptide of 14 amino acid residues encoded by exon 5a disrupts the DNA-binding property of PD5a. (**B**) Transactivation potential of PAX6 and PAX6(5a) at a ratio of 8:1 resulted in robust synergistic interactions from P6CON- and 5aCON- but not from HDCON-driven reporters described in Materials and Methods. The results are shown for CHO-K1 cells as means ± SD (n = 6). (**C**) Transcriptional synergism and its dependence on PAX6 (200 ng) to PAX6(5a) ratios of 16:1, 8:1, 4:1, 2:1 and 1:1. The experimental conditions are described above. (**D**) Western immunoblotting of CHO-K1 cells transiently transfected with both HA-PAX6 and FLAG-PAX6(5a) cDNAs. TBP was detected with an anti-TBP antiserum and used as a loading control. NTC indicates extracts from non-transfected cells.

In contrast to a single mammalian *Pax6* gene, four Pax6-homologous genes, *eyeless* (*ey*), *twin of eyeless* (*toy*), *eye gone* (*eyg*) and *twin of eyegone* (*toe*), are present in the *Drosophila* genome (24–27). These genes play key roles in eye

specification and growth together with a number of distinct functions in the development of the central nervous system. They are capable of inducing ectopic eye formation in abnormal body positions (26). Pax6 is structurally similar to toy and ey proteins, as they contain the canonical PDs (27). In contrast, eyg is a protein with an N-terminally truncated PD (24). The effect of this shortening should be similar to the inactivation of DNA-binding activity of the PAI(5a) subdomain in Pax6(5a). Indeed, Pax6(5a) can replace eyg in a *Drosophila* eye growth assay, confirming this hypothesis (25). It has been also shown that ey and eyg act synergistically in the induction of ectopic eyes (24) which raises the possibility that their mammalian homologs, Pax6 and Pax6(5a), also function together.

In humans, heterozygous mutations in *PAX6* cause a diverse spectrum of ocular abnormalities (6,28) and subtle changes in the olfactory system and brain (29). Nonsense mutations generating truncated Pax6 proteins typically result in aniridia. The hallmark of aniridia is iris hypoplasia, often combined with cataracts, glaucoma, nystagmus and foveal and optic nerve hypoplasia (30,31). In contrast, missense mutations generating single amino acid substitutions cause, in about 50% of cases, less severe phenotypes, e.g. foveal hypoplasia, Peters' anomaly, congenital cataracts and autosomal dominant keratitis (see 3,32 for reviews). A rare case of a human PAX6 compound homozygote resulted in anophthalmia and severe brain defects lethal after birth (28). Based on the above phenotypes, it has been proposed that a PAX6 gene dosage effect is responsible for phenotypes associated with mutations in the one allele of PAX6 (6,32). Nevertheless, molecular details of the haploinsufficiency were probed only superficially. Earlier studies have focused on the possibility that truncated PAX6 proteins might act as dominant negative repressors of the wild-type PAX6 (33,34). Studies of individual human PAX6 and PAX6(5a) mutants revealed that some mutants retain activity in a number of functional assays (35,36).

Here, we tested possible co-activation mechanisms by PAX6 and PAX6(5a) since both proteins are co-expressed *in vivo* (10,14–18), and because of a functional synergism between *Drosophila ey* and *eyg* (24). This revealed a remarkable functional synergism between the alternatively spliced products of the *PAX6* gene. Next, we tested the potential role of this synergism in gene regulation of crystallin promoters. Crystallins are regulated by Pax6 in combination with other DNA-binding transcription factors, namely members of the large Maf family and retinoic acid-activated nuclear receptors RARβ and RXRβ (5). The synergism between Pax6 and Pax6(5a) can have a significant impact under haploinsufficient conditions when mutated forms of these proteins are present. Thus, we examined the mechanism of PAX6 gene dosage effect by testing simultaneously four PAX6 proteins, i.e. wild-type PAX6, mutated PAX6, wild-type PAX6(5a) and mutated PAX6(5a), recapitulating the Pax6 protein composition in heterozygous cells.

MATERIALS AND METHODS

Materials

Oligonucleotides were purchased from Invitrogen (Frederick, MD), and mouse monoclonal antibody against Pax6 from Developmental Biology Hybridoma Bank (Iowa City, IA). Antibodies against Pax2/5/8 (sc-7747), Pax3/7 (sc-7748) and TBP (sc-273) were from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-Flag M2 and anti-HA monoclonal antibodies were from Sigma and Roche Applied Science, respectively. 293T (an adenovirus-transformed human embryonic kidney epithelial) cells were obtained from ATCC (Manassas, VA), rabbit N/N1003A (non-transformed lens epithelial) cells were provided by Dr J. Reddan (Oakland University, Oakland, MI) and CHO-K1 (Chinese hamster ovary cell line) was provided by Dr Jonathan Backer (Department of Molecular Pharmacology).

Reporter genes

Mouse αB- and γF-crystallin gene promoters contained fragments –162 to +45 and –226 to +41, respectively, in pGL3 (Promega, Madison, WI), and were generated from parental plasmids described elsewhere (37,38). Six, four and four copies of PAX6 PD, PD5a (alternatively spliced PD) and HD consensus binding sites, P6CON, 5aCON and HDCON, were cloned 5′ of the E4 TATA minimal promoter in pGL3 (10,11,20,35). The sequences were: P6CON, TTCAGGAAA-AATTTTCACGCTTGAGTTCACAGCTCGAGT; 5aCON, AAATCTGAACATGCTCAGTGAATGTTCATTGACTCT-CGAGGTC and HDCON (P3 site), TCGAGGGCATC-AGGATGCTAATCTGATTAGCATCCGATCGGG (Pax6-binding sites are underlined). Four copies of LSR1 were generated by inserting two copies of an oligonucleotide ctagTGTTTCTCTTTTCTTAGCTCAGTGAGTACCGGGT-ATGTGTCGTGTTTCTCTTTTCTTAGCTCAGTGAGTAC-CGGGTATGTGTC (nucleotides used for subcloning are shown in lowercase letters), between SpeI and EcoRV, and NheI and SmaI sites in E4 TATA minimal promoter described above.

Expression plasmids and site-directed mutagenesis of DNA

Expression plasmids for PAX6 and PAX6(5a) and their mutants (G18W, R26G, G64V, R128C and R317X) were made in the CMV-based vector, pKW10 (20), using the Quick-change mutagenesis kit (Stratagene, La Jolla, CA) as described elsewhere (36). Flag-tagged wild-type and mutated PAX6 and PAX6(5a) cDNAs were generated in a vector p3xFLAG-CMV –10 (Sigma, St Louis, MO). HA-tagged PAX6 was generated using vector pHM6 (Roche, Indianapolis, IN). Transcription factors in pKW10 were obtained from Drs M. Busslinger (Pax2, Pax3 and Pax5), M. Duncan (Otx2) and O. Sundin (Optx2/Six6), respectively. Lhx2, MafA, MafB, c-Maf and Six3a cDNA were obtained from Drs J. Botas, A. Sharma, K. Yoshida and Z. Kozmik, respectively, and subcloned into pKW10. Pitx3 cDNA in pcDNA3 was obtained from Dr E. Semina. Six3b and Msx2 cDNAs were prepared using RT–PCR and mouse lens and embryonic mRNAs (E11.5) and subcloned into pKW10. Expression vectors encoding RARβ and RXRβ were described earlier (39).

Cell cultures, transfections and dual luciferase assays

All cells were grown in DMEM with 10% fetal bovine serum supplemented with 20 μg/ml gentamicin, in a 5% CO_2 incubator at 37°C. CHO-K1, 293T and N/N1003A cells were

seeded at a density of 40–50% confluency in six-well plates the day before transfection. The medium was changed 2–3 h before the cells were transfected by the calcium phosphate co-precipitation procedure. At 60–80% confluency, cells were transfected with 5 μg of firefly luciferase reporter DNA and differing 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 (Promega), was included in all transfections. Experiments including retinoic acid (all-*trans* retinoic acid, Sigma) were performed as described earlier (39). In addition, some experiments were conducted in the presence of pSV40 and pTK *Renilla* luciferase (Promega) to control for possible indirect effects during transfections. The cells were kept in medium with 10% serum for 48 h post transfection. Cells were passively lysed and luciferase activity was measured at room temperature using a dual luciferase reporter assay kit (Promega, Madison, WI). Firefly luciferase activities were normalized relative to *Renilla* luciferase activity. Each experiment was conducted in triplicate and repeated at least twice.

Expression of Pax proteins in cultured cell lines and western blotting

Nuclear extracts from cultured αTN4-1 lens cells (40) and mouse melanoma B16 cells (Geneka, Montreal, Canada) were used as positive controls for Pax6 and Pax3, respectively. Pax2 was expressed in 293T cells using a plasmid as described above. Protein concentrations were determined using the Bio-Rad Coomassie Blue protein assay (Bio-Rad Laboratories, Hercules, CA), and 50 μg of protein was loaded on each lane of a 10% discontinuous SDS–PAGE gel (Bio-Rad). The protein was transferred to nitrocellulose and incubated with 1:2000 dilution of primary serum. Bound antibodies were detected with 1:2000 HRP linked anti-mouse IgG as described earlier (41). Pax2/5/8 (or Pax3/7) antibodies were used at dilutions 1:1000.

RESULTS

Mammalian PAX6 and PAX6(5a) act synergistically to activate gene expression

To test the possibility that mammalian PAX6 and PAX6(5a) (Fig. 1A), like *Drosophila* ey and eyg (24), are actual partners for gene regulation, we performed a series of cotransfections using both PAX6 and PAX6(5a) at ratios relevant to endogenous ratios of these proteins judged from quantitative studies at the mRNA and protein levels (10,14–18). Ratios for PAX6/PAX6(5a) of ~8:1 were found in mouse embryo (10), lens cultured cells (16,18) and 6-week-old mouse lens (42). The experiments were performed with P6CON, 5aCON and HDCON reporters, as described in Materials and Methods, containing consensus binding sites of PAX6 PD, PD5a and HD in CHO-K1 cells. These cells were used because they do not express endogenous Pax6, Pax2/5/8 and Pax3/7 (data not shown). The results of a series of cotransfections are shown in Figure 1B. PAX6 activated transcription from P6CON, while PAX6(5a) had barely any positive effect. However, the combination of PAX6 and PAX6(5a) expression generated robust activation, often exceeding the multiplied effects of individual Pax6 proteins. For example, at a PAX6/PAX6(5a) ratio of 8:1, the activation of transcription using the P6CON-driven reporter was 18.7-fold compared to 7.3- and 1.0-fold changes with PAX6 and PAX6(5a), respectively. A similar result was observed with the 5aCON reporter (Fig. 1B). In contrast, no significant enhancement of reporter gene activation in the presence of both PAX6 and PAX6(5a), compared to PAX6 or PAX6(5a) alone, was seen with HDCON sites (Fig. 1B). Similar results were obtained in 293T and P19 cells (data not shown), indicating that the synergistic effects were not restricted to a single cell line.

To gain further insight into this synergism, we probed different ratios of PAX6 to PAX6(5a), 16:1, 8:1, 4:1, 2:1 and 1:1. The results, shown in Figure 1C, showed that maximum activation was observed at ratios 8:1 and 1:1 of PAX6 to PAX6(5a) for both P6CON- and 5aCON-driven promoters. To demonstrate the expression of alternatively spliced forms of Pax6 proteins in transfected cells, we employed HA-tagged PAX6 and Flag-tagged PAX6(5a) cDNAs because a 5a-peptide specific antibody was not available. The results (see Fig. 1D) showed that PAX6 and PAX6(5a) proteins were both expressed in a dose-specific manner in the transfected cells.

Co-expression of PAX6 and PAX6(5a) and αB- and γF-crystallin gene regulation

To demonstrate a potential role of the PAX6 and PAX6(5a) complex in transcriptional control of natural Pax6-target genes, we used the mouse αB- and γF-crystallin promoters shown earlier to respond to Pax6 (37,39,43). The mouse αB-crystallin promoter contains both Pax6- and Maf-binding sites, T-MARE/αCE2 in the lens-specific enhancer region, LSR1 (Fig. 2A) (5,38). Mouse γF-crystallin promoters contains a single Pax6/Pax6(5a) binding site next to the retinoic acid responsive element (RARE) (37) (Fig. 5A). Targeted deletion of c-Maf in mouse showed reduced expression of both the αB- and γF-crystallins in the embryonic transgenic lenses (44,45), and the remaining expression is thought to be due to the expression of other members of the Maf family of transcription factors, namely MafA, in the lens (46–49).

Thus, we conducted cotransfection experiments involving various combinations of PAX6, PAX6(5a), c-Maf and MafA to assess their regulatory roles on the mouse αB-crystallin promoter (Fig. 2). The experiments were performed in both non-lens (293T) and lens (N/N1003A) cells as the presence of Pax6 proteins in lens cultured cells could influence the activation levels of individual transcription factors and their combinations as well (40). In addition, experiments were performed in 293T cells to show that the potential synergism is not limited to a specific cell line. In lens cells, the basal αB-crystallin promoter activity was 20-times higher relative to the 293T cells when normalized against the promoter-less pGL3 in agreement with earlier studies (38). Cotransfection with PAX6 resulted in weak activations, 2.5- and 3.7-fold, in both cell types in agreement with earlier data (43). In contrast, cotransfection with PAX6(5a) gave a weak repression in non-lens cells and no effect in lens cells (Fig. 2B). Cotransfections with MafA or c-Maf resulted in appreciable activations ranging from 10.2- to 18.1-activation demonstrating, for the first time, that αB-crystallin promoter contains at least one functional T-MARE site. The highest activation in this group, 18.1-fold, was observed with c-Maf in lens cells indicating

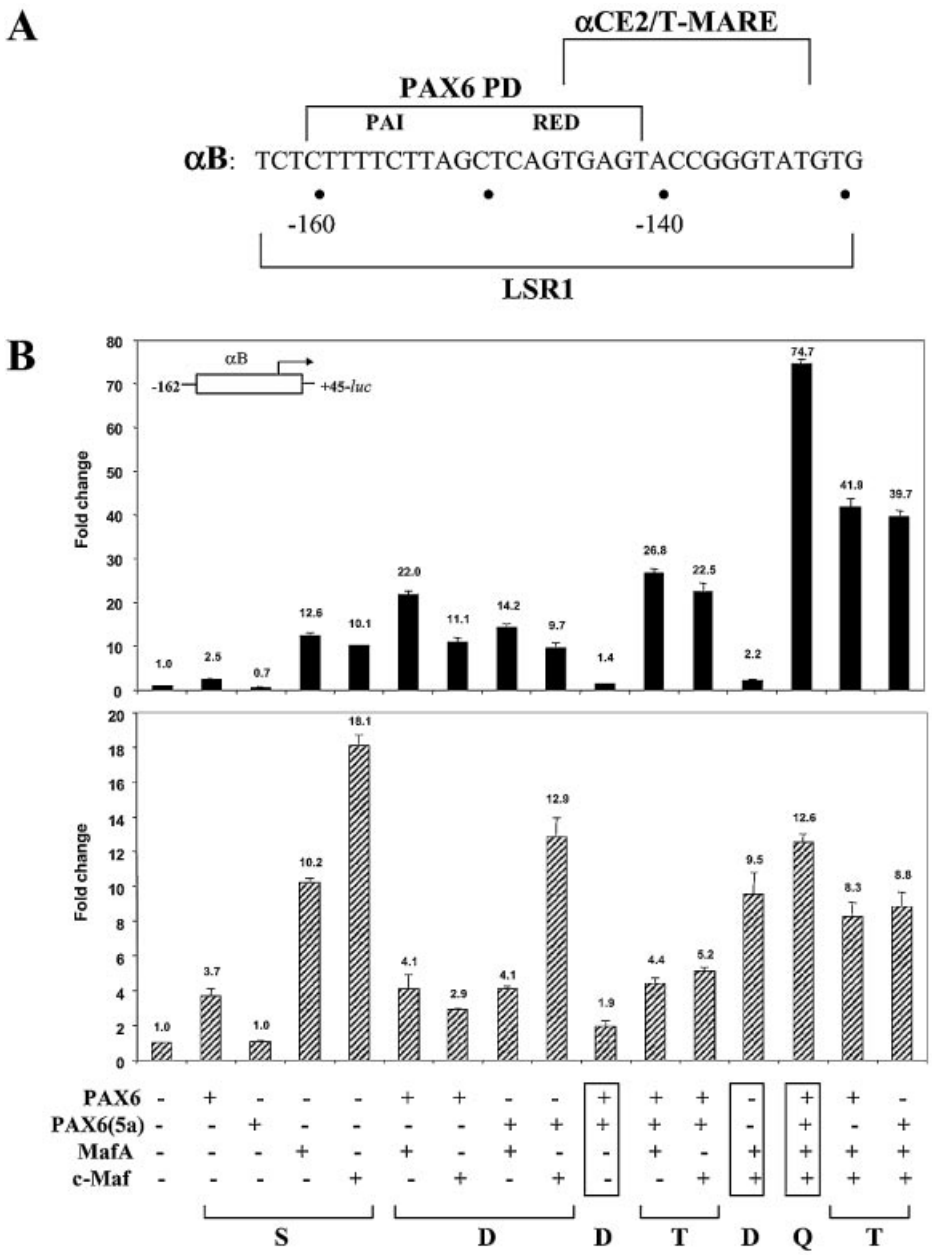

Figure 2. Functional synergism between PAX6 and PAX6(5a) and mouse αB-crystallin promoter. (**A**) Schematic representation of a Pax6-binding site partially overlapping with an αCE2/T-MARE binding site in the lens-specific enhancer region (LSR1) of the mouse αB-crystallin promoter (38,43). (**B**) Mouse αB-crystallin promoter (–162/+45) was cotransfected with cDNAs encoding PAX6 (200 ng), PAX6(5a) (25 ng), c-Maf (80 ng) and MafA (80 ng) alone or in indicated combinations. Results in 293T cells (solid boxes), and in N/N1003A lens (striped boxes) cells. The results are shown as means ± SD (n = 9). The data were normalized to the activity of the reporter plasmids cotransfected with corresponding amounts of empty vector, pKW10. Group of transfections using one (S), two (D), three (T) and four (Q) cDNAs are indicated by horizontal brackets. The experiments highlighting functional significance of Pax6/PAX6(5a) complex are boxed.

that endogenous Pax6 (40) in combination with transfected c-Maf was a more potent activator than with MafA. Next, four possible combinations of two Pax6 variants and two Mafs were tested. In non-lens cells, two combinations, i.e. PAX6/MafA and PAX6(5a)/MafA, resulted in transcriptional synergisms. The other combinations, PAX6/c-Maf and PAX6(5a)/c-Maf showed marginal effects. In contrast, in lens cells all four combinations resulted in reduced activation compared to the activation levels obtained with MafA and c-Maf alone. The simplest explanation for the results in lens cell would be that ectopic expression of either Pax6 or Pax6(5a) combined with the endogenous Pax6 proteins disrupts their 'optimal' ratio as suggested from data shown in Figure 1C.

Thus, based on the synergistic results from Figure 1B and C, we investigated transactivations in the presence of PAX6/PAX6(5a) and MafA/c-Maf, also co-expressed in lens cells (46,49). In non-lens cells, PAX6/PAX6(5a) and MafA/c-Maf complexes elicited only 1.4- and 2.2-fold activations, respectively (Fig. 2B). However, a robust activation of 74.7-fold was mediated by PAX6/PAX6(5a) together with MafA/c-Maf.

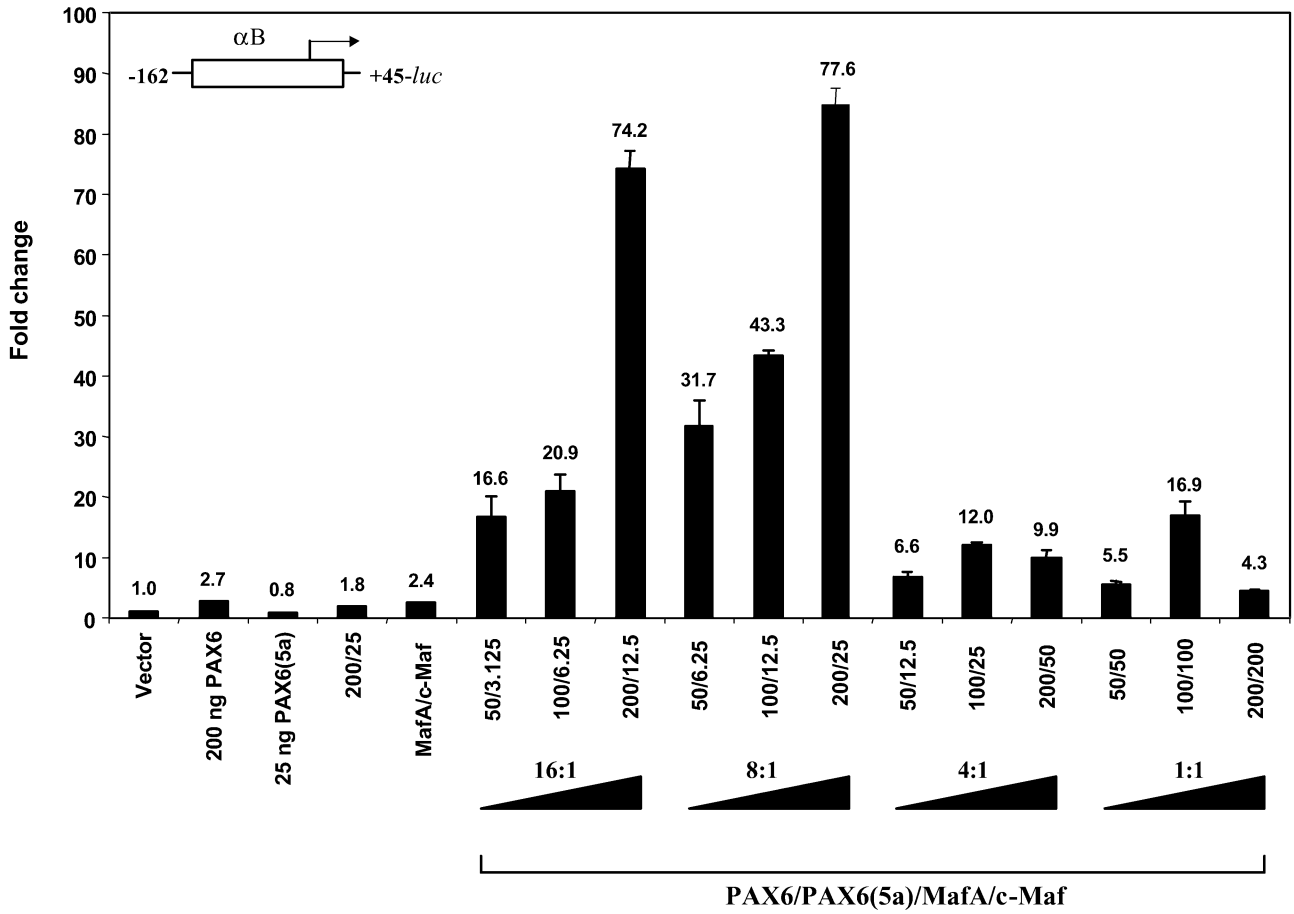

Figure 3. Functional synergism between PAX6 and PAX6(5a) at different ratios and mouse αB-crystallin promoter in 293T cells. The amounts of PAX6 and PAX6(5a) cDNA expression plasmids are shown. The experiment was conducted and evaluated as described in the legend to Figure 2B. The highest activation of the promoter was found at a ratio of 8:1.

Addition of PAX6/PAX6(5a) increased the promoter activity mediated by MafA/c-Maf by a factor of 34-fold. Omission of either PAX6(5a) or PAX6 from the experiment resulted in about half of the activity observed with both PAX6/PAX6(5a). From these data we conclude that maximal αB-crystallin promoter activity requires the presence of both PAX6 and PAX6(5a), in combination with MafA and c-Maf.

In lens cells, the PAX6/PAX6(5a) complex weakly activated the promoter by a factor of 1.9 (Fig. 2B, lower panel). However, the MafA/c-Maf complex activated the promoter 9.5-fold, likely using the endogenous Pax6 proteins (40). Quantitatively, the 5- and 6.6-fold increase between the presence and absence of MafA/c-Maf (9.5/1.9) and addition of MafA/c-Maf to PAX6/PAX6(5a) (12.6/1.9) in lens cells indicate functional interactions between Pax6 and large Maf proteins; however, they do not directly prove synergism observed in non-lens cultured cells. In addition to the effects of endogenous proteins expressed in cultured lens cells, the lack of synergism in lens cells could be caused by the quantities of activators and/or interactions between distal LSR1 and proximal LSR2 of the mouse αB-crystallin promoter (43).

To further clarify the possibility that different amounts and ratios of Pax6 proteins tested in 293T cells lacking the endogenous Pax6 proteins could result in the variabilities observed in lens cells, we tested different concentrations and ratios of PAX6 to PAX6(5a). Herein, we tested ratios 16:1, 8:1, 4:1 and 1:1, and 50, 100 and 200 ng of PAX6 with 6.25, 12.5 and 25 ng of PAX6(5a), respectively, with the mouse αB-crystallin promoter and fixed amounts of MafA/c-Maf, as shown in Figure 3. The results revealed Pax6 dose-dependent transcriptional synergism with MafA/c-Maf with the highest stimulation generated by 200/25 ng (8:1) of PAX6/PAX6(5a).

To test whether LSR1 alone (see Fig. 2A) contains *cis*-elements capable of promoting synergistic interactions between Pax6 and large Maf proteins, we generated a reporter plasmid containing four copies of LSR1 in combination with a heterologous, E4 TATA, 'minimal' promoter, and used this plasmid in a series of transfection tests. As the αB-crystallin is highly expressed in lens epithelium exhibiting high levels of expression of Pax6 and MafB, we tested these proteins together. The results revealed synergism between Pax6/Pax6(5a) and MafB on the LSR1-driven reporter both in non-lens (see Fig. 4A) and lens (see Fig. 4B) cultured cells. Furthermore, αB-crystallin expression *in vivo* is regulated by Pax6 (42) and c-Maf (44,45). Cotransfection of Pax6/Pax6(5a) with c-Maf using the LSR1-driven reporter also showed synergism between these proteins in lens cells (see Fig. 4C).

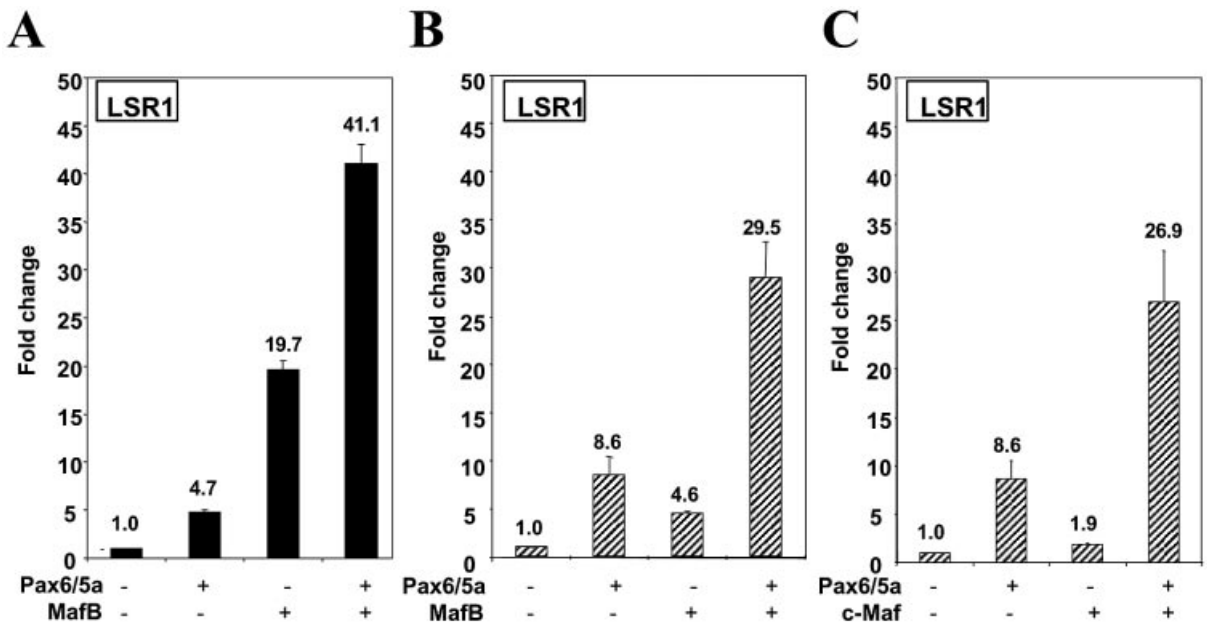

Figure 4. Functional synergism between Pax6 and large Maf proteins. (**A**) LSR1-driven promoter was cotransfected with cDNAs encoding PAX6, PAX6(5a) and MafB in 293T cells. (**B**) LSR1-driven promoter was cotransfected with cDNAs encoding PAX6, PAX6(5a) and MafB in N/N1003A lens cells. (**C**) LSR1-driven promoter was cotransfected with cDNAs encoding PAX6, PAX6(5a) and c-Maf in N/N1003A lens cells. PAX6 (200 ng), PAX6(5a) (25 ng) and 80 ng of the large Maf were tested as indicated. The results were calculated as described in Figure 2B legend.

Collectively, the above transfection studies support the idea that optimal function of the αB-crystallin promoter requires synergism between PAX6, PAX6(5a) and large Maf proteins.

Next, we tested promoter regulation of the mouse γF-crystallin promoter. It has been shown earlier that a specific mutagenesis of the Pax6/Pax6(5a) binding site reduced promoter activity in transfected primary lens cells, however, cotransfections with either Pax6 or Pax6(5a) resulted in 50 and 88% repression of the promoter in lens and non-lens cells, respectively (37). The Pax6-binding site adjacent to a RARE, shown in Figure 5A, is required for the function of the RARE (37). Nevertheless, a potential synergism between Pax6 and proteins interacting with RARE was not found earlier (37). Our data confirmed repression of the γF-crystallin promoter by both PAX6 and PAX6(5a) in CHO-K1 and N/N1003A cells (Fig. 5B). The results showing 50% inhibition agreed for lens cells (37). The quantitative difference between the present data (40% inhibition) and results in chicken primary fibroblasts (88% inhibition) were likely caused by use of different cell types. Next, we showed that either PAX6/PAX6(5a) or RARβ/RXRβ alone in the presence of retinoic acid did not significantly activate the mouse γF-crystallin promoter (Fig. 5B). In contrast, a notable synergism between these proteins was found when cDNAs encoding PAX6/PAX6(5a) and RARβ/RXRβ were simultaneously cotransfected into both lens and non-lens cells in the presence but not in the absence of retinoic acid (Fig. 5B). Taken together, the functional data using two crystallin promoters indicate that PAX6 and PAX6(5a) together are required for optimal function of other crystallin regulatory factors, specifically MafA/c-Maf and RARβ/RXRβ with the mouse αB- and γF-crystallin promoters, respectively.

PAX6 haploinsufficiency and gene regulation

Previous studies on the mechanism of PAX6 haploinsufficiency have analyzed individual mutants and compared these to wild-type PAX6, or a specific mutant was cotransfected with wild-type PAX6, excluding *de facto* the role of PAX6(5a) (34,35,50–52) and the effect of the transcriptional synergism identified here. However, haploinsufficient cells *in vivo* simultaneously express the wild-type and mutated PAX6 and PAX6(5a) proteins (see Fig. 6A), and mutual interaction of the four proteins originating from the normal and mutant allele may be critical for the net effect.

To test effects of individual mutations, we used a representative panel of five naturally occurring PAX6 and PAX6(5a) mutants (36). The mutation G18W is located in the β-turn motif close to the N-terminal end of the PD, the mutation R26G is located within the PAI subdomain, the mutation G64V is located in the linker between PAI and RED subdomains, and the mutation R128C is located in the RED subdomain. These missense mutations substitute critical amino acid residues involved in the three-dimensional structure of Pax6 PD (9) and functionally affect their transactivation properties (36). The nonsense mutation R317X results in premature termination of the C-terminal activation domain in Pax6 and has been shown to significantly reduce PAX6 transactivation of P6CON-, 5aCON- and HDCON-driven promoters (34,36). In order to recapitulate the *in vivo* environment of Pax6-haploinsufficient cells (see Fig. 6A), we tested transactivations by four Pax6 proteins [i.e. PAX6, PAX6(5a) and their mutated counterparts] in individual assays. Parallel experiments were performed to assess their properties in the absence of either PAX6 or PAX6(5a).

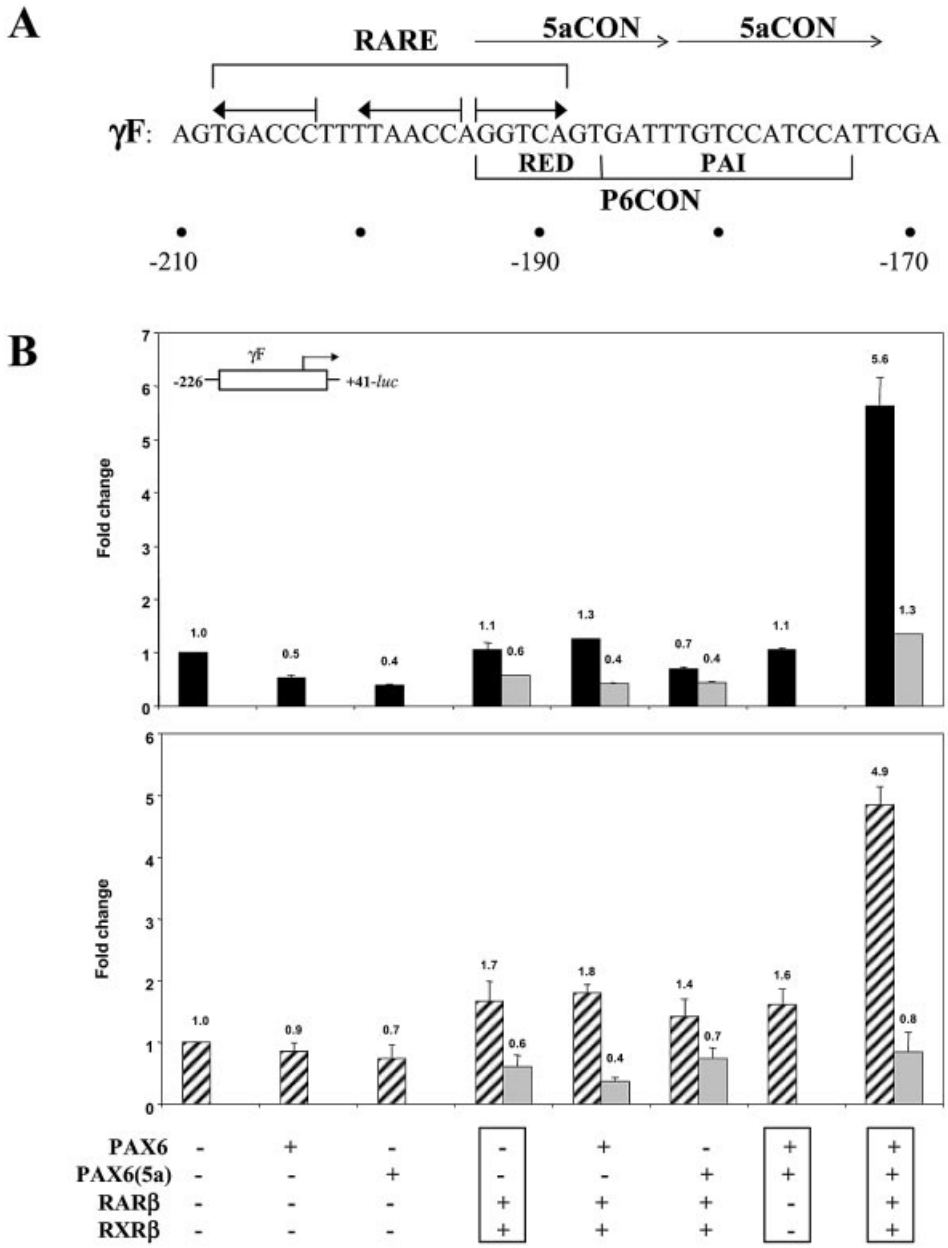

Figure 5. Functional synergism between PAX6 and PAX6(5a) and mouse γF-crystallin regulatory proteins. (**A**) Schematic representation of a Pax6-binding site partially overlapping with a RARE-binding site in the lens-specific enhancer region of the mouse γF-crystallin promoter (5,37). A unique Pax-6 binding site can be recognized by both Pax6 and Pax6(5a) interacting in opposite directions (37). (**B**) Mouse γF-crystallin promoter (–226 to +41) was cotransfected with cDNAs encoding PAX6 (200 ng), PAX6(5a) (25 ng), RARβ (80 ng) and RXRβ (80 ng) alone or in indicated combinations. Results obtained in CHO-K1 (upper panel) and lens N/N1003A (lower panel) are displayed. Experiments conducted in the presence of retinoic acid (solid and striped boxes). The results obtained in the absence of retinoic acid are shown by gray boxes. The results are shown as means ± SD (n = 9). The data were normalized to the activity of the reporter plasmids cotransfected with corresponding amounts of empty vector, pKW10. The experiments highlighting functional significance of the Pax6/PAX6(5a) complex are boxed.

Herein, we used P6CON-driven reporters and tested transactivation of individual wild-type and mutated PAX6 and PAX6(5a) proteins, followed by analysis of simultaneous presence of PAX6 or PAX6(5a) together with each mutant. The cells were cotransfected with 200 ng of individual PAX6 or each PAX6 mutant (Fig. 6B), 25 ng of PAX6(5a) or each PAX6(5a) mutant (Fig. 6D). The results for the PAX6 series are shown in Figure 6B. Mutants G18W and R317X in PAX6 had clearly reduced activities. Mutants R26G and G64V had activities comparable with PAX6. In contrast, mutant R128C elicited more than 3-fold activation compared to wild-type PAX6. These results (Fig. 6B) established reference points for comparisons with the reactions in which wild-type and mutated proteins were mixed together. Next, cotransfections of 100 ng of PAX6 together with 100 ng of each mutant were compared to the activation by 200 ng of PAX6. The data, shown in Figure 6C, resulted in a variety of effects. Although the mutant G18W weakly transactivated alone, a mixture of

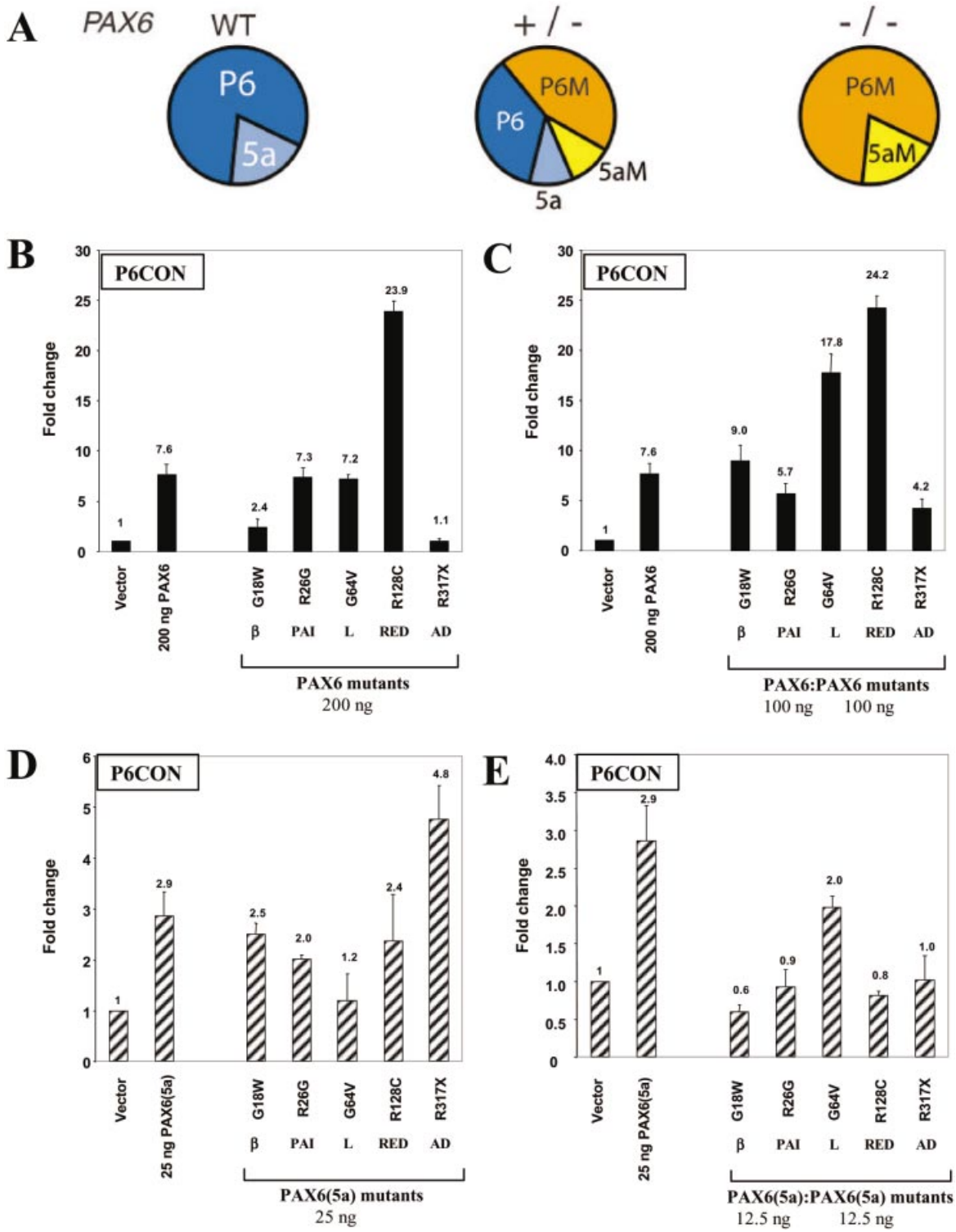

Figure 6. Functional studies of PAX6 mutants alone or pairwise PAX6/mutant, and PAX6(5a)/mutant using P6CON reporter in CHO-K1 cells. (**A**) Diagrammatic representation of protein composition in wild-type (WT), Pax6 heterozygous (+/–) and homozygous (–/–) cells. (**B**) Transactivation by PAX6 and its four missense (G18W, R26G, G64V and R128C) and one nonsense (R317X) mutants. The experiment was performed with 200 ng of individual Pax6 cDNA. (**C**) Transactivation by 100 ng of PAX6 combined with 100 ng of individual mutant. (**D**) Transactivation by PAX6(5a) and its four missense (G18W, R26G, G64V and R128C) and one nonsense (R317X) mutants. The experiment was performed with 25 ng of individual PAX6(5a) cDNA in CHO-K1 cells. (**E**) Transactivation by 12.5 ng of PAX6(5a) combined with 12.5 ng of individual mutant. The original phenotypes and references for human mutations are: G18W, cataract (64); R26G, Peters' anomaly (65); G64V, cataract and foveal hypoplasia (67); R128C, foveal hypoplasia (66); and R317X, aniridia and cataract (64). The G18W mutation is located in the β-turn motif (β) close to the N-terminal end of the PD, the mutation R26G is located within the PAI subdomain (PAI), the mutation G64V is located in the linker (L) between PAI and RED subdomains and the mutation R128C is located in the RED subdomain (RED). The nonsense mutation R317X results in premature termination of the C-terminal activation domain (AD) in Pax6.

PAX6/PAX6(G18W) gave a result similar to that obtained with 200 ng of PAX6 only. Mutants R26G and G64V tested together with PAX6 (Fig. 6C) also did not follow the results obtained in the absence of PAX6 (Fig. 6B). However, mutants R128C and R317X showed correlative properties between these two assays (Fig. 6, compare B and C). Similar experiments comparing mutants in PAX6(5a) are shown in Figure 6D and E. Each mutation tested had different properties when probed alone (Fig. 6D) or in the presence of wild-type PAX6(5a) (Fig. 6E).

Finally, to test the conditions recapitulating Pax6 protein content in haploinsufficient cells, we tested 200 ng of PAX6 and 25 ng PAX6(5a), or 100 ng of each PAX6 and the specific mutant together with 12.5 ng of each PAX6(5a) and the specific mutant. The results are shown in Figure 7. The reference reaction which includes 200 ng of PAX6 together with 25 ng of PAX6(5a) cDNAs resulted in an 18-fold activation (Fig. 7A). Reactions testing mutants G18W and R26G resulted in fold changes statistically similar to the control experiment. In contrast, reactions testing mutants G64V and R128C gave about a 33% increase compared to the wild-type conditions. However, nonsense mutant R317X gave only 16% activation compared to the wild-type Pax6 proteins. Therefore, the results mimicking Pax6 haploinsufficiency showed retention of the activities of each missense mutant, which could not be predicted from their properties tested in the absence of the PAX6(5a) variant (compare Fig. 7A with results in Fig. 6B and C). The data raise the intriguing possibility that the mutated PAX6(5a) proteins, although harboring the same mutations as PAX6, might 'rescue' the deficiency in PAX6, at least under the conditions tested here.

To further examine Pax6 haploinsufficiency, we tested a natural mouse αB-crystallin promoter. This promoter was selected for two reasons. Our earlier study found reduced expression of the αB-crystallin gene in Pax6 heterozygous lenses containing null mutation, i.e. 50% gene dosage (42), showing that the αB-crystallin is an *in vivo* Pax6-dosage sensitive gene. In addition, the present data (see Figs 2 and 3) established a functional synergism with c-Maf/MafA, allowing one to test these mutants in the presence of other specific DNA-binding transcription factors. First, we tested Pax6 haploinsufficiency in the absence of c-Maf/MafA as shown in Figure 7B. The results showed reduced activation of the αB-crystallin promoter with all mutants (Fig. 7B). The simplest interpretation of the data would be that both DNA-binding and transcriptional activities of these diverse Pax6 mutants evenly reduced the promoter activity mediated by the normal Pax6 proteins. Next, we tested this promoter using conditions recapitulating Pax6 haploinsufficiency in the presence of MafA/c-Maf. Cotransfections of PAX6/PAX6(5a)/c-Maf/MafA yielded ~75-fold relative transactivation (Fig. 7C). Two mutants, G18W and R26G, resulted in statistically significant reductions of overall transcriptional activities of the αB-crystallin promoter. In contrast, mutants G64V and R128C gave moderate transcriptional enhancements of 47 and 63%, respectively. Surprisingly, missense mutant R317C yielded 82% of activity compared to the wild-type control, although it retained only 18% of activity tested with P6CON (Fig. 6C). Hence, our data raised the possibility that Maf proteins can significantly 'rescue' R317X truncated Pax6 proteins, but not Pax6 missense mutants G18W and R26G, on the αB-crystallin promoter. Finally, we show that all Pax6 mutants accumulate in transfected cells to similar levels (Fig. 8).

DISCUSSION

The present study had two goals: (i) to test possible synergism between PAX6 and PAX6(5a), alternatively spliced products of the same gene; and (ii) to determine activity of Pax6 proteins at experimental conditions that recapitulate PAX6 haploinsufficiency *in vivo*. An understanding of these issues is critical 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 the molecular mechanism of the gene-dosage effect in human and mouse developmental abnormalities. A significant proportion of genes encoding transcription factors (e.g. FOXC1, PAX2, PAX3, PAX6, PITX2, PITX3, SIX6 and TBX1) are dosage-sensitive (3,32,53–56). In addition, some of them, e.g. PAX2, PAX3 and PAX6, are expressed in multiple isoforms (10–14), and the different isoforms may be employed together in specific gene regulation.

PAX6 and PAX6(5a) functionally interact to promote transcription of crystallin genes

A recent report has shown that two *Drosophila Pax* genes, *eyeless* and *eye gone*, act cooperatively to induce compound eye development (24). *Eyeless* is a *Drosophila* homolog of *Pax6*. Although *eye gone* does not have a vertebrate homolog, its encoded protein appears to be structurally/functionally analogous to Pax6(5a), as it contains only a partial PAI subdomain (24,25).

Here, we show that human PAX6 and PAX6(5a) synergistically stimulate transcription from both natural and synthetic promoters. Synthetic promoters were initially tested in the cell lines that did not express endogenous Pax6 proteins. Importantly, the synergism was observed only for Pax6-binding sites using the PD and PD5a DNA-binding domains, but not the HD-binding sites. This finding, together with concentration-dependent effects, showing two peaks with a P6CON-driven promoter (see Fig. 1C) and a single peak with αB-crystallin promoter (see Fig. 3), and cDNA concentration-dependent expression of Pax6 variants detected by westerns (see Fig. 1D) argues against non-specific and indirect effects. Furthermore, the key transfections were repeated and normalized with SV40- and tk-driven *Renilla* luciferase, confirming the results normalized using the CMV-driven luciferase. Finally, studies of natural crystallin promoters (Figs 2–5 and 7) revealed a potential for the synergism *in vivo* (see below).

Here, we have found that synergism between PAX6 and PAX6(5a) is required for the strong activation of the mouse αB-crystallin promoter (Figs 2 and 3) in non-lens cells in combination with members of the large Maf family of transcription factors co-expressed *in vivo* in the lens with Pax6 (46–49), and known to regulate the endogenous αB-crystallin (42,44,45). Although the individual MafA and c-Maf proteins activated the αB-crystallin promoter (12.5- and 10.1-fold), their simultaneous presence in the absence of Pax6 proteins in 293T cells resulted in only 2.2-fold activation. This contrasted with robust activity (74.7-fold activation) of this promoter in the presence of PAX6, PAX6(5a), MafA and

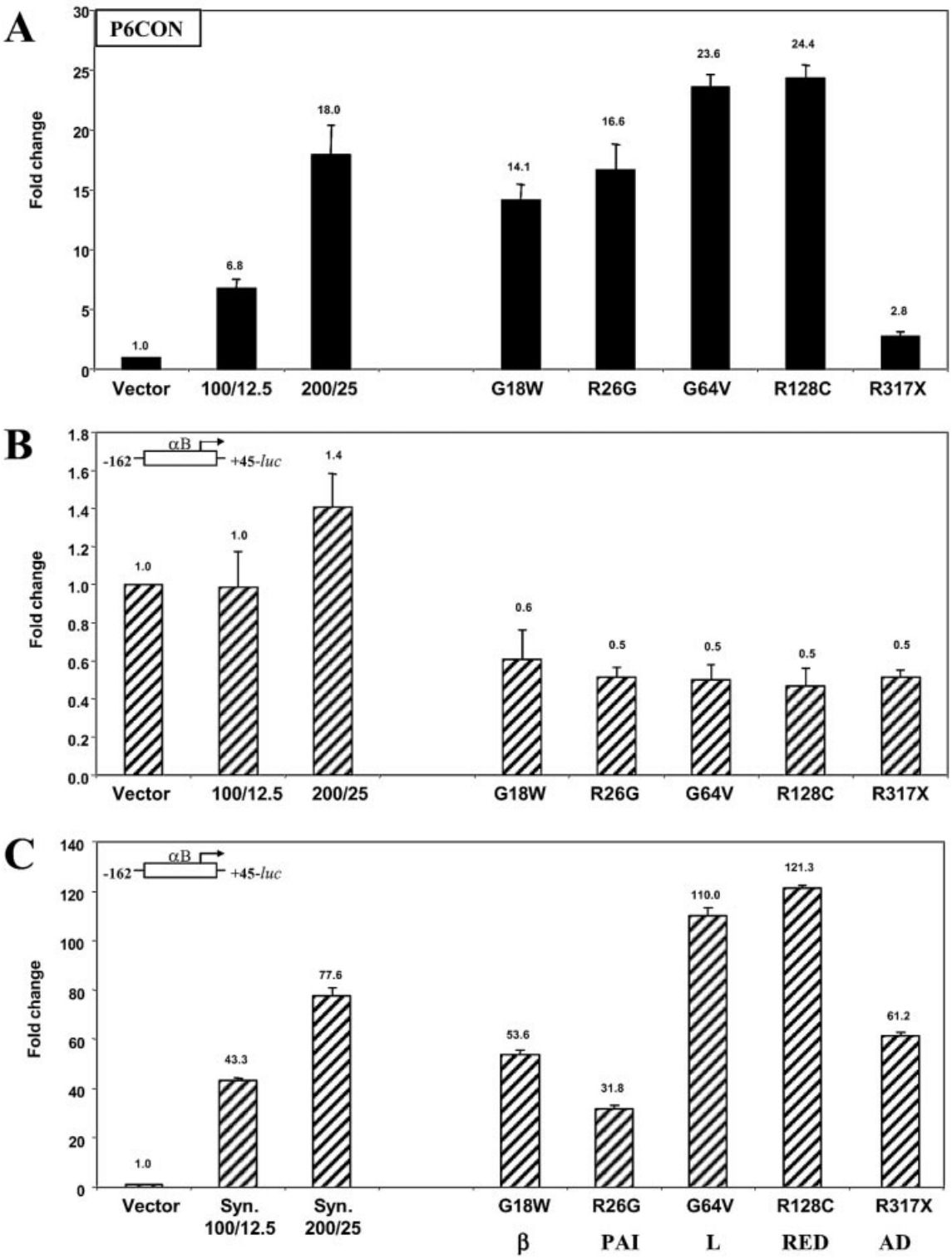

Figure 7. Haploinsufficiency of PAX6 mutants G18W, R26G, G64V, R128C and R317X. (**A**) Combined transactivation potential of PAX6, PAX6(5a) and their mutants with P6CON reporter. P6CON reporters were cotransfected with 100 ng of PAX6 and 12.5 ng of PAX6(5a), and 200 ng of PAX6 and 25 ng of PAX6(5a). The 200/25 experiment represents the wild-type situation as shown in Figure 6A. The 100/12.5 experiment represents a null mutation in one Pax6 allele. A mixture of four cDNAs mimicking Pax6 haploinsufficiency as illustrated in Figure 6A, i.e. 100 ng of PAX6, 100 ng of mutated PAX6, 12.5 ng of PAX6(5a) and 12.5 ng of mutated PAX6(5a) were tested. Compare results with Figure 6. (**B**) Haploinsufficiency of Pax6 on the mouse αB-crystallin promoter in the presence of PAX6/PAX6(5a) in 293T cells. Compare results with (C). (**C**) Haploinsufficiency of Pax6 on the mouse αB-crystallin promoter in the presence of PAX6, PAX6(5a), c-Maf and MafA. Mouse αB-crystallin promoters were cotransfected with a mixture of four cDNAs mimicking Pax6 haploinsufficiency [100 ng of each PAX6 and mutated PAX6, and 12.5 ng of each PAX6(5a) and mutated PAX6(5a)] and 80 ng of each c-Maf and MafA in 293T cells. The results are shown as means ± SD (n = 6). Mutants are described in the text and legend to Figure 6.

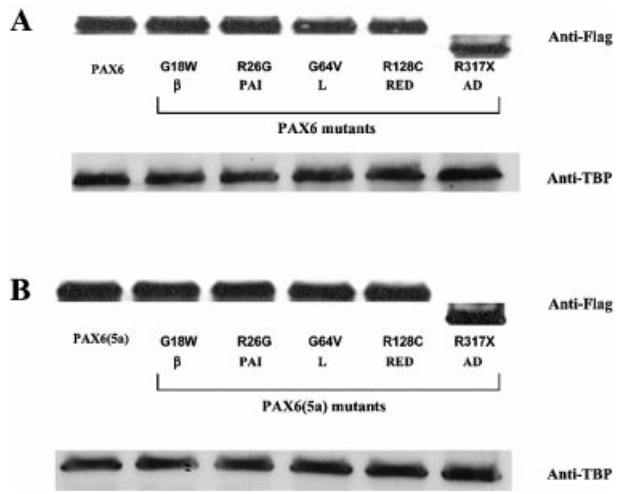

Figure 8. Western immunoblotting of PAX6 and PAX6(5a) mutants expressed in transiently transfected CHO-K1 cells. (**A**) The proteins were expressed as Flag-PAX6 with PAX6(5a) and probed with Flag M2 antibody for PAX6 series, or (**B**) as PAX6 with Flag-PAX6(5a) and probed with the same antibody to reveal PAX6(5a). TBP was detected with an anti-TBP antiserum and used as a loading control.

c-Maf. The likely mechanism for this activation involves recruitment of transcriptional co-activators p300/CBP by Pax6 and/or large Maf proteins reported earlier (57,58). In addition, physical interactions between Pax6 and c-Maf or MafA on the rat glucagon promoter have already been documented (59). Simultaneous transcriptional co-activation of the αB-crystallin gene by Pax6 and large Maf proteins correlates well with strong expression of αB-crystallin in lens epithelium (60), and in secondary lens fibers, that parallels the expression of these transcription factors in lens (44,45,61).

The present data on the mouse γF-crystallin promoter provide the answer to why an intact Pax6/Pax6(5a) binding site is required for retinoic acid-mediated activation of this promoter (37). The synergism is found only if PAX6, PAX6(5a) and RARβ/RXRβ are simultaneously tested in the experiment (Fig. 5). Synergistic interactions between Pax6, large Maf proteins, retinoic acid-activated nuclear receptors and Sox proteins are essential for the understanding of tissue-specific and extremely high expression of crystallin genes in the lens (5). None of these genes are tissue-specific, however, their unique combination in lens provides the optimal activation conditions.

Molecular mechanism of synergism between PAX6 and PAX6(5a)

The identification of synergism between PAX6 and PAX6(5a) in this report opens research avenues to understand its molecular mechanism. A direct physical interaction between PAX6 and PAX6(5a) has not been confirmed in the nuclear extracts using immunoprecipitations of FLAG- and HA-tagged co-expressed Pax6 proteins (data not shown). In addition, no ternary complexes between PAX6/PAX6(5a) and P6CON oligonucleotide used as a specific probe were detected in gel shift assays using the full-length recombinant Pax6 proteins (data not shown). These negative results are consistent with the reporter assays using the HDCON (see Fig. 1B) driven by a 'natural' PAX6 dimeric binding site (20). Thus, it is likely that on promoters where PAX6 directly interacts with DNA (e.g. P6CON and αB-crystallin promoter; 39,43), PAX6(5a) does not enter the assembling transcriptional machinery as a direct partner of PAX6, nor forms a stochiometric complex with PAX6 indirectly via another protein. Fractionation of crude lens nuclear extracts revealed the presence of Pax6 proteins in a large number of fractions, indicating that Pax6 associates with a number of other proteins (A.Cvekl, unpublished data). To address the specificity of the PAX6 interaction with PAX6(5a), we tested a number of HD-containing transcription factors co-expressed with Pax6 in mammalian embryonic eye and brain including Lhx2, Optx2/Six6, Six3a, Six3b, Msx2, Otx2, Pax2 and Pax3 (4,5,23) using P6CON-reporter and none of them could functionally synergize with PAX6. In contrast, two HD proteins, Msx2 and Otx2, actually reduced PAX6 activity (data not shown). Nevertheless, synergistic effects between PAX6 and PAX6(5a) agree well with the initial prediction that the Pax6 proteins are potent activators of transcription (20). When a C-terminal domain of Pax6 was tested with a GAL4 DNA-binding subdomain, the protein had 25% of the activity of GAL4 VP16, indicating the potency of the isolated Pax6 activation domain (20). However, transcriptional studies using Pax6 alone rarely exceeded 8-fold activation (23,33,35,40,43,62).

Haploinsufficiency of PAX6

Structural and functional studies of Pax6 mutants are essential for our understanding of human ocular, brain and olfactory abnormalities in Pax6 heterozygotes (3,32). These defects are thought to originate from aberrant expression of direct Pax6-target genes. However, only a fraction of these target genes may be compromised in the presence of missense PAX6 mutations as phenotypes associated with these mutations are, in about half of cases, moderate compared to the panocular aniridia (6). Employment of four Pax6 proteins simultaneously expressed in Pax6 heterozygous cells have shown here, for the first time, that the 'net effect' of Pax6 mutations cannot be predicted from the property of specific mutations tested only in the context of PAX6 (35,50–52,63). In addition, our recent DNA-binding studies of G18W, R26G, G64V and R128C mutants using gel shift assays revealed that these mutants possess a broad ability to interact with P6CON, 5aCON and HDCON binding sites (36), though the mutants clearly had either impaired or improved transactivation properties using the same binding sites (36). Thus, to examine the function of these mutants, one has to employ a physiologically relevant target gene and probe its regulation considering other regulatory proteins. Since αB-crystallin gene mRNA is reduced in Pax6 heterozygous (42) and c-Maf homozygous (45,46) lenses, it is the right dosage-sensitive gene to be tested with the mutants. Here, we have shown that the αB-crystallin promoter is particularly sensitive to mutants G18W and R26G, but not to G64V or R128C (Fig. 7B). Indeed, G18W and R26G may only affect *in vivo* the embryonic lens and cornea (64,65), where αB-crystallin is strongly expressed (60). In contrast, mutant R128C caused a specific retinal defect, a foveal hypoplasia (66). Further progress in understanding of abnormal ocular development in Pax6 heterozygous eyes and Pax6 homozygous ocular precursor tissues will depend on the identification and

functional characterization of Pax6 target genes followed by probing these genes with Pax6 missense mutants at haploinsufficient conditions.

ACKNOWLEDGEMENTS

We thank Drs Barbara Birshtein and Jonathan Warner for critical reading of the manuscript. We are grateful to Drs Jonathan Backer, Meinrad Busslinger, Juan Botas, Melinda Duncan, Tom Harris, Zbynek Kozmik, Richard Maas, Keiko Ozato, Elena Semina, Arun Sharma, Olof Sundin and Kazuhiko Yoshida for reagents used in this study. We thank Drs Ronald Burde, Harry Engel, Raju Kucherlapati and Scott Emmons for their encouragement during the course of this work. We are grateful to members of DNA Core Sequencing of the AECOM for their excellent services. Supported by NIH grant EY12200. A.C. was a recipient of a Research to Prevent Blindness Inc. (RPB) Career Development Award.

REFERENCES

1. Chi,N. and Epstein,J.A. (2002) Getting your Pax straight: Pax proteins in development and disease *Trends Genet.*, **18**, 41–47.
2. Simpson,T.I. and Price,D.J. (2002) Pax6: a pleiotropic player in development. *Bioessays*, **24**, 1041–1051.
3. Van Heyningen,V. and Williamson,K.A. (2002) PAX6 in sensory development. *Hum. Mol. Genet.*, **11**, 1161–1167.
4. Chow,R.L. and Lang,R.A. (2001) Early eye development in vertebrates. *Annu. Rev. Cell. Dev. Biol.*, **17**, 255–296.
5. Cvekl,A. and Piatigorsky,J. (1996) Lens development and crystallin gene expression: many roles for Pax-6. *Bioessays*, **18**, 621–630.
6. Glaser,T., Walton,D.S., Cai,J., Epstein,J.A., Jepeal,L. and Maas,R.L. (1995) PAX6 gene mutations in aniridia. In Wiggs,J. (ed.), *Molecular Genetics of Ocular Disease*. Wiley, New York, NY, pp. 55–81.
7. Mansouri,A., St-Onge,L. and Gruss,P. (1999) Role of genes in endoderm-derived organs. *Trends Endocrinol. Metab.*, **10**, 164–167.
8. Jun,S. and Desplan,C. (1996) Cooperative interactions between paired domain and homeodomain. *Development*, **122**, 2639–2650.
9. Xu,H.E., Rould,M.A., Xu,W., Epstein,J.A., Maas,R.L. and Pabo,C.O. (1999) Crystal structure of the human Pax6 paired domain–DNA complex reveals specific roles for the linker region and carboxy-terminal subdomain in DNA binding. *Genes Dev.*, **13**, 1263–1275.
10. Kozmik,Z., Czerny,T. and Busslinger,M. (1997) Alternatively spliced insertions in the paired domain restrict the DNA sequence specificity of Pax6 and Pax8. *EMBO J.*, **16**, 6793–6803.
11. Epstein,J.A., Glaser,T., Cai,L., Jepeal,L., Walton,D.S. and Maas,R.L. (1994) Two independent and interactive DNA-binding subdomains of the Pax6 paired domain are regulated by alternative splicing. *Genes Dev.*, **8**, 2022–2034.
12. Vogan,K.J., Underhill,D.A. and Gros,P. (1996) An alternative splicing event in the Pax-3 paired domain identifies the linker region as a key determinant of paired domain DNA-binding activity. *Mol. Cell. Biol.*, **16**, 6677–6686.
13. Kozmik,Z., Kurzbauer,R., Dörfler,P. and Busslinger,M. (1993). Alternative splicing of pax-8 gene transcripts is developmentally regulated and generates isoforms with different transactivation properties. *Mol. Cell. Biol.*, **13**, 6024–6035.
14. Carriere,C., Plaza,S., Martin,P., Quatannens,B., Bailly,M., Stehelin,D. and Saule,S. (1993) Characterization of quail Pax-6 (Pax-QNR) proteins expressed in the neuroretina. *Mol. Cell. Biol.*, **13**, 7257–7266.
15. Koroma,B., Yang,,J. and Sundin,O. (1997) The Pax-6 homeobox gene is expressed throughout the corneal and conjunctival epithelia. *Invest. Ophthalmol. Vis. Sci.*, **38**, 108–120.
16. Richardson,J., Cvekl,A. and Wistow,G. (1995) Pax-6 is essential for lens-specific expression of ζ-crystallin. *Proc. Natl Acad. Sci. USA*, **92**, 4676–4680.
17. Zhang,W., Cveklova,K., Oppermann,B., Kantorow,M. and Cvekl,A. (2001) Quantitation of PAX6 and PAX6(5A) transcript levels on adult human lens, cornea and monkey retina. *Mol. Vis.*, **7**, 1–5.
18. Jaworski,C., Sperbeck,S., Graham,C. and Wistow,G. (1997) Alternative splicing of Pax6 in bovine eye and evolutionary conservation of intron sequences. *Biochem. Biophys. Res. Commun.*, **240**, 196–202.
19. Singh,S., Mishra,R., Arango,N.A., Deng,J.M., Behringer,R.R. and Saunders,G.F. (2002) Iris hypoplasia in mice that lack the alternatively spliced Pax6(5a) isoform. *Proc. Natl Acad. Sci. USA*, **99**, 6812–6815.
20. Czerny,T. and Busslinger,M. (1995) DNA-binding and transactivation properties of Pax-6: three amino acids in the paired domain are responsible for the different sequence recognition of Pax-6 and BSAP (Pax-5). *Mol. Cell. Biol.*, **15**, 2858–2871.
21. Eberhard,D. and Busslinger,M. (1999) The partial homeodomain of the transcription factor Pax-6 (BSAP) is an interaction motif for the retinoblastoma and TATA-binding proteins. *Cancer Res.*, **59S**, 1716s–1724s.
22. Cvekl,A., Kashanchi,F., Brady,J.N. and Piatigorsky,J. (1999) Pax-6 interactions with TATA-box binding protein and retinoblastoma protein. *Invest. Ophthalmol. Vis. Sci.*, **40**, 1343–1350.
23. Mikkola,I., Bruun,J.-A., Holm,T. and Johansen,T. (2001) Superactivation of Pax6-mediated transactivation from paired domain-binding sites by DNA-independent recruitment of different homeodomain proteins. *J. Biol. Chem.*, **276**, 4109–4118.
24. Jang,C.-C., Chao,J.-L., Jones,N., Yao,L.-C., Bessarab,D.A., Kuo,Y.M., Jun,S., Desplan,C., Beckendorf,S.K. and Sun,Y.H. (2003) Two Pax genes, *eye gone* and *eyeless*, act cooperatively in promoting *Drosophila* eye development. *Development*, **130**, 2939–2951.
25. Dominguez,M., Ferres-Marco,D., Gutierrez-Aviño,F.J., Speicher,S.A. and Beneyto,M. (2004) Growth and specification of the eye are controlled independently by eye gone and eyeless in *Drosophila melanogaster*. *Nature Genet.*, **36**, 31–39.
26. Gehring,W.J. and Ikeo,K. (1999) Pax-6: mastering eye morphogenesis and eye evolution. *Trends Genet.*, **15**, 371–377.
27. Czerny,T., Halder,G., Kloter,U., Souabni,A., Gehring,W.J. and Busslinger,M. (1999) Twin of eyeless, a second Pax-6 gene of *Drosophila*, acts upstream of eyeless in the control of eye development. *Mol. Cell*, **3**, 297–307.
28. Glaser,T., Jepeal,L., Edwards,J.G., Young,R.S., Favor,J. and Maas,R.L. (1994) *PAX6* gene dosage effect in a family with congenital cataracts, aniridia, anophthalmia and central nervous system defects. *Nature Genet.*, **7**, 463–471.
29. Sisodiya,S.M., Free,S.L, Williamson,K.A., Mitchell,T.N., Willis,C., Stevens,J.M., Kendall,B.E., Shorvon,S.D., Hanson,I.M., Moore,A.T. and van Heyningen,V. (2001) PAX6 haploinsufficiency causes cerebral malformation and olfactory dysfunction in humans. *Nature Genet.*, **28**, 214–216.
30. Nelson,L.B., Spaeth,G.L., Nowinski,T.S., Margo,C.E. and Jackson,L. (1984) Aniridia. A review. *Surv. Ophthalmol.*, **28**, 621–642.
31. Hittner,H.M. (1989) Aniridia. In Ritch,R., Shields,M.B. and Krupin,T. (eds), *The Glaucomas*. The C.V.Mosby Comp., St Louis, MO, pp. 869–884.
32. Prosser,J. and van Heyningen,V. (1998) PAX6 mutations reviewed. *Hum. Mutat.*, **11**, 93–108.
33. Duncan,M.K., Cvekl,A., Li,X. and Piatigorsky,J. (2000) Truncated forms of Pax-6 disrupt lens morphology in transgenic mice. *Invest. Ophthalmol. Vis. Sci.*, **41**, 464–473.
34. Singh,S., Tang,H.K., Lee,J.-Y. and Saunders,G.F. (1998) Truncation mutations in the transactivation region of PAX6 result in dominant-negative mutants. *J. Biol. Chem.*, **273**, 21531–21541.
35. Yamaguchi,Y., Sawada,J., Yamada,M., Handa,H. and Azuma,N. (1997) Autoregulation of Pax6 transcriptional activation by two distinct DNA-binding subdomains of the paired domain. *Genes Cells*, **2**, 255–262.
36. Chauhan,B.K., Yang,Y., Cveklova,K. and Cvekl,A. (2004) Functional properties of natural human PAX6 and PAX6(5a) mutants. *Invest. Ophthalmol. Vis. Sci.*, **45**, 385–392.
37. Kralova,J., Czerny,T., Spanielova,H., Ratajova,V. and Kozmik,Z. (2002) Complex regulatory element within the γE- and γF-crystallin enhancers mediates Pax6 regulation and is required for induction by retinoic acid. *Gene*, **286**, 271–282.
38. Gopal-Srivastava,R. and Piatigorsky,J. (1994) Identification of a lens-specific regulatory region (LSR) of the murine αB-crystallin gene. *Nucleic Acids Res.*, **22**, 1281–1286.
39. Gopal-Srivastava,R., Cvekl,A. and Piatigorsky,J. (1998) Involvement of retinoic acid/retinoid receptors in the regulation of murine αB-crystallin/small heat shock protein gene expression in the lens. *J. Biol. Chem.*, **273**, 17954–17961.

40. Cvekl,A., Kashanchi,F., Sax,C.M., Brady,J.N. and Piatigorsky,J. (1995) Transcriptional regulation of the mouse αA-crystallin gene: activation dependent on a cyclic AMP-responsive element (DE1/CRE) and a Pax-6-binding site. *Mol. Cell. Biol.*, **15**, 653–660.
41. Baulmann,D.C., Ohlmann,A., Flügel-Koch,C., Goswami,S., Cvekl,A. and Tamm,E.R. (2002) Pax6 heterozygous eyes show defects in chamber angle differentiation that are associated with a wide spectrum of the other anterior eye segment abnormalities. *Mech. Dev.*, **118**, 3–17.
42. Chauhan,B.K., Zhang,,W., Cveklova,K., Kantorow,M. and Cvekl,A. (2002) Identification of differentially expressed genes in mouse Pax6 heterozygous lenses. *Invest. Ophthalmol. Vis. Sci.*, **43**, 1884–1890.
43. Gopal-Srivastava,R., Cvekl,A. and Piatigorsky,J. (1996) Pax-6 and αB-crystallin/small heat shock protein gene regulation in the murine lens. Interaction with the lens-specific regions, LSR1 and LSR2. *J. Biol. Chem.*, **271**, 23029–23036.
44. Ring,B.Z., Cordes,S.P., Overbeek,P.A. and Barsh,G.S. (2000) Regulation of mouse lens fiber cell development and differentiation by the Maf gene. *Development*, **127**, 307–317.
45. Kawauchi,S., Takahashi,S., Nakajima,O., Ogino,H., Morita,M., Nishizawa,M., Yasuda,K. and Yamamoto,M. (1999) Regulation of lens fiber cell differentiation by transcription factor c-Maf. *J. Biol. Chem.*, **274**, 19254–19260.
46. Kataoka,K., Han,S., Shioda,S., Hirai,M., Nishizawa,M. and Handa,H. (2002) MafA is a glucose-regulated and pancreatic β-cell-specific transcriptional activator for the insulin gene. *J. Biol. Chem.*, **277**, 49903–49910.
47. Benkhelifa,S., Provot,S., Nabais,E., Eychene,A., Calothy,C. and Felder-Schmittbuhl,M.P. (2001) Phosphorylation of MafA is essential for its transcriptional and biological properties. *Mol. Cell. Biol.*, **21**, 4441–4452.
48. Olbrot,M., Rud,J., Moss,L.G. and Sharma,A. (2002) Identification of β-cell-specific insulin gene transcription factor RIPE3b1 as mammalian MafA. *Proc. Natl Acad. Sci. USA*, **99**, 6737–6742.
49. Lecoin,L., Sii-Felice,K., Pouponnot,C., Eychene,A. and Felder-Schmittbuhl,M.P. (2004) Comparison of maf gene expression patterns during chick embryo development. *Gene Expr. Patterns*, **4**, 35–46.
50. Tang,H.K., Chao,L.-Y. and Saunders,G.F. (1997) Functional analysis of paired box missense mutations in the PAX6 gene. *Hum. Mol. Genet.*, **6**, 381–386.
51. Chao,L.-Y., Mishra,R., Strong,L.C. and Saunders,G.F. (2003) Missense mutations in the DNA-binding region and termination codon in PAX6. *Hum. Mutat.*, **2**, 138–145.
52. Singh,S., Stellrecht,C.M., Tang,H.K. and Saunders,G.F. (2000) Modulation of PAX6 homeodomain function by the paired domain. *J. Biol. Chem.*, **275**, 17306–17313.
53. Dressler,G.R., Wilkinson,J.E., Rothenpieler,U.W., Patterson,L.T., Williams-Simons,L. and Westphal,H. (1993) Deregulation of Pax-2 expression in transgenic mice generates severe kidney abnormalities. *Nature*, **362**, 65–67.
54. Funke,B., Epstein,J.A., Kochilas,L.K., Lu,M.M., Pandita,R.K., Liao,J., Bauerndistel,R., Schuler,T., Schorle,H., Brown,M.C., Adams,J. and Morrow,B.E. (2001) Mice overexpressing genes from the 22q11 region deleted in velo-cardio-facial syndrome/DiGeorge syndrome have middle and inner ear defects. *Hum. Mol. Genet.*, **10**, 2549–2556.
55. Schedl,A., Ross,A., Lee,M., Engelkamp,D., Rashbass,P., van Heyningen,V. and Hastie,N. (1996) Influence of Pax6 gene dosage on development: overexpression causes severe eye abnormalities. *Cell*, **86**, 71–82.
56. Semina,E.V., Ferrell,R.E., Mintz-Hittner,H.A., Bitoun,P., Alward,W.L.M., Reiter,R.S., Funkhauser,C., Daack-Hirsch,S. and Murray,J.C. (1998) A novel homeobox gene PITX3 is mutated in families with autosomal-dominant cataracts and ASMD. *Nature Genet.*, **19**, 167–170.
57. Hussain,M.A. and Habener,J.F. (1999) Glucagon gene transcription activation mediated by synergistic interactions of pax-6 and cdx-2 with the p300 co-activator. *J. Biol. Chem.*, **274**, 28950–28957.
58. Chen,Q., Dowhan,D.H., Liang,D., Moore,D.D. and Overbeek,P.A. (2002) CREB-binding protein/p300 co-activation of crystallin gene expression. *J. Biol. Chem.*, **277**, 24081–24089.
59. Planque,N., Leconte,L., Coquelle,F.M., Benkhelifa,S., Martin,P., Felder-Schmittbuhl,M.P. and Saule,S. (2001) Interaction of Maf transcription factors with Pax-6 results in synergistic activation of the glucagon promoter. *J. Biol. Chem.*, **276**, 35751–35760.
60. Robinson,M.L. and Overbeek,P.A. (1996) Differential expression of αA- and αB-crystallin during murine ocular development. *Invest. Ophthalmol. Vis. Sci.*, **37**, 2276–2284.
61. Duncan,M.K., Haynes,J.I., Cvekl,A. and Piatigorsky,J. (1998) Dual roles for Pax-6: a transcriptional repressor of lens fiber cell-specific β-crystallin genes. *Mol. Cell. Biol.*, **18**, 5579–5586.
62. Goudreau,G., Petrou,P., Reneker,L.W., Graw,J., Loster,J. and Gruss,P. (2002) Mutually regulated expression of Pax6 and Six3 and its implications for the Pax6 haploinsufficient lens phenotype. *Proc. Natl Acad. Sci. USA*, **99**, 8719–8724.
63. Mishra,R., Gorlov,I.P., Chao,L.Y., Singh,S. and Saunders,G.F. (2002) PAX6, paired domain influences sequence recognition by the homeodomain. *J. Biol. Chem.*, **277**, 49488–49494.
64. Wolf,M.T.F., Lorenz,B., Winterpacht,A., Drechsler,M., Schumacher,V., Royer-Pokora,B., Blankenagel,A., Zabel,B. and Wildhardt,G. (1998) Ten novel mutations found in Aniridia. *Hum. Mutat.*, **12**, 304–313.
65. Hanson,I.M., Fletcher,J.M., Jordan,T., Brown,A., Taylor,D., Adams,R.J., Punnett,H.H. and van Heyningen,V. (1994) Mutations at the *PAX6* locus are found in heterozygous anterior segment malformations including Peters' anomaly. *Nature Genet.*, **6**, 168–173.
66. Azuma,N., Nishina,S., Yanagisawa,H., Okuyama,T. and Yamada,M. (1996) PAX6 missense mutation in isolated foveal hypoplasia. *Nature Genet.*, **13**, 141–142.
67. Hanson,I., Churchill,A., Love,J., Axton,R., Moore,T., Clarke,M., Meire,F. and van Heyningen,V. (1999) Missense mutations in the most ancient residues of the PAX6 paired domain underlie a spectrum of human congenital diseases. *Hum. Mol. Genet.*, **8**, 165–172.