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Overexpression of PAX6(5a) in lens fiber cells results in cataract and upregulation of α5β1 integrin expression

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SUMMARY

The *PAX6* gene, a key regulator of eye development, produces two major proteins that differ in paired domain structure: PAX6 and PAX6(5a). It is known that an increase in the PAX6(5a) to PAX6 ratio leads to multiple ocular defects in humans. Here, transgenic mice were created that overexpress human PAX6(5a) in the lens. These mice develop cataracts with abnormalities in fiber cell shape as well as fiber cell/lens capsule and fiber cell/fiber cell interactions. While the structure of the actin cytoskeleton appeared relatively normal, the cataractous lens expresses increased amounts of paxillin and p120^{ctn} as well as large aggregates of α5β1 integrin in the dysgenic fiber cells. The elevated amounts of these proteins in the transgenic lens correlated well with elevated levels of their respective mRNAs. To investigate the role of Pax-6(5a) in the upregulation of these genes, a series of gel shift experiments using truncated proteins and consensus oligonucleotides demonstrated the complexity of Pax-6 and Pax-6(5a) binding to DNA, aiding our identification of potential binding sites in the human α5- and β1-integrin promoters. Consequent gel shift analysis demonstrated that these putative regulatory sequences bind Pax-6 and/or Pax-6(5a) in lens nuclear extracts, suggesting that the human α5 and β1 integrin promoters contain PAX6/PAX6(5a) binding sites and maybe directly regulated by this transcription factor in the transgenic lens. We conclude that these transgenic mice are good models to study a type of human cataract and for identifying batteries of genes that are directly or indirectly regulated by both forms of Pax-6.

Key words: Paired domain, Homeodomain, Lens, Transgenic, Cataract

INTRODUCTION

The Pax genes are transcriptional regulators that play diverse roles in development and disease (Stuart and Gruss, 1996). All members of the Pax family have an N-terminal 128 amino acid paired domain (PD) that contains two independent helix-turn-helix globular DNA-binding subdomains (Wilson et al., 1995), the N-subdomain (PAI) and the C-subdomain (RED) (Jun and Desplan, 1996). In Pax-3, 4, 6 and 7, the paired domain is followed by a second DNA-binding helix-turn-helix motif comprising a paired-type homeodomain, HD (Wilson et al., 1995). The Pax genes are often alternatively spliced and generate multiple proteins, each with a potentially different biological function (Epstein et al., 1994b; Kozmik et al., 1997).

Heterozygous mutations in the gene for one Pax family member, *PAX6*, result in the human eye defects aniridia, autosomal dominant keratitis, Peter's anomaly, congenital nystagmus (Hanson et al., 1999), foveal hypoplasia, and cataract (Glaser et al., 1995). Homozygous *Pax-6* mutations result in the absence of eyes and pancreatic alpha cells as well as defective central nervous system and facial development (Grindley et al., 1995; Onge et al., 1997). Alternative splicing generates two major forms of Pax-6: the predominant 46 kDa species and Pax-6(5a), a 48 kDa protein containing an additional 14 amino acid peptide (Epstein et al., 1994b; Jaworski et al., 1997; Richardson et al., 1995). Since the 5a-encoded peptide disrupts the ability of PAI to bind DNA, Pax-6(5a) binding depends only on RED (Epstein et al., 1994b; Kozmik et al., 1997) and HD (Sheng et al., 1997a,b). In contrast, Pax-6 can interact with DNA via either PAI, RED, HD or combinations thereof. Thus, Pax-6 may recognize a wider spectrum of DNA sequences than Pax-6(5a).

While some direct targets for Pax-6 function are known, the majority of Pax-6 subordinated genes remain to be discovered. In this study, we generated a transgenic mouse that specifically overexpresses PAX6(5a) in the lens. Our initial hypothesis was that overexpressed PAX6(5a) would activate genes that contain binding sites recognized by RED and/or HD. Indeed, the balance between PAX6 and PAX6(5a) expression is essential for normal lens physiology in vivo as evidenced by the

congenital cataracts seen in humans with either mutations causing the relative overexpression of PAX6(5a) to PAX6 (Epstein et al., 1994b) or harboring a missense mutation in the 5a exon (Azuma et al., 1999). Our data show that overexpression of PAX6(5a) in lens fiber cells results in anomalous lens morphology and overexpression of proteins involved in cell signaling and adhesion, including α5β1 integrin, a fibronectin receptor (Johansson et al., 1997). Finally, we provide Pax-6-DNA-binding data suggesting that PAX6(5a)/PAX6 participates in the transcriptional regulation of the α5- and β1-integrin genes.

MATERIALS AND METHODS

Recombinant plasmids

Bacterial expression plasmids producing truncated human Pax-6 or Pax-6(5a) proteins fused to glutathione-S-transferase (GST) (Epstein et al., 1994a,b) were provided by Drs J. Epstein and R. L. Maas (Harvard Medical School, Boston, MA). The GST-PAX6 (PD/HD) expression plasmid (aa 1-285) was as described (Cvekl et al., 1999). The GST-PAX6(5a) (PD5a/HD; aa 1-294) expression plasmid was generated by PCR amplification of the human Pax-6 (5a) cDNA (Epstein et al., 1994b) using the following primers; forward 5′ ACT GCC GGA TCC ATG CAG AAC AGT CAC AGC GGA 3′; and reverse, 5′ ACT GCC GAT ATC TTA AGG AAT GTG ACT AGG AGT GTT GCT GGC 3′. The resulting product was gel purified, digested with *Bam*HI and *Eco*RV, ligated into the *Bam*HI/*Sma*I site of pGEX-2T (Amersham-Pharmacia Biotech, Piscataway, NJ), and confirmed by DNA sequencing. The construct used to overexpress PAX6(5a) in the lens of transgenic mice, pACP3-PAX6(5a), was generated by digesting the human PAX6(5a) expression vector, pCMVPAX6(5a), (Epstein et al., 1994b) with *Not*I and ligating the PAX6(5a) cDNA into pACP3 which was partially digested with *Bsp*120I. pACP3 contains the mouse αA-crystallin promoter (−342/+49), a polylinker, and the SV40 small-T antigen intron and polyadenylation signal (Duncan et al., 2000).

Generation and screening of transgenic mice

The fragment containing the αA-crystallin promoter, PAX6(5a) coding sequence, and the SV40 small T antigen intron and polyadenylation sequence was liberated from pACP3-PAX6(5a) by digestion with *Not*I and *Pvu*I and purified as described (Duncan et al., 1995). The fragment was used by the National Eye Institute transgenic mouse facility to generate transgenic mice (strain FVB/N). Transgenic animals were identified by PCR analysis of DNA obtained by tail biopsy using primers derived from the SV40 small T intron (Reneker and Overbeek, 1996). All experiments utilizing animals were approved by the National Eye Institute and University of Delaware institutional review boards. Gross anatomy and histology of lenses were analyzed as described (Duncan et al., 2000).

Analysis of transgene expression

RNA was purified from the lenses of 16-week-old transgenic mice using the SV total RNA isolation kit (Promega) and analyzed by RT-PCR using the SV40 small T antigen derived primers described previously (Reneker and Overbeek, 1996). The ratio of PAX6(5a) to Pax-6(con) expression levels were measured by RT-PCR using PD primers flanking the 5a exon whose sequence is identical in both mouse and human Pax-6 (5′ CCA GCA TGC AGA ACA GTC ACA G 3′; 5′ TGG GCT ATT TTG CTT ACA ACT T 3′).

Western blot analysis

Lenses were homogenized in 200 μl of a buffer containing 20 mM sodium phosphate, 1 mM EGTA, pH 7.0. The soluble and insoluble fractions were separated and the insoluble fraction was solubilized in 1% SDS and its concentration determined by the D_c protein assay (Bio-Rad, Hercules, CA). Fifty micrograms of solubilized protein was separated on a 6% SDS-PAGE gel and blotted onto nitrocellulose filters. Blots were incubated with rat monoclonal antibodies against either α5, α6 or β1-integrin diluted according to the manufacturer's recommendations (PharMingen International, San Diego, CA). Bound antibody was detected with horseradish peroxidase-linked sheep anti-rat IgG (Amersham/Pharmacia) and a chemiluminescent substrate (DuoLux, Vector Laboratories).

Expression of N-cadherin, E-cadherin, paxillin, β-catenin, α-catenin, and $p120^{ctn}$ was analyzed by preparing protein extracts with TRIzol (Life Technologies) from lenses of three week or four month old mice and using reference extracts from Transduction Laboratories (Lexington, KY). Following SDS-PAGE and transfer to Hybond-ECL membrane (Amersham/Pharmacia Biotech), the membrane was incubated with mouse monoclonal antibodies against these proteins (Transduction Laboratories). Note that the $p120^{ctn}$ antibody is listed as pp120 <src substrate> also known as p120cas in the Tranduction Laboratories catalog (Zondag et al., 2000). Bound antibody was detected with horseradish peroxidase-linked sheep anti-mouse IgG (Amersham/Pharmacia Biotech) and a chemiluminescent substrate (SuperSignal, Pierce,).

RT-PCR

The following specific primers were used: α5 integrin (TCC TGC ATC AAC CTT AGC TTC and TTC TTC TCC CCA TAC ACA TCC, GenBank accession no. X79003), β1 integrin (ATG GGA CAG GAG AAA ATG GAC and TCT CTC TTC CTA CAC ACA CAC; GenBank accession no.Y00769), paxillin (AAC AAG CAG AAG TCA GCA GAG CC and CTA GCT TGT TCA GGT CGG AC; Mazaki et al., 1998), and $p120^{ctn}$ (AGG AGC TTC GGA AGC CAC TG and GCG AAG AAA GGA AAA AAA TC; vanHengel et al., 1999), giving specific products of 431, 409, 582, and 347 bp, respectively. Control sets of primers amplifying β-actin (TGT GAT GGT GGG AAT GGG TCA G and TTT GAT GTC ACG CAC GAT TTC C; Clontech Laboratories) and GAPDH (CCC TTC ATT GAC CTC AAC and TTC CAC CCA TCA CAA AC; Tamm et al., 1999) were used giving specific products of 514 and 301 bp, respectively. RT-PCR reactions (total volume of 25 μl) were performed using a SuperScript II One-step RT PCR kit (Life Technologies), RT step was conducted at 50°C for 30 minutes and a specific number of PCR cycles at 94/55/72°C each for 45 seconds using the Eppendorf Mastercycler gradient cycler. Specificity of primers and reaction conditions were tested first using E18.5 day mouse embryonic RNA. Next, DNA-free total RNAs isolated from wild-type and transgenic lenses were titrated to yield similar amounts of β-actin and GAPDH products within the range of 22-25 cycles. β1-integrin products were detectable with 22 to 25 cycles while α5-integrin, paxillin, and $p120^{ctn}$ specific products were visualized after 31 to 34 cycles. All agarose gels used for the semiquantitative analysis of PCR products were stained with EtBr after the electrophoresis according to FMC BioProducts instructions. PCR products were isolated using GFX columns (Pharmacia), subcloned into pT7Blue-3 vector (Novagen) and sequenced using the ABI370 Sequencer (Perkin Elmer) at the AECOM DNA Core Sequencing Facility.

Confocal microscopy

In order to visualize the actin cytoskeleton in four-week-old mice, lenses were isolated, fixed 18 hours in neutral buffered formalin, embedded in 3% agarose and 150 μm thick vibratome sections prepared. Sections were then incubated with a mixture of Texas red phalloidin (1:100 dilution, Molecular Probes, Eugene OR) to stain actin and SYTO13 (Ankarcrona et al., 1995; 1:1000 dilution, Molecular Probes) to visualize nuclei.

For immunocytochemistry, unfixed lenses were embedded in TFM (Triangle Biomedical, Durham, NC) and 12-15 μm frozen sections

prepared. The sections were collected on silanized glass slides and stored at –80°C until use. The slides were fixed in 1:1 acetone/methanol at –20°C for 20 minutes then allowed to air dry. Integrins were detected with the antibodies described above, Pax-6/PAX6 was detected by using an anti-Pax-6 paired domain mouse monoclonal antibody (Ericson et al., 1997) (Developmental Studies Hybridoma Bank, Iowa City, IA), paxillin was detected using a fluorescein labeled anti-paxillin monoclonal antibody (Transduction Laboratories) and fibronectin was detected with a polyclonal antisera (Chemicon International, Temecula, CA). The bound, non-fluoresceinated antibodies were visualized following incubation with the appropriate secondary antibody (anti-rabbit, anti-mouse or anti-rat IgG) conjugated to Rhodamine Red X (1:50 dilution; Jackson Immunologicals, West Grove, PA) and the cell nuclei were detected by counterstaining with SYTO13 (1:1000 dilution in PBS; Molecular Probes). All confocal microscopy was performed on a Zeiss 510 LSM confocal microscope configured with an Argon/Krypton laser (488 nm and 568 nm excitation lines).

Expression of proteins in *E. coli* and protein purification

GST-fusion proteins were produced as previously described (Cvekl et al., 1999). Nuclear extracts were prepared from the mouse lens derived cell line αTN4-1 as previously described (Cvekl et al., 1995a).

Electrophoretic mobility shift assays (EMSA)

The following oligonucleotides were synthesized, radiolabeled with ^{32}P using T4 polynucleotide kinase, and annealed with their complement; 5aCON (Epstein et al., 1994b), 5′ AAT AAA TCT GAA CAT GCT CAG TGA ATG TTC ATT GAC TCT CTC GAG GTC A 3′; P6CON (Epstein et al., 1994a), 5′ GGA TGC AAT TTC ACG CAT GAG TGC CTC GAG GGA TCC ACG TCG A 3′; α5-integrin (+196/+225), 5′ CCT TCT TCG GAT TCT CAG TGG AGT TTT ACC 3′; α5-integrin (–451/–422), 5′ TCT CCT CCC TAA AGC ACT GAG GGA GAC CCC 3′; α5-integrin (–701/–672), 5′ ATC CTC CTG ATG AAT GAC CCC AAA CTC CTG 3′; β1-integrin (–1398/–1358), 5′ AAA TTC AAG AAT GTT GAG TTC TAA TGG CAG TTT GAC CAT TGA GAG ATT AAT A 3′ and β1-integrin (–521/–492), 5′ AAA CGG GAC ATC AGT GGA CAA ACG GGA GCG 3′. Oligonucleotides containing Pax-6 binding sites from crystallin promoters, site δ1-crystallin IIa (Cvekl et al., 1995b), 5′ GAT CCC TAT ACA ATA TTG TGC ATT GTC TTC TAC TG 3′; and αA-crystallin site E (Cvekl et al., 1994), 5′ GAT CCC CAC TAA TGC CTT CAT TCT GCG AGA GCA GTA TAG 3′ were synthesized, annealed with their complement and cloned into the *Bam*HI/*Sal*I site of pGEM-4Z (Promega, Madison, WI). For EMSAs, *Eco*RI-*Hin*dIII fragments were generated from the respective plasmids, end labeled with ^{32}P using Klenow enzyme, and gel purified.

EMSAs with purified Pax-6 derived proteins were conducted as described previously (Cvekl et al., 1994) using the probes described above in the presence of 1 μg of poly[d(A-T)] as a non-specific competitor. The amount of each recombinant protein used (typically between 200 and 400 ng) was determined by titration in order to obtain protein/DNA interactions of intensities and patterns comparable to those reported previously (Epstein et al., 1994a,b) with consensus Pax-6 sites. EMSAs with αTN4-1 nuclear extracts were as described (Cvekl et al., 1995a) except either 1 or 2 μg of poly[d(A-T)] was used as non-specific competitor. The identity of the Pax-6 complex was determined by preincubation with antisera 11 raised against the PD of quail Pax-6 (Carriere et al., 1993).

RESULTS

αA-crystallin/PAX6 (5a) transgenic mice develop cataracts

Three independent founders harboring the αA-crystallin/PAX6(5a) transgene were generated. Two of these founders had severe, bilateral cataracts (Fig. 1) while the third was morphologically normal (data not shown). One of the affected founders did not transmit the transgene to its progeny and was not analyzed further. The other affected founder transmitted the transgene and the penetrance of the cataract phenotype relative to the presence of the transgene was 100%. RNA was isolated from lenses harvested from mice of the two established lines and subjected to RT-PCR analysis using primers derived from sequence flanking the SV40 small T intron. Mice from the line affected with cataracts expressed the transgene (Fig. 2A) while mice from the unaffected line did not (data not shown). Thus, further analysis was only conducted on the line expressing the transgene.

The amount of PAX6(5a) mRNA produced from the transgene relative to the amount of Pax-6 produced endogenously was studied by RT-PCR performed on total lens RNA using primers that recognize the paired domains of both the human derived PAX6(5a) transgene and endogenous mouse Pax-6 (Fig. 2B). This analysis confirms previous studies that show that the wild-type lens expresses much higher levels of canonical Pax-6 than Pax-6(5a) (Jaworski et al., 1997; Richardson et al., 1995) and strongly suggests that the transgenic lens is expressing approximately equal amounts of PAX6(5a) and canonical Pax-6. Immunohistochemistry was then performed on lenses using a monoclonal antibody that recognizes both the Pax-6 PD and PD(5a) (Ericson et al., 1997; Nishina et al., 1999). In the lens of wild-type mice, Pax-6 protein was detected predominately in the nuclei of epithelial cells with protein levels decreasing in newly differentiated fiber cells (Fig. 2C) as previously reported in the avian lens (Li et al., 1994). In the 3-week-old transgenic lens, Pax-6 protein is still detected in the epithelium as expected. In addition, Pax-6 levels remained elevated in the nuclei of newly differentiated lens fiber cells of the transgenic mice consistent with the expression pattern expected from the αA-crystallin promoter (Fig. 2D; Robinson et al., 1995). Qualitatively, Pax-6 expression levels also appeared elevated in the lens epithelium, however, western blot analysis was not sufficiently sensitive to quantify this difference.

Overexpression of Pax-6 disrupts normal lens structure

Lenses from newborn mice overexpressing PAX6(5a) are slightly smaller than normal and stain unevenly with eosin, however, their overall morphology is relatively normal (data not shown). By three weeks of age, the transgenic lens is noticeably smaller than wild type (Fig. 1B,D; Table 1) and exhibits a number of morphological alterations (Fig. 1D,F). Notably, cells in the epithelial to fiber transition zone are less elongated and much wider than normal, deeper in the lens these cells take on a swollen, balloon shaped appearance.

Since cytoskeletal rearrangements seemed possible, the actin cytoskeleton was stained with Texas Red phalloidin (Fig. 3). This stain not only clearly reveals the cell shape changes, but also demonstrates that the filamentous actin cytoskeleton of the PAX6(5a) transgenic lens still localizes to the cell membranes (Fig. 3B), a distinctive feature of normal fiber cell morphology (Lo et al., 1997). Counterstaining of phalloidin stained lenses with Syto13, a DNA stain used to detect pycnotic nuclei (Ankarcrona et al., 1995), revealed that the nuclear

morphology of the swollen lens fibers was relatively normal (Fig. 3B). The nuclei of balloon shaped cells were somewhat abnormal (Fig. 3D) consistent with the observation that some regions of the lens contained vacuoles and regions apparently filled with cell debris (Fig. 1D). By twenty weeks of age, the lens structure of the PAX6(5a) transgenics is highly disordered and characterized by a breakdown of much of the cellular structure. Commonly, the posterior capsule ruptures and the lens contents extrude into the posterior chamber (data not shown).

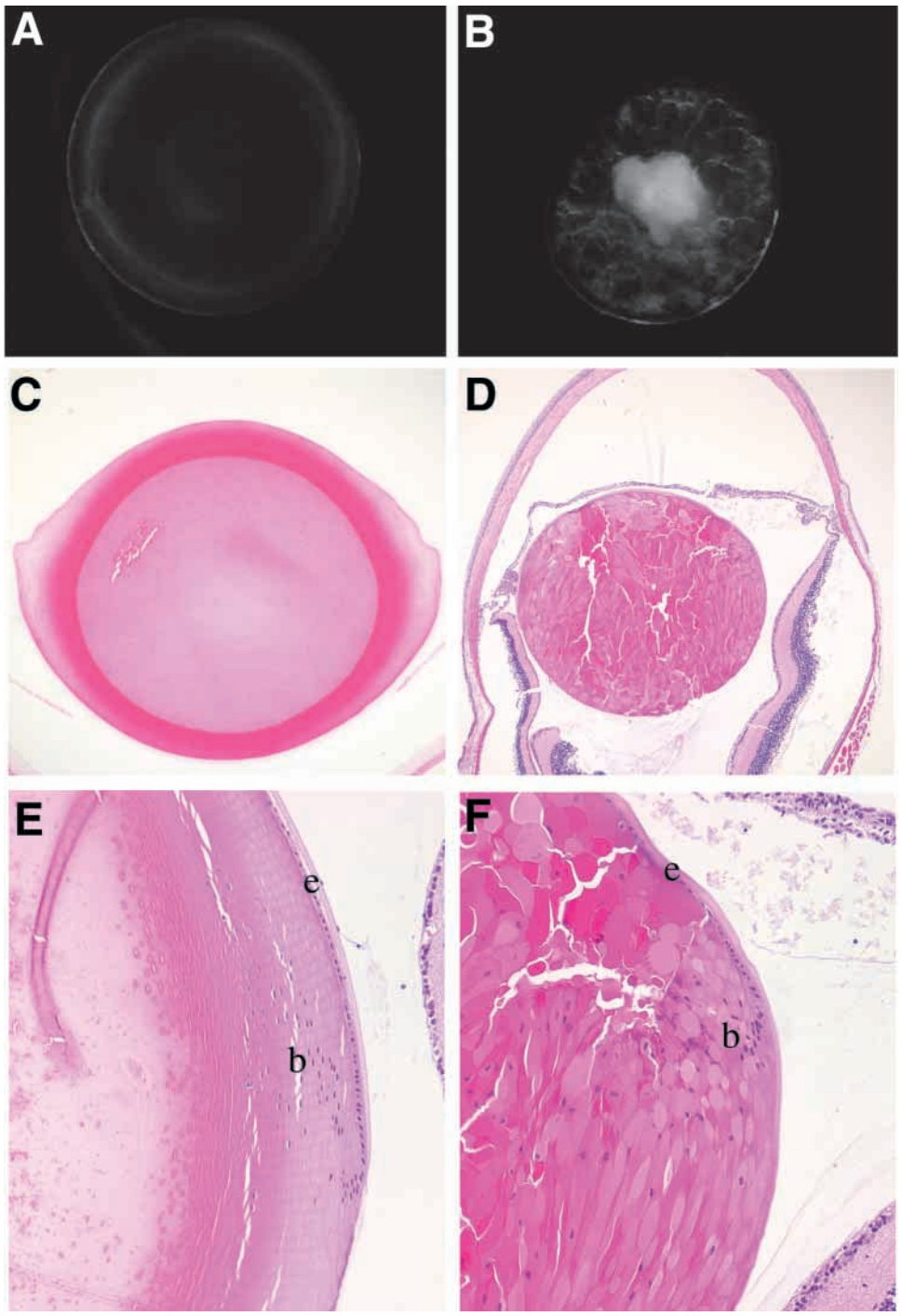

Fig. 1. Morphology of lenses from αA-crystallin/PAX6(5a) transgenic mice. (A) A normal lens visualized with darkfield microscopy. The faint ring around the edge is an internal reflection. (B) A lens from a transgenic mouse. Note the prominent nuclear cataract and cortical light scatter. (C,D) hemotoxylin and eosin stained lens from a wild-type mouse (C) and transgenic (D) mouse. (E,F) Higher magnification of the equator of the wild-type (E) lens shown in C and transgenic lens (F) shown in D. Note the swollen appearance, relatively loose packing of the elongating fiber cells and the persistence of nuclei deep into the lens. Abbreviations: e, lens epithelium; b, lens 'bow' consisting of young, newly differentiated lens fiber cells.

Molecular analysis of cell adhesion molecules

Since the lens fiber cells of mice expressing PAX6(5a) are abnormally shaped and may have defects in cell packing, the expression pattern of a number of proteins involved in cell adhesion and shape were compared between normal and transgenic mouse lenses. Integrins were initially considered because they appear to be critical in fiber cell:fiber cell and fiber cell:capsule interactions in the lens (Bassnett et al., 1999; Menko et al., 1998; Walker and Menko, 1999). The expression pattern of three integrin subunits expressed in the lens (Menko et al., 1998), α5-, α6- and β1-integrin, were investigated by both western blot analysis and confocal immunolocalization. α5- and β1-integrin subunits accumulated in the 5a transgenic lens as indicated by western blotting (Fig. 4A,G). Much of the accumulated protein was found in large aggregates associated with the dysgenic lens fiber cells (Fig. 4C,K). In contrast, α6-integrin expression levels were unaffected (Fig. 4D) and the protein localization appeared relatively normal (Fig. 4F).

Since α5β1-integrin is a fibronectin receptor (Johansson et al., 1997), and upregulation of fibronectin expression is associated with upregulation of α5β1-integrin during the epithelial to mesenchymal transition of lens epithelial cells to myofibroblasts (Zuk and Hay, 1994), fibronectin localization was investigated. In the anterior lens capsule of wild-type mice (Fig. 5A), this protein was predominately found at the outer surface of the capsule that faces the aqueous humor. In the anterior capsule of the transgenic lens (Fig. 5B), this pattern was preserved, but additional fibronectin surrounded the lens epithelial cells. In the

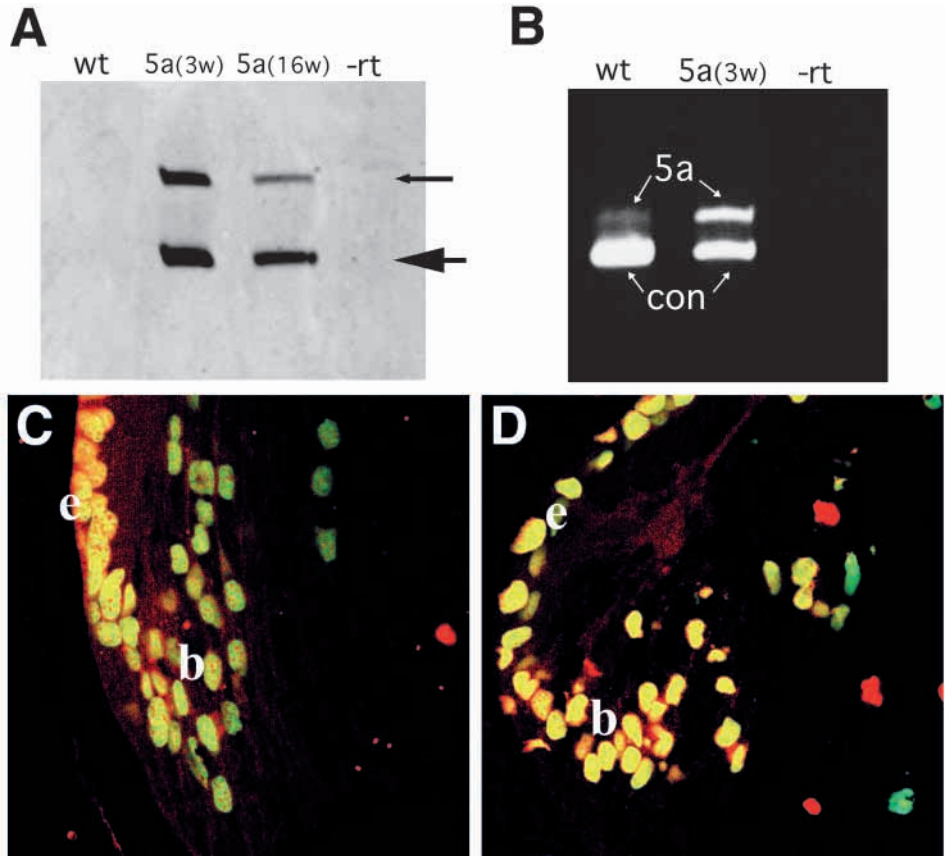

Fig. 2. Analysis of transgene expression in the lens of αA-crystallin/PAX6(5a) mice. (A) RT-PCR using primers derived from SV40 small T antigen. Spliced (large arrow) and unspliced (small arrow) transcripts were detected. (B) RT-PCR analysis of Pax-6 expression using primers that flank the 5a exon. Note that the transgenic lens is expressing approximately a 1:1 ratio of Pax-6 and Pax-6(5a). (C and D) Immunolocalization of Pax-6 protein in the lens of wild-type (C) or transgenic (D) 3-week-old mice. The Pax-6 protein is stained red, the nuclei are stained green and co-localization appears yellow-orange. Note that abundant amounts of Pax-6 protein are detected in older fiber cells of transgenic mice as compared to wild type. wt, lens RNA from a 3-week-old wild-type mouse; 5a(3w), lens RNA from a 3-week-old transgenic mouse; 5a(16w), lens RNA from a 16-week-old transgenic mouse; –rt, lens RNA from a 3-week-old transgenic mouse amplified without reverse transcriptase; 5a, lens RNA from a 3-week-old transgenic mouse; 5a(–rt), lens RNA from a 3-week-old transgenic mouse amplified without reverse transcriptase; e, lens epithelium; b, lens 'bow' consisting of young, newly differentiated lens fiber cells.

posterior capsule of both transgenic and wild-type mice (Fig. 5C,D), fibronectin staining was detected throughout the capsule. Little to no fibronectin was detected in or among the fiber cells of wild-type mice (Fig. 5A,C), while some punctate signals were detected near the dysgenic fiber cells of transgenic mice (Fig. 5B,D).

Next, the expression levels of other known cell adhesion molecules were tested by western blotting. Expression levels of E-cadherin were unaffected in the 3 week and 4 month old lens while those of N-cadherin, α-catenin and β-catenin expression levels were slightly elevated in the 3-week-old but not the 4 month old transgenic lens. In contrast, the protein levels of $p120^{ctn}$, which mediates the cell signaling initiated by cadherins (Steinberg and McNutt, 1999) as well as paxillin, which is involved in signaling via integrins (Miyamoto et al., 1995), are highly elevated in the lens (Fig. 6A) of 3-week-old transgenic mice. At 4 months of age, the levels of both of these proteins are still upregulated, but the increased $p120^{ctn}$ expression was less pronounced. The cellular localization of paxillin was then determined (Fig. 6B,C,D,E). In the wild-type lens, paxillin is detected at high levels in the lens epithelium with a downregulation of expression early in lens fiber cell

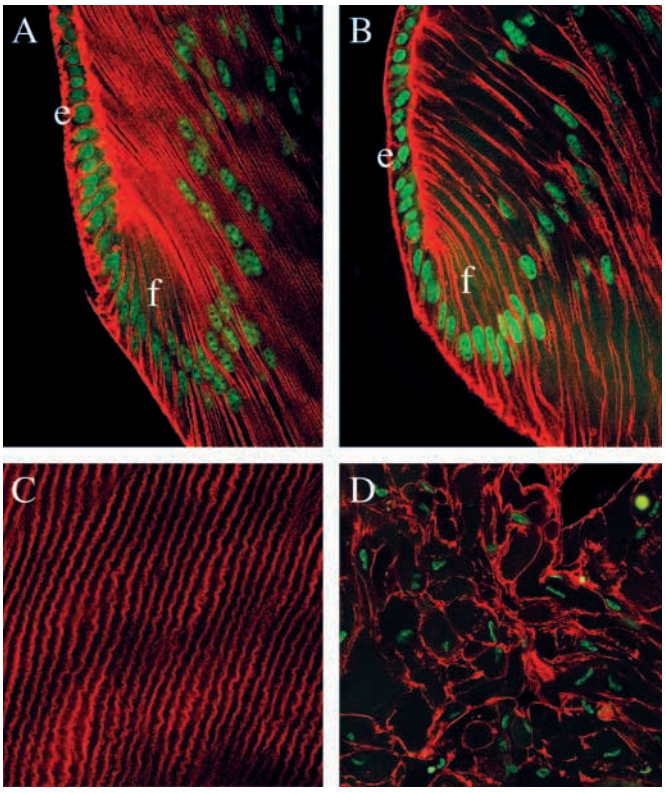

Fig. 3. Localization of the actin cytoskeleton in the lens by staining with Texas red phalloidin. (A) Lens equator of a 3-week-old wild-type mouse. (B) Lens equator of a 3-week-old of αA-crystallin/PAX6(5a) mice transgenic mouse. Note that the newly elongating fiber cells are swollen and less tightly packed than seen in the non-transgenic lens. (C) Nucleus of the wild-type lens shown in A. (D) Nucleus of the αA-crystallin/PAX6(5a) transgenic lens shown in B. Note the complete loss of regular fiber cell organization and the persistence of the cell nuclei. e, lens epithelium; f, lens fibers.

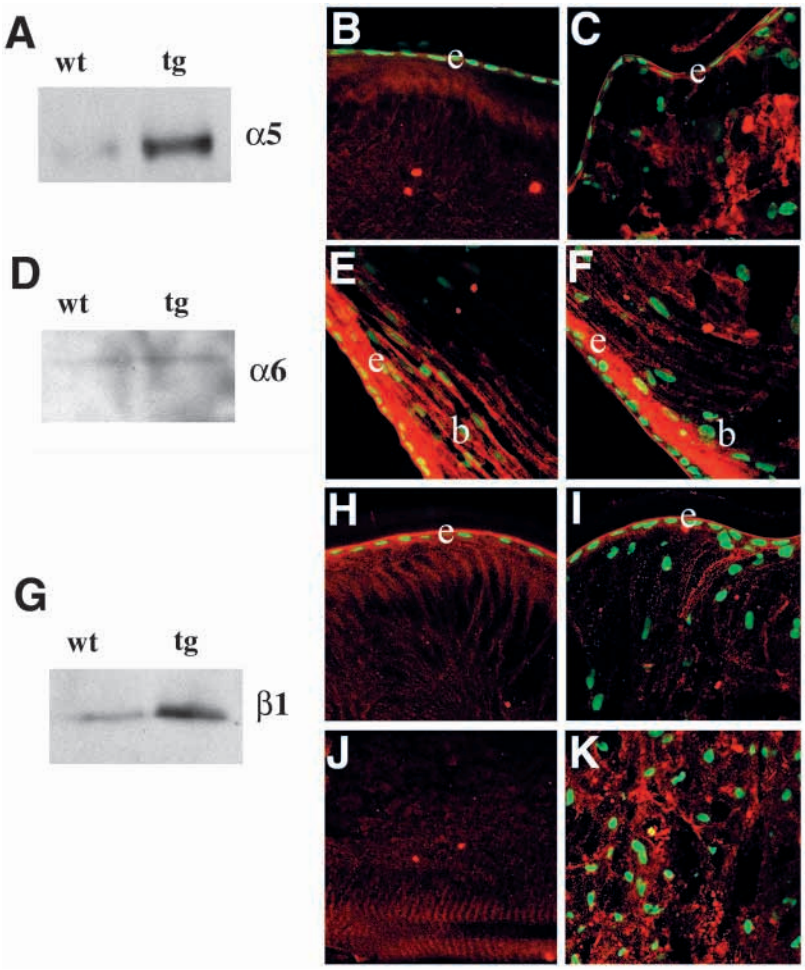

Fig. 4. Expression pattern of integrins in the lens of 3-week-old mice. (A) Western blot of α5-integrin expression. (B,C) Immunohistochemistry of α5-integrin expression in the lens. (B) Wild-type lens. (C) αA-crystallin/PAX6(5a) transgenic lens. Note that aggregates of α5-integrin immunoreactivity are seen in dysgenic lens fiber cells. (D) Western blot analysis of α6-integrin expression in the lens. (E,F) Immunohistochemistry of α6-integrin expression in the lens. Note that α6-integrin is localized to the lens epithelium and newly differentiating fiber cells. The levels of α6-integrin are downregulated during fiber cell differentiation in both the wild-type and transgenic lens. (G) Western blot of β1-integrin expression in the lens. (H,I,J,K) Immunohistochemistry of β1-integrin expression in the lens. (H) Wild-type lens. Note that the expression levels of β1-integrin are higher in the lens epithelium as compared to the lens fiber cells. (I) αA-crystallin/Pax-6(5a) transgenic lens. Note that aggregates of β1-integrin immunoreactivity are seen in dysgenic lens fiber cells. (J) Nucleus of a wild-type lens. Note that relatively low amounts of β1-integrin are localized to fiber cell membranes. (K) Nucleus of a αA-crystallin/PAX6(5a) lens. Note that large aggregates of β1-integrin immunoreactivity are seen in nucleated, highly dysgenic fiber cells. wt, wild type; tg, αA-crystallin/PAX6(5a) transgenic; α5, α5-integrin; α6, α6-integrin; e, lens epithelium; b, lens 'bow'.

differentiation (Fig. 6B,D, data not shown). In the transgenic lens, lens epithelial expression is maintained (Fig. 6C) but paxillin levels remain high in dysgenic fiber cells (Fig. 6E).

Elevated expression of mRNAs encoding α5 and β1 integrins, paxillin, and p120^{ctn} in transgenic lenses

Next, we performed a semiquantitative RT-PCR analysis of mRNAs encoding those proteins found upregulated in PAX6(5a) transgenic lenses (Fig. 7). From the data shown in Fig. 7C, we concluded that ectopic PAX6(5a) expression in transgenic lenses not only elevated expression of α5 and β1 integrins, paxillin, and p120^{ctn} proteins but also their mRNAs. The elevated levels of α5- and β1 integrin, paxillin, and p120^{ctn} expression in PAX6(5a) transgenic mice can be explained by a model in which PAX6(5a) directly regulates transcription from their promoters. Alternatively, PAX6(5a) may activate expression of other regulatory proteins that, downstream of Pax-6, activate transcription of those genes.

Pax-6 and Pax-6(5a)DNA-binding properties re-examined using a panel of known binding sites

The prerequisite for direct role of PAX6(5a) in this process is the presence of at least one PAX6(5a) binding site. The DNA-binding properties of Pax-6 proteins were previously examined using recombinant PD and PD5a and nuclear extracts containing endogenous Pax-6 and Pax-6(5a) proteins (Cvekl et al., 1994; Czerny and Busslinger, 1995; Epstein et al., 1994a,b; Kozmik et al., 1997). While several levels of complexity of Pax-6 binding to DNA were revealed, these studies oversimplified the problem of Pax-6 binding to DNA in vivo. For instance, isolated PD and PD5a may have different DNA-binding properties when linked to the HD since the HD may both expand the 'core' PD or PD5a binding region by adding ATTA/TAAT to the consensus and/or alter the binding properties of PAI and RED. Thus, in order to better predict potential Pax-6(5a) binding sites in the promoters of naturally occurring genes, we first re-examined the binding properties of Pax-6 and Pax-6(5a) using natural binding sites to better understand contributions of their PAI and RED subdomains and significance of one or more missmatches between the target sequence and 'optimal' binding site. Thus, PD (canonical paired domain), PD/HD (canonical paired domain with homeodomain), PD5a (5a containing paired domain), and PD(5a)/HD (5a containing paired domain with homeodomain) proteins were incubated with 5aCON and P6CON (Fig. 8A). The 5aCON oligonucleotide contains up to four binding sites for PD5a, with two sites preferentially occupied (Fig. 8A, lane 4). In contrast, PD5a/HD predominately binds to this probe as a monomer (Fig. 8A, lane 5). The 5aCON probe can also interact with PD and PD/HD proteins to form mono- and dimeric complexes (Fig. 8A, lanes 2 and 3). In contrast, P6CON binds monomers of PD and PD/HD (Fig. 8A, lanes 7 and 8) but binds neither PD5a nor PD5a/HD (Fig. 8A, lanes 9 and 10). From these data, we concluded that P6CON can interact only with PD and PD/HD while 5aCON can bind all four Pax-6 forms.

Next, the binding of PD and PD5a to the naturally occurring

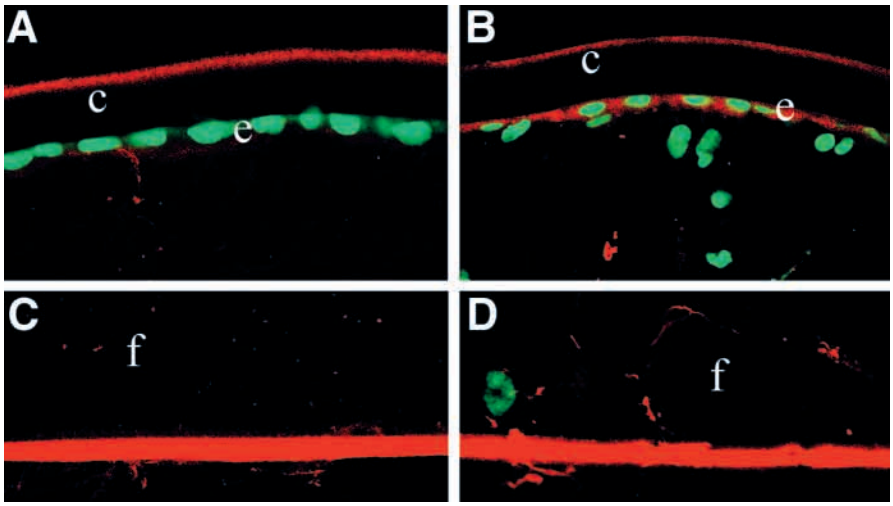

Fig. 5. Localization of fibronectin in the 3-week-old mouse lens. (A,B) Anterior capsule of a wildype (A) and αA-crystallin/PAX6(5a) (B) lens. The laminar distribution of fibronectin seen in the wild-type lens is maintained in the transgenic lens but relatively higher amounts of fibronectin are associated with the lens epithelial cells. (C,D) Posterior capsule of a wild-type (C) and αA-crystallin/PAX6(5a) (D) lens. The overall pattern of fibronectin immunoreactivity is normal in the transgenic lens, however, the capsule is less regular and some loci of immunoreactivity are observed among the dysgenic fiber cells.

Pax-6 binding sites, δ1/IIa (+1827/+1857, chicken δ1-crystallin; Cvekl et al., 1995b) and αA/E (–60/–29, chicken αA-crystallin; Cvekl et al., 1994) was tested by EMSA using truncated PD proteins with known binding properties to P6CON and 5a CON (Epstein et al., 1994b). The δ1/IIa and αA/E sites were recognized by both PD and PD5a, forming

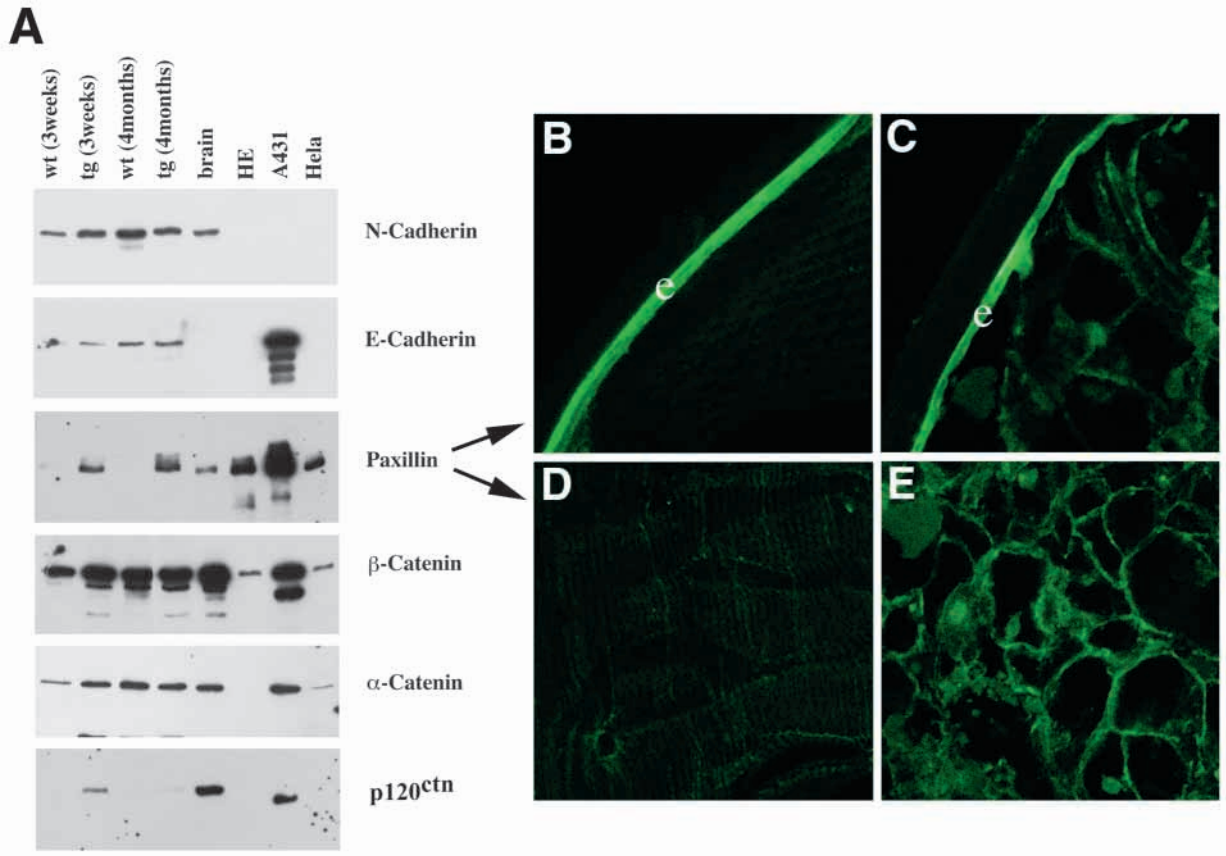

Fig. 6. Expression of various proteins involved in cell adhesion in the lens of αA-crystallin/PAX6(5a) mice. (A) Western blot analysis of proteins derived from wild-type and transgenic lenses. (B,C,D,E) Immunohistochemistry of paxillin expression in the lens. (B) Central epithelium of a 3-week-old wild-type lens showing that paxillin is mainly found in the lens epithelium. (C) Central epithelium of a 3-week-old αA-crystallin/PAX6(5a) transgenic lens. Note that appreciable amounts of paxillin expression are observable in dysgenic fiber cells. (D) The lens nucleus of the wild-type lens shown in B. Note the low level of paxillin staining in differentiated fiber cells. (E) The lens nucleus of the transgenic lens shown in C. Note that the dysgenic fiber cells have appreciable levels of paxillin that localizes to the cell membrane. wt, wildtype; tg, αA-crystallin/PAX6(5a) transgenic; HE, human aortic endothelium A431, human epidermoid carcinoma, HeLa, human cervical carcinoma; e, lens epithelium; f, lens fibers.

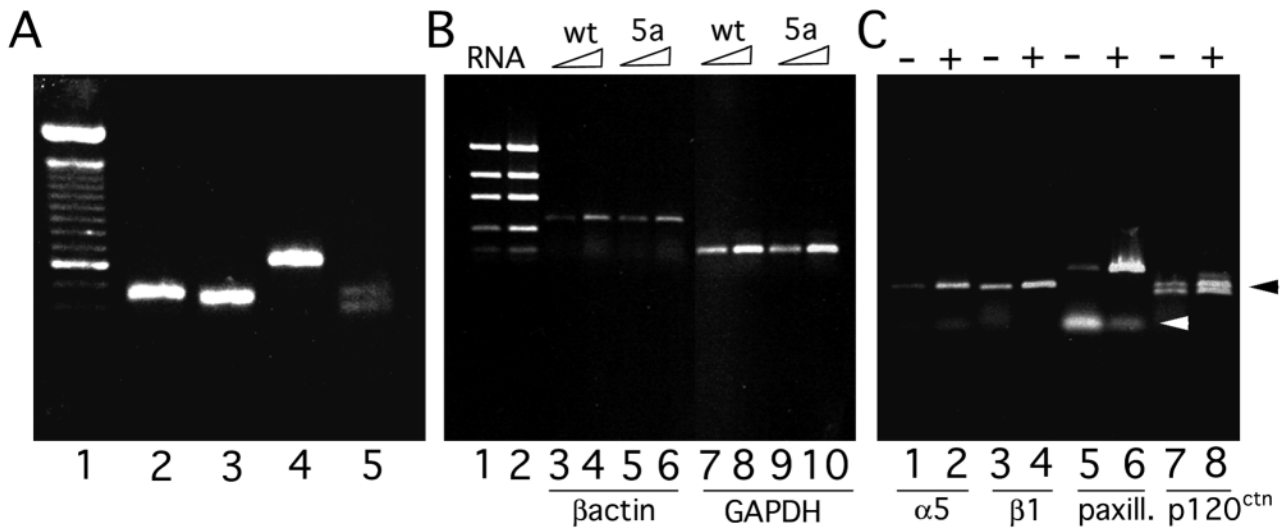

Fig. 7. RT-PCR analysis of gene expression in normal and transgenic lenses. (A) Specificity of amplification product generated on mouse 18.5 dpc total RNA and analyzed on 1.2% agarose gel, 100 bp MW marker (Life Technologies); (B) Normalization experiment analyzed on 1% agarose gel post-electrophoresis stained with EtBr of wild-type and PAX6(5a) transgenic total lens RNA (62.5 ng, lanes 3, and 5, 7, and 9; 125 ng, lanes 4, 6, 8, and 10) using β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as indicated. Low DNA mass ladder showing staining of 5, 10, 20, 40, 60, and 100 (lane 1) and 10, 20, 40, 80, 120, and 200 ng (lane 2) of individual DNA fragments (Life Technologies); and (C) amplification of α5 and β1 integrins, paxillin, and p120^{ctn} using total RNA prepared from wild-type (lanes 1, 3, 5, and 7) and transgenic (2,4,6, and 8) lenses and analyzed on 1% agarose gel post-electrophoresis stained with ethidium bromide. Black arrow points to specific products, white arrow points to unincorporated primers.

strong monomeric and dimeric complexes (Fig. 8B,C, lanes 2 and 3). Deletion of the 14 N-terminal aa (ΔN14) which comprise the N-terminal β-turn (Xu et al., 1999; Fig. 8D**)** reduced the formation of dimeric complexes but did not abolish binding to either site (Fig. 8B,C; lane 4). Deletion of the 24 N-terminal aa (ΔN24) which additionally comprise a portion of α helix one (Xu et al., 1999; Fig. 8D) restored the ability of the PD to form both mono- and dimeric complexes with both crystallin derived sites (Fig. 8B,C; lane 5) even though this truncated protein is unable to interact with P6 CON (Epstein et al., 1994b). Deletion of the N-terminal 34 aa (ΔN34), which comprise both the β-turn and the entirety of α helix one (Xu et al., 1999), abolishes the ability of the PD to bind either δ1/IIa, αA/E (Fig. 8B,C; lane 6) or P6CON (Epstein et al., 1994b) while it still can interact with PD5a (Epstein et al., 1994b).

Deletion of 33 aa from the RED domain (ΔC33) which comprise α-helices 5 and 6 (Xu et al., 1999) abolishes binding to δ1/IIa (Fig. 8C, lane 7) and 5a CON (Epstein et al., 1994b). However, many copies of this truncated protein appear to bind to αA/E (Fig. 8C, lane 7), most likely due to the presence of several TTC/GA sequence motifs (targets for the PAI helix-turn-helix domain; Xu et al., 1999) in this oligonucleotide. This assertion is consistent with the ability of ΔC33 to bind P6 CON (Epstein et al., 1994b). Deletion of 48 aa from the C terminus of PD (ΔC48) almost completely abolishes the RED domain (Xu et al., 1999) and virtually abolished the DNA-binding capacity of these truncated proteins to the crystallin sites (Fig. 8B,C; lane 8) and 5a CON (Epstein et al., 1994b) even though this protein can still bind to P6 CON (Epstein et al., 1994b).

Binding of truncated Pax-6 PD proteins to sites δ1/IIa and αA/E suggested that the RED domain is critical for binding while the PAI domain likely played only a supportive role. The above data also indicate that several mismatches between 'consensus' and actual binding site may frequently occur making the prediction of Pax-6 binding sites in genomic DNA somewhat difficult. The elevated levels of α5- and β1 integrin, paxillin and p120^{ctn} expression in PAX6(5a) transgenic mice can be explained by a model in which PAX6(5a) directly regulates transcription from their promoters. Alternatively, PAX6(5a) may activate expression of other regulatory proteins that, downstream of Pax-6, activate transcription of these genes.

Prediction and in vitro confirmation of Pax-6 and Pax-6(5a) binding sites in the 5′ flanking sequence of the α5 and β1 integrin genes

In order for PAX6(5a) to directly regulate the expression of the paxillin, p120^{ctn} α5-integrin and β1-integrin genes, it must be able to bind directly to their promoters. However, to date, promoter studies of the paxillin and p120^{ctn} genes have not been reported. While some functional characterization of the α5 (Birkenmeier et al., 1991) and β1 (Cervella et al., 1993) integrin promoters has been performed, no information is available on the regulatory sites controlling developmental expression of those genes in lens and other tissues. In an attempt to address the question of whether Pax-6(5a) could directly regulate α5- and β1-integrin, we examined promoter regions of the human α5- and β1-integrin genes (GenBank accession numbers U48214, X68969 and M74954) for potential Pax-6 binding regions using 'consensus' binding sites. In view of the complexity of Pax-6 protein interactions with DNA presented above, we allowed two mismatches in our search for potential binding sites. From this search, three regions of the α5- and two regions of the β1-integrin promoters were selected as candidate Pax-6 binding regions (Fig. 9a) and

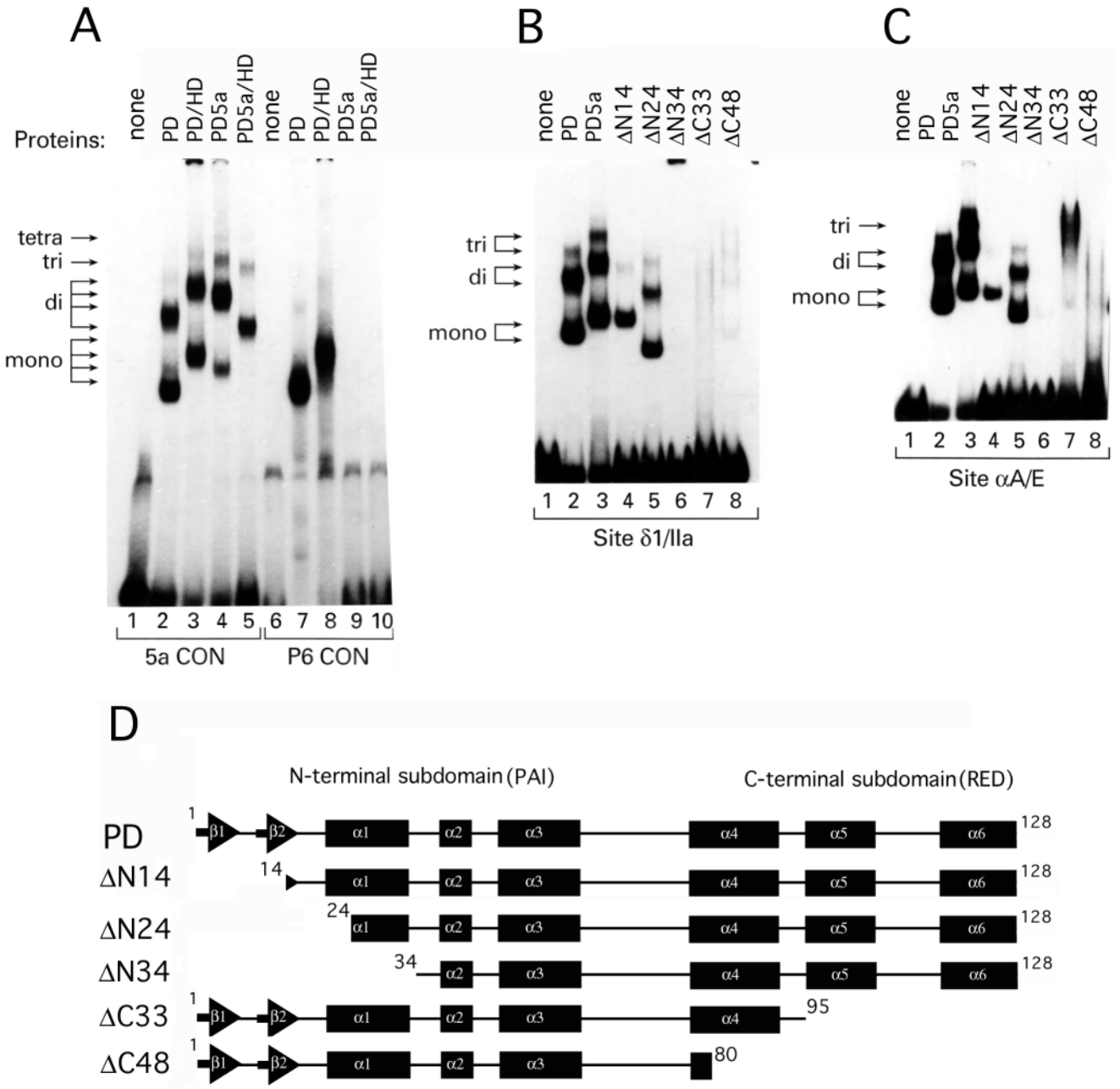

Fig. 8. Pax-6 and Pax-6(5a) binding to synthetic and known Pax-6 binding sites. (A) EMSA to investigate the role of the homeodomain in 5a CON and P6 CON recognition by Pax-6 and Pax-6(5a). (B,C) EMSA to investigate the mechanism of Pax-6 binding to the known Pax-6 site found in the δ1-crystallin (B) (Cvekl et al., 1995b) and αA-crystallin (C) promoter (Cvekl et al., 1995a). (D) Diagrammatic representation of the proteins used in B and C. mono, monomeric protein:DNA complexes; di, dimeric protein:DNA complexes; tri, trimeric protein:DNA complexes; tetra, tetrameric protein:DNA complexes; PD, paired domain; PD/HD, paired domain and homeodomain; PD5a, paired domain with 5a exon insert; PD5a/HD paired domain with 5a exon insert with homeodomain.

oligonucleotide probes prepared for EMSA. A region of the α5-integrin promoter (–701/–672) bound recombinant PD, PD/HD, and PD5a but not PD5a/HD proteins (Fig. 9b) while a sequence derived from the β1-integrin promoter (–1398/–1358) bound all four forms of Pax-6 (Fig. 8d). The remaining three probes tested (Fig. 9a) bound recombinant Pax-6 proteins with much lower affinity and were not examined further. Finally, we tested whether α5- (–701/–672) and β1-integrin (–1398/–1358) promoter-derived oligonucleotides bound to native Pax-6 protein found in nuclear extracts prepared from a lens-derived cell line. Both probes generated several distinct protein-DNA complexes (Fig. 9b,d). Preincubation of the lens nuclear extract with Pax-6 antiserum inhibited the formation of a single complex on both probes (Fig. 9b,d; arrow) while other protein-DNA complexes were unaffected. From these experiments, we conclude that the human α5- and β1-integrin promoters contain Pax-6 binding sites, a result that directly supports our model implicating Pax-6 (5a) as a directly acting

regulatory factor controlling expression of these genes in the transgenic lens.

DISCUSSION

The present study had two goals. First, to determine whether overexpression of PAX6(5a) in the lens could yield a phenotype that mimics the pathogenesis of cataract in humans expressing an altered ratio of PAX6(5a) to PAX6 (Epstein et al., 1994b). Second, to determine whether this model can be used to identify novel Pax-6 regulated genes.

Overexpression of PAX6(5a) in a wild-type lens leads to cataract

Indeed, transgenic mice overexpressing PAX6(5a) in the lens develop a grossly observable, juvenile onset nuclear cataract with associated cortical changes. Since Pax-6 protein levels are elevated in the lens fiber cell nuclei of transgenic mice and this report as well as others demonstrated that endogenous Pax-6 expression in the lens is predominately localized to the lens epithelium, it is likely that nearly all of the Pax-6 protein expressed in the fiber cells of transgenic mice is PAX6(5a). However, since the promoters of both the human and quail PAX6/Pax-6 genes are autoregulated by PAX6/Pax-6 (Okladnova et al., 1998; Plaza et al., 1993), it is possible that the lens fiber cells of transgenic mice are expressing elevated levels of endogenous PAX6 and PAX6(5a) protein as well as transgene derived PAX6(5a). However, the high levels of sequence conservation seen in mammalian PAX6 proteins make this possibility difficult to test. Nevertheless, even if the transgene derived PAX-6(5a) is upregulating the production of Pax-6/Pax-6(5a), the observed phenotype is comparable to the cataracts seen in humans with an altered ratio of PAX6(5a) to PAX6 (Epstein et al., 1994b).

Previously, it was reported that transgenic mice harboring a YAC containing the entire human PAX6 locus and expressing human PAX6 in the eye develop severe ocular

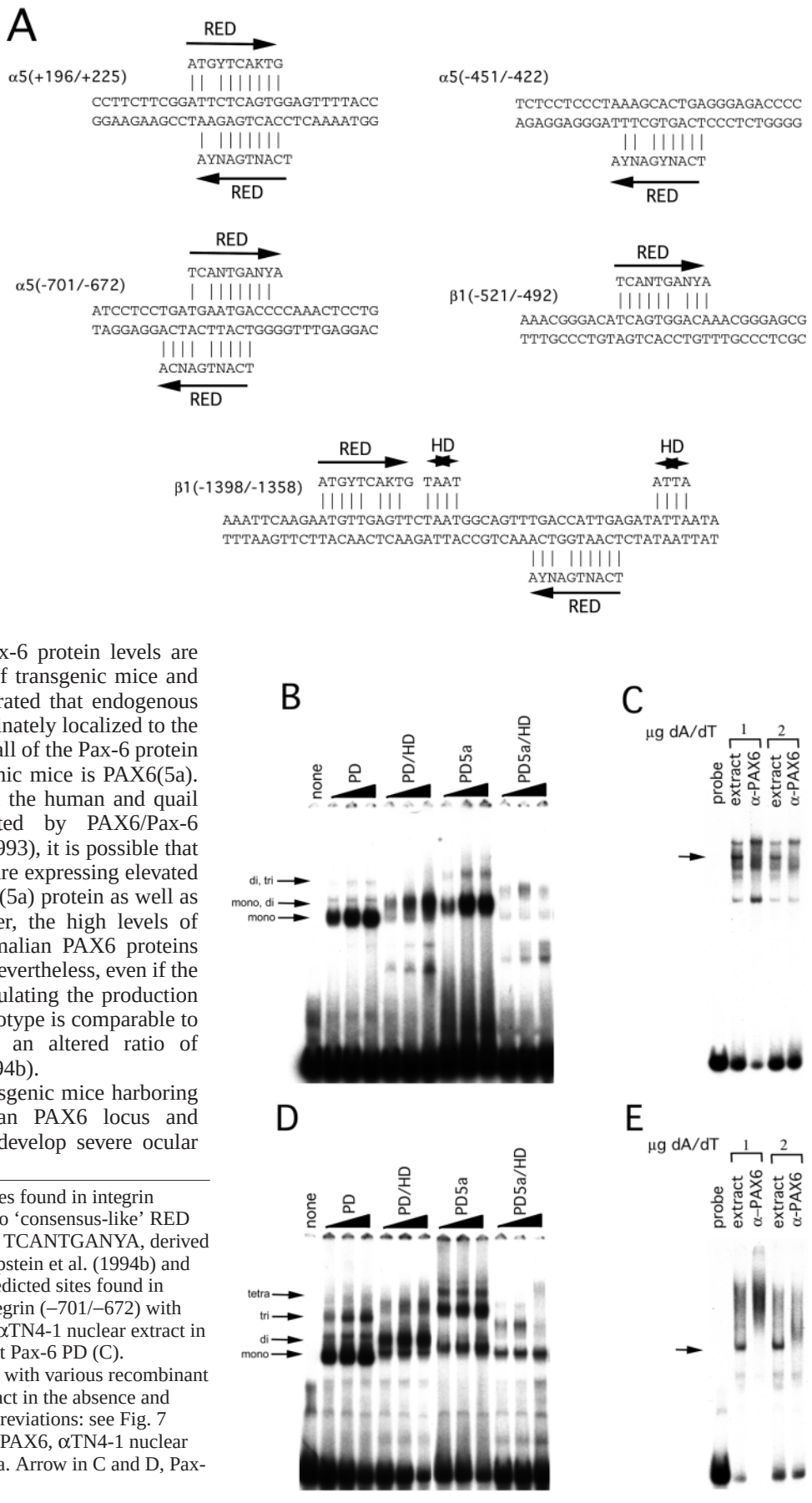

Fig. 9. EMSA of putative Pax-6 binding sites found in integrin promoters. (A) Sequence comparison of two 'consensus-like' RED domain binding sites, ATGYTCAKTG and TCANTGANYA, derived from a combination of data published by Epstein et al. (1994b) and Kozmik et al. (1997), respectively; with predicted sites found in integrin promoters. (B,C) EMSA of α5-integrin (–701/–672) with various recombinant Pax-6 proteins (B) or αTN4-1 nuclear extract in the absence and presence of antisera against Pax-6 PD (C). (D,E) EMSA of β1 integrin (–1398/–1358) with various recombinant Pax-6 proteins (D) or αTN4-1 nuclear extract in the absence and presence of antisera against Pax-6 PD. Abbreviations: see Fig. 7 legend; extract, αTN4-1 nuclear extract; α-PAX6, αTN4-1 nuclear extract preincubated with Pax-6 PD antisera. Arrow in C and D, Pax-6 containing complex.

abnormalities including microophthalmia and cataract (Schedl et al., 1996). Since the lens phenotype of these mice was only partially described and neither the expression pattern or levels of Pax-6 or Pax-6(5a) were determined in the lens, it is not possible to directly compare the phenotype of these mice with those reported here. Further confounding the issue, the ocular phenotype in the YAC transgenics was only partially penetrant and often only unilateral, consistent with both environmental and strain effects (Schedl et al., 1996). In contrast, the phenotype of the expressing line of PAX6(5a) transgenics is completely penetrant and highly consistent animal to animal.

Standard histological analysis and localization of the filamentous actin cytoskeleton of αA-crystallin/PAX6(5a) transgenic lenses revealed that the cataract morphology was characterized by swollen fibers that finally roundup into balloon shaped cells. Interestingly, the shape of the dysgenic fiber cells is reminiscent of the 'Wedl Cells' often seen in human posterior subcapsular cataract (Eshaghian and Streeten, 1980). Since humans that preferentially express PAX6(5a) suffer from cortical and diffuse posterior polar lens opacities (Epstein et al., 1994b), it is possible that these mice mimic the lens defects of these patients. One weakness in this argument is that Pax-6 mRNA is normally only expressed in the lens epithelium even though Pax-6 protein is still detectable in newly differentiating lens fiber cells of adults. Thus, the cataracts found in the present transgenic mice may occur by a mechanism distinct from the cataracts seen in the humans.

Identification of genes upregulated in Pax-6(5a) transgenic lenses

Pax-6 is essential for normal eye development and function. It has been postulated that Pax-6 may regulate expression of cell adhesion molecules (Edelman and Jones, 1998) as well as transduce the signals generated in cell signaling cascades (Mikkola et al., 1999). Currently, about a dozen genes regulated by Pax-6 in the eye (Cvekl and Piatigorsky, 1996; Duncan et al., 1998), brain (Meech et al., 1999; Stoykova et al., 1997), and pancreas (Andersen et al., 1999; Ritz-Laser et al., 1999) are known. This study has identified four genes whose expression levels are altered in the PAX6(5a) overexpressing lens, all of which are known to be involved in either cell adhesion, cell/extracellular matrix adhesion and/or adhesion dependent cell signaling. Thus, these genes can be considered to be potentially involved in the pathogenesis of posterior subcapsular cataract as well as potential in vivo targets of PAX6(5a) function. However, these genes may also be regulated by PAX6 since 5a-type DNA binding sites are also recognized by PAX6.

The present data showed increased expression of α5β1 integrin in the Pax-6(5a) transgenic mouse lens. Previously, increased levels of α5 and β1 integrin were seen in lens epithelial cells undergoing an epithelial to mesenchyme transition to myofibroblasts in response to suspension in collagen gels (Zuk and Hay, 1994). However, a number of lines of evidence suggest that the cataract phenotype of the PAX6(5a) transgenic is not an epithelial to mesenchyme transition. First, myofibroblasts have a distributed actin cytoskeleton (Schmitt-Graff et al., 1990) while dysgenic fiber cells expressing elevated amounts of α5β1 integrin have an actin skeleton that localizes under the cell membrane, a normal feature of lens cells (Lo et al., 1997). Second, myofibroblasts sharply downregulate the expression of α6 integrin while the transgenic lens has normal patterns of α6 integrin expression. Third, myofibroblasts secrete large amounts of fibronectin into their environment (Zuk and Hay, 1994) which was not observed in the transgenic lens. Lastly, myofibroblasts lose the ability to express crystallins (Zuk and Hay, 1994) while the PAX6(5a) transgenic lens maintained this ability (data not shown).

Since PAX6(5a) transgenic lenses overexpressed one type of cell adhesion molecule, the expression levels of others were investigated. While levels of the tested cadherins and catenins were only slightly elevated in the 3-week-old transgenic lens, paxillin and $p120^{ctn}$ expression levels were highly elevated in the lens of both three week and four month old transgenic mice. Paxillin serves as a linker molecule between the cytoskeleton, integrin cytoplasmic domains, catenins and other cell signaling molecules (Turner, 1998). In the wild-type lens, this protein was found predominantly in the lens epithelium while lower levels of expression were seen at the fiber membrane borders in a pattern similar to that of F-actin. Notably, paxillin expression levels in the normal lens correlate well with the expression pattern of Pax-6. In the PAX6(5a) transgenic lens, increased amounts of paxillin and its mRNA are seen in dysgenic fiber cells consistent with the idea that this gene is also directly upregulated by Pax-6(5a) in the transgenic lens. Alternatively, this increase in paxillin expression could be a secondary effect caused by changes in cell signaling due to increased α5β1 integrin expression or other as yet unidentified molecules.

Since α5β1 integrin, paxillin, and $p120^{ctn}$ are known to participate in cell adhesion, cell/extracellular matrix adhesion and cell signaling, the alterations in expression level of one or all of these proteins could easily be the initiating event in the PAX6(5a) transgenic cataract. The increased levels of clustered α5β1-integrin in association with fibronectin detected in dysgenic fiber cells could indicate that this integrin is actively sending signals to the ERK/MAP kinase pathway resulting in as yet unidentified changes in gene expression which result in cataract (Schlaepfer and Hunter, 1998). Alternatively, α5β1-integrin could be sending signals via PI 3-kinase, focal adhesion kinase or integrin linked kinase which could more directly result in alterations in cell shape or cell adhesion leading to cataract (Longhurst and L. J, 1998; Plopper et al., 1995). These effects could be further amplified by the observed upregulation of paxillin and $p120^{ctn}$ expression since these two proteins have been found to be associated with α5β1-integrin at focal adhesions. However, all of these proteins perform multiple functions which are regulated in complex ways including alternative splicing, phosphorylation and protein/protein interaction (Brown et al., 1998; Miyamoto et al., 1995; Walker and Menko, 1999) so a definitive determination of the role of α5β1 integrin, paxillin, and $p120^{ctn}$ in the pathogenesis of cataract will require further study.

While the expression levels of all of these genes were upregulated in response to the overexpression of PAX6(5a), it is still possible that one or all of these genes are not direct Pax-6(5a) targets. Thus, the next logical step in these studies is the prediction and confirmation of candidate Pax-6 binding sites in the promoters of these genes. While information on the paxillin and $p120^{ctn}$ promoters was unavailable, the sequence of the α5-integrin (Birkenmeier et al., 1991) and β1-integrin (Cervella et

al., 1993) promoters was known. The known 5′ flanking sequences of α5-integrin gene (–926/+23) can drive promoter activity in an α5-integrin expressing cell line and deletion analysis suggested that this promoter contains a silencer between –657/–178 while a number of activating sequences reside between –178 and –27 (Birkenmeier et al., 1991). β1-integrin expression is controlled by two different promoters separated by 261 nucleotides that produce mRNAs with alternative 5′ flanking sequences but identical coding regions (Cervella et al., 1993). The proximal β1-integrin promoter appears to be more responsive to transforming growth factor β1 induction (Cervella et al., 1993) while the distal promoter may be more responsible for β1-integrin expression during embyonic development, including low levels of expression in the embryonic eye (Hirsch et al., 1993). The most promising potential binding site identified (β1 integrin, –1398/–1358 of the distal promoter), using the known consensus Pax-6 binding sites with two mismatches, appears to contain PD, PD5a and HD recognition sequences. Moreover, the 5′ flanking sequence of both α5-integrin (–701/–672) and β1 integrin (–1398/–1358) are recognized by full length Pax-6 present in a lens nuclear extract consistent with the idea that both of these integrin genes can be regulated by Pax-6(5a) in transgenic lens fibers. Confirmation that the α5 and β1 integrin promoters are directly regulated by Pax-6 or Pax-6(5a) will require a detailed analysis of the functional requirements of the integrin promoters in the lens using transgenic mice coincident with co-transfection analyses using Pax-6 expression vectors in cultured cells.

Our study tested tools to successfully predict naturally occurring Pax-6 binding sites in genomic sequences and emphasized the need to examine any candidate sites with recombinant PD/HD and PD5a/HD proteins as well as full length Pax-6. Our data also provide evidence that some naturally occurring Pax-6 sites (e.g. δ1/IIa and αA/E) interact preferentially with the intact RED and not PAI subdomain of Pax-6. In addition, RED domain binding can be further modulated by the presence of the HD when the site contains ATTA/TAAT sequences (such as β1 integrin site, –1398/–1358). We propose that PAX6 RED domain in conjunction with the PAI, linker domain, and HD adopts much broader spectrum of binding conformations requiring future studies. This may play important role in vivo to modulate PAX6/PAX6(5a) activation potentials depending on the actual binding conformation (Yamaguchi et al., 1997).

In conclusion, the formation of cataract and overexpression of signaling proteins, including paxillin and α5β1 integrin, make the Pax-6(5a) transgenic mouse a valuable new model for simultaneously exploring target genes of Pax-6(5a), the function of Pax-6 gene expression in the postnatal lens and cataractogenesis, the most common form of ocular pathology worldwide that results in blindness.

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