Identification of Genes Downstream of Pax6 in the Mouse Lens Using cDNA Microarrays

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Pax6 is a transcription factor that regulates the development of the visual, olfactory, and central nervous systems, pituitary, and pancreas. Pax6 is required for induction, growth, and maintenance of the lens; however, few direct Pax6 target genes are known. This study was designed to identify batteries of differentially expressed genes in three related systems: 8-week old Pax6 heterozygous lenses, 8-week old Pax6 heterozygous eyes, and transgenic lenses overexpressing Pax6(5a), using high throughput cDNA microarrays containing about 9700 genes. Initially, we obtained almost 400 differentially expressed genes in lenses from mice heterozygous for a Pax6 deletion, suggesting that Pax6 haploinsufficiency causes global changes in the lens transcriptome. Comparisons between the three sets of analyses revealed that paralemmin, molybdopterin synthase sulfurylase, Tel6 oncogene (ETV6), a cleavage-specific factor (Cpsf1) and tangerin A were abnormally expressed in all three experimental models. Semiquantitative reverse transcription (RT)-PCR analysis confirmed that all five of these genes were differentially expressed in Pax6 heterozygous and Pax6(5a) transgenic lenses. Western blotting and immunohistochemistry demonstrated that paralemmin is found at high levels in the adult lens and confirmed its down-regulation in the Pax6(5a)-transgenic lenses. Collectively, our data provide insights into the genetic programs regulated by Pax6 in the lens.

Pax6 is among the most widely studied transcription factors because of its participation in the organogenesis of the eye, brain, head, and pancreas (1–4). The essential role of Pax6 in early eye induction is conserved throughout the evolution of vertebrates, relatively few genes have been shown to be directly regulated by Pax6. In Drosophila, Pax6/ey directly regulates the transcription of rhodopsins (19) and sine oculis (20). In vertebrates, Pax6 directly affects expression of Pax2 in the developing optic cup and stalk (21). Genetic evidence suggests that the genes for the eye development regulators Eya1 and -2 (22), Sox-2 (7), and c-Maf (23) are also direct targets. In addition to these developmental regulators, Pax6 can directly regulate the insulin, glucagon, and somatostatin genes expressed in the pancreas (22); L1-CAM expression in the brain (25); keratin K12 (26) and gelatinase B (27) expression in the cornea; and αA-, αB-, β1-, and γ-crystallin expression in the lens (28–30). Although the mechanism of Pax6 function has not been studied in detail in many of these cases, it appears that it can function both as a transcriptional activator and repressor in vitro assays.

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★ The abbreviations used are: HD, homeodomain; RT, reverse transcription; EST, expressed sequence tag; MOCS3, molybdopterin synthase sulfurylase.

The diverse functions of Pax6 appear to originate from both the complex regulatory mechanisms controlling the tissue-specific transcription and splicing of the Pax6 mRNA as well as its ability to participate in multiple molecular interactions. A prevailing form of Pax6 in mouse embryos contains two DNA-binding domains, the paired domain and homeodomain (HD),1 which can interact both independently and cooperatively with DNA, whereas the C terminus comprises the transcriptional activation domain (9, 10). The paired domain contains two subdomains, PAI and RED, each of them capable of binding independently to DNA (9). A splice variant, Pax6(5a), has an additional 14 amino acids inserted into the PAI subdomain. This results in its recognition of only a subset of Pax6 binding sites (11–14). Recent evidence suggests that Pax6 function is further modulated by interactions of its homeodomain with a diverse set of proteins, including the homeodomain-containing proteins Six3, Prox1, and Lhx2 (15) and the transcription factors TFIID and pRb (16). Pax6 also physically interacts with c-Maf/Maf A (17) and MitF (microphthalmia) (18), two important transcription factors controlling lens differentiation, and retinal development, respectively.

While Pax6 is clearly a central player in many developmental processes, relatively few genes have been shown to be directly regulated by Pax6. In Drosophila, Pax6/ey directly regulates the transcription of rhodopsins (19) and sine oculis (20). In vertebrates, Pax6 directly affects expression of Pax2 in the developing optic cup and stalk (21). Genetic evidence suggests that the genes for the eye development regulators Eya1 and -2 (22), Sox-2 (7), and c-Maf (23) are also direct targets. In addition to these developmental regulators, Pax6 can directly regulate the insulin, glucagon, and somatostatin genes expressed in the pancreas (22); L1-CAM expression in the brain (25); keratin K12 (26) and gelatinase B (27) expression in the cornea; and αA-, αB-, β1-, and γ-crystallin expression in the lens (28–30). Although the mechanism of Pax6 function has not been studied in detail in many of these cases, it appears that it can function both as a transcriptional activator and repressor in vitro assays.

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used to study Pax6 function by determining which genes are affected by both Pax6 haploinsufficiency in the eye (1, 6–8) and Pax6(5a) overexpression (14) in the lens. Our studies demonstrate the usefulness of microarray analysis for the analysis of gene expression in pathological conditions and give some insight into the function of Pax6 in the mature lens.

**EXPERIMENTAL PROCEDURES**

**Mice**—NMRI mice heterozygous for a Pax6 knockout/lacZ knock-in allele were generously provided by Dr. Peter Gruss (Max Planck Institute of Biophysical Chemistry, Gottingen, Germany) (34), while wild type NMRI mice were obtained from Charles River Laboratories (L’Arbresle, France). FVB/N mice overexpressing Pax6(5a) in lens fibres under the control of the mouse a-crystallin promoter and wild type strain matched controls were described previously (14).

**RNA Preparation**—Lenses and eyes from which lenses were surgically removed were isolated from 8-week-old Pax6 heterozygous and wild type mice, and stored in RNA later (Ambion, Woodlands, TX) until RNA isolation procedures were performed using the Total RNA Kit (Ambion). The genotype of Pax6 heterozygous lenses was confirmed by assaying the expression of Pax6 and lacZ using RT-PCR with primers designed to amplify Pax6 (5'-TTT AAC CAA GGG CGG TGA GCA G-3' and 5'-TCT CCC TGT AGT ATG ACG TGG G-3') and lacZ (5'-GTC TGG TCA TTC AAG TCT TCA A-3' and 5'-GTC TGG TCA TTC AAG TCT TCA A-3'). All primers used in this study (Table I) were designed to cross intron-exon boundaries and were tested in the absence of reverse transcriptase step. The hybridizations were performed for 30 min. The initial reverse transcriptase step was conducted at 50 °C for 30 min, and amplifications were conducted at the annealing temperature of 58 °C.

Lenses were isolated from 3- and 8-week mice overexpressing Pax6(5a) in lens fibres and strain-matched controls as described (14). RT-PCR—Microarray Procedures—DNAs were generated using 2–5 μg of total RNA and indirectly labeled with Cy3- and Cy5-specific dendrimers (Bio-Rad). Glass slide microarrays containing about 9700 mouse sequence verified genes were prepared and immobilized using the 3DNA detection system from Genisphere, Inc. The hybridizations were performed at 50 °C, with three subsequent washes of the slides performed in 2× SSC, 0.1% SDS; 2× SSC; and 0.2× SSC buffers. The chips were scanned using the GenePix 4000A scanner (Axon Instruments, Union City, CA), and primary data were analyzed using the GenePix 3.02 software. Each experiment was conducted in triplicate. Control self-hybridizations were performed using wild type RNA to determine S.D. values that were used to determine the eventual cut-off values.

**Quality Control, Data Analysis, and Statistics**—Primary data were flagged using four default parameters set in the Genepix 3.0 program. Intensity data for both channels were normalized by the widely used global intensity normalization method (37). The intensity of each spot in each channel was adjusted by subtracting the local background from the observed intensity (Ij) = Iij − Bij, where Iij, Iij, and Bij are the adjusted intensity, observed intensity, and background for the jth gene in the ith channel (i = 1, 2, … n) in the ith channel (i = 1, 2, respectively), and then subjected to log transformation (ln(Ij)). The overall intensity for each channel was calculated by taking the power of the average of the log of the adjusted intensity for all genes

\[ I_{Ch1} = e^{\frac{1}{n} \sum_{j=1}^{n} \ln(I_{Ch1,j})} \]

\[ I_{Ch2} = e^{\frac{1}{n} \sum_{j=1}^{n} \ln(I_{Ch2,j})} \]

where n is the number of genes, and \( I_{Ch1} \) and \( I_{Ch2} \) are the overall intensity for channel 1 and channel 2, respectively. The intensities for both channels were then balanced by multiplying the adjusted intensity of each spot in channel 2 by the ratio of the overall intensity in channel 1 over that in channel 2 (r = \( I_{Ch1,j}/I_{Ch2,j} \)). Means and S.D. values were calculated for those genes with no more than one flagged data point. For normalized data tables, see the Supplemental Material. Genes were classified into 12 functional groups (38) using annotations from the Swissprot data base (available on the World Wide Web at expasy.org/sprot). The tangerin A was identified from EST E2A174755 using the Gencarta data base (Compgen Ltd., Tel Aviv, Israel).

**Western Blotting**—Lenses were dissected from 6-week-old Pax6(5a) transgenic and wild type litter mates and homogenized in radioimmune precipitation buffer (1× phosphate-buffered saline, 1% Nonidet P-40, 0.1% deoxycholate, 0.1% SDS, 0.575 μg/ml phenylmethylsulfonyl fluoride, 45 μg/ml aprotinin, 1 mM sodium orthovanadate). Supernatants were collected following two spins at 10,000 g. Protein concentrations were immediately determined using Bio-Rad DC protein assay (Bio-Rad, and 56 μg of protein were loaded on each lane of a 10% gel, with 0.2% SDS; 2× SSC; and 0.2× SSC buffers. The chips were scanned using the GenePix 4000A scanner (Axon Instruments, Union City, CA), and primary data were analyzed using the Genepix 3.02 software. Each experiment was conducted in triplicate. Control self-hybridizations were performed using wild type RNA to determine S.D. values that were used to determine the eventual cut-off values.

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**ET-PCR**—All transcripts studied were reverse-transcribed and amplified using the One Step RT-PCR system (Invitrogen). The initial reverse transcriptase step was conducted at 50 °C for 30 min. The annealing temperatures used for individual experiments are indicated in Table I. All amplifications shown here were performed at 29 cycles. All primers used in this study (Table I) were designed to cross intron-exon boundaries and were tested in the absence of reverse transcriptase. Reaction control reactions were performed initially to ensure linearity of amplification over concentrations of total RNA ranging from 5 to 100 ng.

**Immunohistochemical Labeling and Preparation**—Mouse eyes were enucleated and embedded in tissue freezing medium (Triangle Bio-Medical Sciences, Durham, NC), and 16-μm frozen sections were prepared. Sections were then fixed in 1:1 acetone/methanol for 10 min at −20 °C and blocked with 1% bovine serum albumin/phosphate-buffered saline for 1 h at RT. Paralammin and preimmune paralammin primary antibodies (39) were prepared in 1% bovine serum albumin/phosphate-buffered saline at dilutions of 1:150. The bound primary antibodies were visualized following incubation with anti-rabbit IgG conjugated with Alexa Fluor 568 (1:50 dilution in 1% bovine serum albumin/
of mouse lens cDNAs from normal (labeled with Cy-3 dendrimers) and Pax6 heterozygous (labeled with Cy-5 dendrimers). Horizontal and vertical axes represent Cy-3- and Cy-5-generated signals in logarithmic scale shown in green and red, respectively. Shown are cut-off points (indicated by horizontal and vertical dashed lines) for genes producing signals below 100. Genes used for subsequent analysis are located in the upper right quadrant, labeled by a gray arrow. The upper and lower boundaries (blue lines) represent a 1.6-fold difference in the expression between Pax6 heterozygous and normal lenses, and the inner slope (blue line) indicates a ratio of 1.0 as described under “Experimental Procedures.” The yellow signal represents unchanged genes. B, a diagrammatic representation of six randomly selected genes annotated with their GenBank™ accession numbers (see “Experimental Procedures”), from the pool of unchanged, down- and up-regulated genes. The horizontal axis represents three independent microarrays, labeled 1, 2, and 3; and the vertical axis represents the ratio of relative Cy-5/Cy-3 intensities for each independent hybridization.

RESULTS

Genes Abnormally Expressed in Pax6 Heterozygous Lenses—Initially, lens RNA obtained from 8-week-old NMRI mice was labeled with either Cy5- or Cy3-labeled dendrimers (35) and self-hybridized to cDNA microarrays containing about 9700 sequence-verified genes (36) to determine the S.D. value of the hybridization ratios (Cy5/Cy3). Since the S.D. obtained from three independent hybridizations was 0.28–0.31, expression ratios more than 1.60 for up-regulated and less than 0.63 for down-regulated genes are statistically significant, since they represent values that differ by two S.D. values. Genes differentially expressed in Pax6 heterozygous lenses as compared with normal lenses were determined by labeling cDNAs from the two samples with Cy3- and Cy5-specific dendrimers before simultaneously probing onto the cDNA microarrays described above. A representative scatter plot from one experiment showing the distribution of hybridization signals generated using GeneSpring 3.02 software (Silicon Genetics, San Carlos, CA) is shown in Fig. 1A. To demonstrate the reproducibility of data using the 3DNA labeling technology (35), we randomly selected six genes (GenBank™ accession numbers AA387340, AA120030, AA445775, AA238399, AA260490, and AA000249), and their ratios of expression from triplicate microarrays are given in Fig. 1B. This is the first report, to our knowledge, using the 3DNA detection system (Genisphere) and polyllysine-coated slides used to print the microarrays, allowing one to work with 2–5 μg of total RNA without any mRNA amplification step (33). In addition, the standard deviation of the control experiment, 0.28–0.31, was comparable with direct incorporation methods employing Cy3’ and Cy5’ modified UTP, which typically yielded values between 0.19 and 0.21.2 Normalized data tables can be obtained on the World Wide Web at www.aecom.yu.edu/thecvekllab. Some of the data were flagged due to the unacceptable signal intensity above background intensity (i.e. if the signal intensity above background intensity was less than 100, then the spot would be flagged). This resulted in the identification of more than 400 differentially expressed genes; the vast majority of them were down-regulated, consistent with Pax6 roles as an activator of transcription. From these data, three lists of genes were generated. The first list, shown in Table II, includes genes with known functions classified into 12 subcategories (38), flagged no more than once, and expressed in Pax6 heterozygous lenses at reduced levels up to a factor of 0.63 and up-regulated genes by a factor of at least 1.6. When a single flag was found, we included the data if mean and median values were similar. The second list, shown in Table III, includes known genes that could not be classified into one of the 11 functional categories. The third list, shown in Table IV, includes ESTs showing strong and moderate similarities with genes deposited in public data bases and contained no more than one flagged value, as described above. Two genes down-regulated in Pax6 heterozygous lenses and relevant to known lens biology are homeodomain-containing transcription factor Pitx3 and structural βA4-crystallin (41).

Genes Abnormally Expressed in Pax6 Heterozygous Lenses, Eyes, and PAX6(5a) Transgenic Lenses to Identify Most Commonly Differentially Expressed Genes—Since such a large number of genes were differentially expressed, it was difficult to select which genes were appropriate for further analysis. Thus, a parallel series of microarray hybridizations were conducted using RNAs obtained from normal and Pax6 heterozygous eyes that have had the lenses surgically removed. This analysis

2 B. K. Chauhan and A. Cvekl, unpublished data.
### Genes Regulated by Pax6 in Mouse Lens

The table shows a list of known genes, arranged into functional categories, that were found to be either up- or down-regulated in Pax6 heterozygous lenses.

| Gene                                      | Description                                                                 | GenBank™ accession no. | Change |
|-------------------------------------------|------------------------------------------------------------------------------|------------------------|--------|
| **Cell growth, division, and DNA synthesis**                                             |                                                                              |                        |        |
| Akr2                                      | Thymoma viral proto-oncogene 2                                              | W82557                 | –2.1   |
| Ccne2                                     | Cyclin E 2                                                                  | AA414283               | –7.7   |
| Lrp1                                      | Ligase I, DNA, ATP-dependent                                                | W66626                 | –7.9   |
| Ptprn1                                    | Protein-tyrosine phosphatase, nonreceptor type 16                           | AA125367               | –7.3   |
| Rab19                                     | RAB19, member ras oncogene family                                           | AA118762               | –1.6   |
| Rho                                       | Stathmin                                                                    | AA260396               | –1.7   |
| Rf1                                        | Replication factor C, 140 kDa                                              | AA011737               | –2.1   |
| **Cell rescue, defense, and death**                                                    |                                                                              |                        |        |
| Bcl10                                     | B-cell leukemia/lymphoma 10 (CARD-containing proapoptotic protein)          | W47752                 | –2.2   |
| Brf1                                      | Butyrate response factor 1                                                  | AA060205               | –1.7   |
| F9                                        | Coagulation factor IX                                                        | AA209911               | –3.6   |
| H2-Ob                                     | Histocompatibility 2, O region β locus                                      | AA145469               | –2.4   |
| Msa2a                                     | Membrane-spanning 4 domains, subfamily A, member 2                          | AA183871               | –2.0   |
| **Cellular organization**                 |                                                                              |                        |        |
| mACF7                                     | Mouse actin cross-linking factor, neural isoform 2                          | W56652                 | –1.9   |
| Adam9                                     | A disintegrin and metalloproteinase domain 9 (meltrin γ)                    | AA210306               | –3.3   |
| Cappa1                                    | Capping protein α 1                                                         | AA414612               | –4.8   |
| Cdh3                                      | Cadherin 3                                                                  | W12889                 | –2.0   |
| Cryba4                                    | Crystallin, β A4                                                           | W92104                 | –1.6   |
| Don                                       | Destrin                                                                     | W17549                 | –1.9   |
| Img                                       | Integral membrane glycoprotein                                              | W83922                 | –1.5   |
| Palrn                                     | Paralemin                                                                   | Y14771                 | –3.6   |
| Ptx2                                      | Flexin 2                                                                    | AA511430               | –2.5   |
| Ptx3                                      | Flexin 3                                                                    | W76838                 | –1.8   |
| Ptn                                        | Profilin 2                                                                  | AA139628               | –8.9   |
| Smoc1                                     | Secreted modular calcium-binding protein 1                                  | AA000223               | –2.3   |
| Sptbn3                                    | β-Spectrin 3                                                                | AA049581               | –1.9   |
| Tbx4                                      | Thrombospondin 4                                                            | AA003452               | –1.9   |
| Tubb5                                     | Tubulin, β 5                                                                | W16254                 | –1.5   |
| **Energy**                                |                                                                              |                        |        |
| Slc25a10                                   | Mitochondrial solute carrier 25a10 (adenine nucleotide translocator)       | W86635                 | –1.6   |
| **Intracellular transport**               |                                                                              |                        |        |
| Nxt1-pending                             | NTP2-related export protein 1                                              | W13971                 | –3.9   |
| PMCA1                                     | Plasma membrane calcium ATPase isoform 1 (Rattus norvegicus)                | AA125425               | +1.8   |
| Sec23a                                    | SEC23A (Saccharomyces cerevisiae)                                           | AA213082               | –3.1   |
| **Ionic homeostasis**                     |                                                                              |                        |        |
| Cln7                                      | Chloride channel 7                                                          | W80488                 | –1.7   |
| **Metabolism**                            |                                                                              |                        |        |
| Asns                                      | Asparagine synthetase                                                       | W29492                 | –1.6   |
| Dhod                                      | Dihydropyruvate dehydrogenase                                               | AA298398               | –2.6   |
| DRP3                                      | Dihydropyrimidinase-related protein 3                                       | W18828                 | –1.8   |
| Gnm1                                      | Glycine N-methyltransferase                                                  | W83078                 | –5.0   |
| Hmgcs2                                    | 3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 2                            | AA030116               | –2.9   |
| Hsd3b1                                    | Hydroxysteroid dehydrogenase-1, 5/5/3-β                                     | AA028760               | –4.5   |
| Mocs3                                     | Molybdenopterin synthase sulfurylase (Homosapiens)                         | W99918                 | –4.2   |
| Mor2                                      | Malate dehydrogenase, soluble                                              | W13686                 | –2.2   |
| Pahx                                      | Phytanoyl-CoA o-hydroxylase                                                  | W82212                 | –1.8   |
| Pcdh11                                    | Pycs (P物arylethylamine-coenzyme A isomerase)                               | AA030780               | –2.2   |
| Pcs                                        | Pyrroline-5-carboxylate synthetase                                           | W41878                 | –1.6   |
| **Protein synthesis and degradation**      |                                                                              |                        |        |
| Cpxf1                                     | Cleavage and polyadenylation-specific factor                               | W41928                 | –2.3   |
| Ef10a                                     | Eukaryotic translation initiation factor 1A                                 | W41459                 | –1.9   |
| Ef10a.2x                                  | Eukaryotic translation initiation factor 2, subunit 3                       | W89599                 | –1.8   |
| FbW5                                      | F-box and WD-40 domain protein 5                                            | W79991                 | –2.1   |
| Prxs20/klk11                              | Hippoastatin                                                                | W13212                 | –6.0   |
| L7                                        | Ribosomal protein, mitochondrial                                           | AA028752               | –2.0   |
| Rnase12                                   | Ribonuclease P2                                                             | W71337                 | –1.7   |
| Ube2g2                                   | Ubiquitin-conjugating enzyme E2G 2                                          | AA237660               | –11.2  |
| Ube4                                      | Ubiquitin-conjugating enzyme 4                                              | AA108185               | –1.8   |
| **Signal transduction**                   |                                                                              |                        |        |
| Arhn                                      | Aplysia Ras-related homolog N (RhsN)                                       | W64242                 | –2.7   |
| Bmp1                                      | Bone morphogenetic protein 1                                               | W82677                 | –1.7   |
| CD97                                      | CD97 antigen                                                                 | AA118715               | –1.8   |
| Drk1                                      | Receptor-like tyrosine kinase                                               | W98395                 | –5.8   |
| Fm1fb2                                    | Mouse feminization 1b homolog (Caenorhabditis elegans)                      | AA030393               | –14.1  |
| Igf1                                      | Interferon-inducible GTPase                                                | AA260490               | +2.0   |
| Mapkt5                                   | Mitogen-activated protein kinase 6                                          | W64920                 | –2.7   |
| **Transcription**                         |                                                                              |                        |        |
| Aebp2                                     | AE-binding protein 2                                                       | AA416308               | –6.1   |
| Atf1                                      | Activating transcription factor 1                                           | W87965                 | –2.1   |
| Eif1                                      | Eif1 repressor factor                                                       | W79066                 | –2.2   |
| Esr1                                      | Estrogen receptor 1                                                         | AA026625               | –1.6   |
| Ets6                                      | ETS variant gene 6 (Tel oncogene)                                          | AA260520               | –5.5   |
| Fkhr1                                     | Forkhead protein 1                                                          | W36356                 | –2.4   |
| Hoxa2                                     | Homeobox A2                                                                | W68322                 | –2.6   |
| Klf1                                      | Kruppel-like factor 1 (erythroid)                                          | W97446                 | –1.7   |
| Nup9                                       | Nuclear FMRP-interacting protein                                           | AA139817               | –1.6   |
| Pitx3                                     | Paired-like homeodomain transcription factor 3                              | AA062140               | –3.9   |
revealed that 79 genes were differentially expressed in Pax6 heterozygous eye, with 14 of these genes being common with the Pax6 heterozygous lens (Fig. 2). Since important genes expressed predominantly in the lens could have been missed in this analysis, a final set of microarray hybridizations were also performed using wild type FVB/N lenses and transgenic lenses expressing the PAX6(5a) splice variant in lens fiber cells (14) in the same mouse strain, and five genes were differentially expressed. These genes are paralemmin (39), molybdopterin synthase sulfurylase (MOCO3),3 the Tel6 oncogene (ETV6) (43), a cleavage-specific factor (Cpsf1), and tangerin A4 encoding a large protein found in brain cDNA libraries. In the group of nine down-regulated genes between Pax6 heterozygous lenses (Fig. 3B), a set of specific primers, we then showed that expression of paralemmin, MOCO3, Tel6 oncogene, a cleavage-specific factor (Cpsf1), and tangerin A was indeed reduced both in the Pax6 heterozygous lenses (Fig. 3C) and in transgenic lenses overexpressing Pax6(5a) (Fig. 4C).

**Paralemmin Protein Levels Are Decreased in the Pax6(5a) Transgenic Lens but Not the Pax6 Heterozygous Lenses—**Immunohistochemical labeling was performed to confirm the differential gene expression of paralemmin at the protein level. In normal lenses, paralemmin protein is detected at the cell membrane in all lens cells (Fig. 5). In Pax6(5a) transgenic lenses, its expression was decreased throughout the fiber mass while appearing normal in the germinative and transition zone (Fig. 5).

In lenses from Pax6 heterozygous mice, its expression appeared to be slightly increased in the lens epithelium as well as primary and secondary fibers (Fig. 5). Western blot confirmed reduced level of paralemmin in the extracts of 5a-transgenic lenses (Fig. 5).

*Candidate Pax6 Binding Sites in Paralemmin, MOCO3, *Pitx3*, and *βA4-Crystallin Genes—*To examine the possibility that Pax6 directly binds to regulatory regions of paralemmin, MOCO3, Pitx3, and βA4-crystallin, we found other regulatory genes and various structural genes. To date, genes directly regulated by Pax6 were either identified by educated guesses (14, 19–30) or from in situ studies of gene expression in Pax6 homozygous embryos (47–49). Multiple functions of Pax6 during the organogenesis of the eye, brain, and pancreas indicate that the majority of genes regulated by Pax6 remain to be discovered.

**Pax6-heterozygous lenses** (Fig. 3B). Using a set of specific primers, we then showed that expression of paralemmin, MOCO3, Tel6 oncogene, a cleavage-specific factor (Cpsf1), and tangerin A was indeed reduced both in the Pax6 heterozygous lenses (Fig. 3C) and in transgenic lenses overexpressing Pax6(5a) (Fig. 4C).

**RT-PCR Analysis of Differentially Expressed Genes—**The differential expressions of mRNAs encoding paralemmin, MOCO3, Tel6 oncogene, a cleavage-specific factor, and tangerin A were confirmed using semiquantitative RT-PCR. As a control, the genes encoding argininosuccinate synthetase and uridine monophosphate kinase and encoding ubiquitin-conjugating enzyme 4 and ubiquinone biosynthesis gene *coq7*/*clkl* were selected from the pool of unchanged genes obtained from the microarray experiments with Pax6 heterozygous and Pax6(5a) transgenic lenses, respectively. As expected, no difference in expression of these genes was found (Figs. 3B and 4B) using the same amounts of RNA of the compared samples. Using these conditions, we also found reduced expression of Pax6 mRNA in

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3 The GenBank™ accession number for mocs3 is AF102544.

4 The GenBank™ accession number for tangerin A is AF305087.
The genes discovered to be differentially expressed in the Pax6 heterozygous lenses are likely to fall into three classes. First are genes whose expression is directly controlled by Pax6 whose reduced expression is the result of a reduction in Pax6 expression (Fig. 3). The genes in the second group are reduced, since their expression is controlled by transcription factors (such as c-Maf, Eya1 and -2, or Sox2) whose expression is reduced, whose reduced expression is the result of a reduction in Pax6 expression (Fig. 3). The genes in the second group are reduced, since their expression is controlled by transcription factors (such as c-Maf, Eya1 and -2, or Sox2) whose expression is controlled by Pax6 (7, 22, 23). The third group of genes are those differentially expressed as an indirect result of the phenotypic alterations seen in Pax6 heterozygote/wild type chimeras undergo preferential lens fiber cell differentiation (53). While the phenotypic alterations in the Pax6 heterozygous lenses are typically mild, the gene expression profiling described here suggests that the transcriptome is globally disrupted. Since the current estimations of genes in the mouse and human genome are between 40,000 to 70,000, and the cDNA microarrays used here contain over 9000 independent genes, it is possible to conservatively predict that at least 2000 genes are differentially expressed in the Pax6 heterozygous lenses.

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Since the gene profiling described here was performed to discover both direct Pax6 target genes and those whose expression is controlled by Pax6-dependent pathways in the lens, the set of genes differentially expressed in the Pax6 heterozygous lens was compared with those whose expression was altered in the remainder of the eye. While this observation reduced the number of genes to be analyzed to 19, this left the concern that important Pax6-dependent genes expressed predominantly in the lens would be improperly sidelined. Thus, expression profiling was also performed on lenses from transgenic mice overexpressing the Pax6(5a) splice form in fiber cells, which normally contain reduced amounts of Pax6 compared with the lens epithelium (54). While a remarkably small number of genes were found to be differentially expressed in Pax6(5a) lenses in light of the drastic alterations in lens fiber cell morphology reduction in the number of cells incorporated into the lens vesicle, perhaps due to decreased cell division in the lens plaque. However, cell division rates appear to be normal after lens formation (6). In contrast, the cataracts appear to develop due to impaired responses of the lens epithelium to the eye environment (52), which may explain why Pax6 +/− epithelial cells in Pax6 heterozygote/wild type chimeras undergo preferential lens fiber cell differentiation (53).
caused by this ectopic expression (14), it was remarkable that 13 of the 27 genes were also differentially expressed in either Pax6 heterozygous lenses or eyes, whereas five were differentially expressed in all three data sets. While the Pax6(5a) transgenic mice express additional Pax6(5a) protein in the lens, and the Pax6 heterozygous lenses express reduced levels of both Pax6 and Pax6(5a), it is interesting to note that additional copies of the entire Pax6 locus caused ocular and lens abnormalities similar to the Pax6 haploinsufficiency (55). In addition, many reporter genes activated by low concentrations of Pax6 in transient transfections are repressed as Pax6 concentrations increase (28). Thus, it is possible that overexpression of Pax6(5a) in the lens could cause repression of a small group of genes that are otherwise activated by Pax6/Pax6(5a) in normal lenses. We focused on these five genes, and using semiquantitative RT-PCR analysis, we showed that expression of paralemmin, MOCS3, Tel6 oncogene, a cleavage-specific factor (Cpsf1), and tangerin A was indeed reduced both in the Pax6 heterozygous lenses (Fig. 3C) and in transgenic lenses overexpressing PAX6(5a) (Fig. 4C). We also found significantly reduced paralemmin in 5a-transgenic but not in Pax6 heterozygous lenses (Fig. 5). We do not know the reason for this unex-
Paralemmin is down-regulated at the protein level in the Pax6(5a) lens. A, Western blot of paralemmin expression in 6-week Pax6(5a) transgenic and wild type mouse lens. Note that paralemmin protein levels are significantly reduced in the lenses of Pax6(5a) transgenic mice. *Paralemmin expression in a 4-week-old wild type lens. Note that high levels of paralemmin protein are associated with fiber cell membranes. Magnification was ×200. C, paralemmin expression in a 4-week-old Pax6(5a) transgenic lens. Note that the paralemmin staining seen in lens fibers is greatly reduced. Magnification was ×200. D, paralemmin expression in a 3-week-old Pax6 heterozygous lens. Magnification was ×200. E, central lens epithelium and fiber cells of a 4-week-old wild type mouse. Magnification was ×630. F, central lens epithelium and fiber cells of a 4-week-old Pax6(5a) transgenic mouse. Magnification was ×630. G, central lens epithelium and fiber cells of a 3-week-old Pax6 heterozygous mouse. Magnification was ×630. H, interface between the zone of denucleation and nucleated lens fibers in a 4-week-old wild type mouse. Magnification was ×630. I, central dysgenic fibers of a 4-week-old Pax6(5a) transgenic mouse. Magnification was ×630. J, 3-week-old wild type lens stained with preimmune serum. Magnification was ×630, ict, wild type; 5a-t, Pax6(5a) transgenic; e, lens epithelium; f, fiber cells; sf, secondary fiber cells; t, transition zone; zd, zone of denucleation. Red, paralemmin; green, DNA.

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expected discrepancy between RNA and protein data; however, several factors could contribute to this finding. First, there may be differences in paralemmin protein turnover between these lenses. Second, Pax6 haploinsufficiency may cause reduced transcription of paralemmin but increased translation and accumulation of the protein. For example, little Dach mRNA is detected in lens fibers by in situ hybridization (56, 57), but protein accumulation is easily detected by immunohistochemistry (58). Finally, it is possible that paralemmin mRNA levels are abundant in the lens epithelium and activated by Pax6, but lower levels of paralemmin transcription normally occur in fibers that are further repressed by high levels of Pax6(5a). These possibilities are not mutually exclusive, since a combination of differential RNA abundance and differential transcription in epithelial and fiber cells may best explain these data.

Our finding of paralemmin expression in the lens raises the possibility of its specific role in the shape of lens fiber cells. Paralemmin is a putative new morphoregulatory protein highly expressed in the forebrain and cerebellum, two prominent tissues expressing Pax6 (59), but also less abundantly in many other tissues (39). The known expression pattern of Pax6 and paralemmin combined with the presence of candidate Pax6 binding site in its putative promoter region raises the possibility that the Pax6 directly regulates its expression. Future studies will be aimed at addressing roles of paralemmin in lens morphology and its transcriptional control.

MOCS3 catalyzes one of the final steps in the formation of the organic complex of molybdenum (Moco) (60). Moco is a cofactor essential to the function of three enzymes: sulfite oxidase, xanthine dehydrogenase, and aldehyde oxidase. Deficiency of Moco results in neurological abnormalities, mental retardation, and, in some cases, dislocated lenses (61, 62). Comparably, dislocated lenses are also found in some aniridia patients (63), suggesting indeed the possibility that MOCS3 is downstream of Pax6. Our data show that the expression levels of MOCS3 are reduced, but not abolished, in the lens and indicate the presence of several Pax6 binding sites (Fig. 6). Hence, the data raise the possibility that Pax6 may act as a modulatory transcription factor fine tuning expression of these genes in the lens, eye, and possibly other tissues in mammalian brain. However, the expression pattern of MOCS3 in the mammalian models is not known due to the lack of antibodies.

We also confirmed reduced expression of three remaining genes from the pool of five genes down-regulated in all three models studied (see Fig. 2): Tel6 oncogene, a cleavage-specific factor (Cpsf1), and tangerin A in both Pax6 heterozygous lenses and Pax6(6a)-transgenic lenses. Tel6/ETV6 encodes a widely expressed transcriptional repressor that is often rearranged in human leukemias (43). Tangerin A is a novel large protein with a calponin homology domain (4). The calponin homology domain is present in many actin binding cytoskeletal and signal-transducing proteins (64). Interestingly, there are more genes with reduced expression in Pax6 heterozygous lenses (i.e. mACF7, Cappa1, Dsn, and Phn2) that also bind actin and are shown in Table II (group Cellular organization). In the absence of relevant information about these genes and/or specific reagents, we did not study them further; nevertheless, their confirmed abnormal expression supports the reliability of the cDNA microarray technology.

While not differentially expressed in Pax6(5a) lenses or Pax6 heterozygous eyes, two genes differentially expressed in Pax6 heterozygous lenses, β4A-crystallin and Pitx3, require special attention. The known expression of β4A-crystallin and of Pitx3 in the lens, supported by phenotypes of mouse and human Pitx3 mutants, makes these two genes prime candidate direct targets for Pax6-mediated regulation of transcription. Pitx3, a homeobox-containing gene, is required for normal lens develop-
opment (65, 66), and mutations in PITX3 cause congenital cataract (67). The expression pattern of the Pitx3 gene in the mouse lens supports the possibility that Pax6 might directly control its expression (48), and we have confirmed reduced expression of Pitx3 in Pax6 heterozygous lenses by RT PCR analysis as described elsewhere.5 Pitx3 expression in lens (from E11) found in the lens vesicle and later in the entire lens with the highest, the developmental patterns of Pax6 and Pitx3 spatially overlap with Pitx3 expression following Pax6 (59, 65). βA4-crystallin appears to be highly expressed only in the lens. In the absence of published data, we searched human and mouse TIGR expression data bases (available on the World Wide Web at www.tigr.org/db) and found that human βA4-crystallin was predominantly expressed in the lens (22 clones) and that mouse βA4-crystallin clones were found mainly in total embryos (11 clones) but not in individual tissue-specific libraries. Only two clones were found in human retina and one clone in human placenta- and mouse skin and epidermis-de- stined libraries, respectively. Two putative Pax6 binding sites were found in the human βA4-crystallin 5′-flanking region, and two evolutionary conserved Pax6 binding sites were found in human and mouse 5′-flanking sequences of PITX3/Pitx3 genes.

Several genes found in the present study are good candidates to explain abnormal lens development in Pax6 heterozygous lenses (6) and properties of chimeric lenses from wild type and Pax6+/− cells (53). The selective exclusion of Pax6 heterozygous lens cells from the E16.5 lens was probably due to the aberrant expression of cell adhesion and extracellular matrix proteins (53). Our list of differentially expressed genes in 8-week-old Pax6 heterozygous lenses includes several membrane-associated proteins (i.e. Adam9, cadherin 3, Img/LIG-1, parallemmin/Palm, Snp63, and Thbs4 (cellular organization genes in Table II) and a receptor-like tyrosine kinase Ddr1 (signal transduction genes in Table II). Some of these proteins may also play critical roles during the lens embryogenesis. Our data also show reduced level of expression of cyclin E2 (69, 70). It may also play critical roles during the lens embryogenesis. Our

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ectopic Pax6(5a) in the lens have revealed a population of 10 genes for future study that have the potential to be direct or indirect Pax6 targets. Our data base will serve as an important gateway for future systemic analysis of genes acting in the same genetic pathway as Pax6, not only in the lens but in other tissues expressing Pax6. In the long term, such differential gene expression information will be useful in understanding how Pax6 mutations result in the diverse eye and brain diseases observed in humans.
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