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The *Optimedin* Gene Is a Downstream Target of Pax6*

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The *Optimedin* gene, also known as *Olfactomedin 3*, encodes an olfactomedin domain-containing protein. There are two major splice variants of the *Optimedin* mRNA, *Optimedin A* and *Optimedin B*, transcribed from different promoters. The expression pattern of the *Optimedin A* variant in the eye and brain overlaps with that for *Pax6*, which encodes a protein containing the paired and homeobox DNA-binding domains. The *Pax6* gene plays a critical role for the development of eyes, central nervous system, and endocrine glands. The proximal promoter of the *Optimedin A* variant contains a putative Pax6 binding site in position −86/−70. Pax6 binds this site through the paired domain *in vitro* as judged by electrophoretic mobility shift assay. Mutations in this site eliminate Pax6 binding as well as stimulation of the *Optimedin* promoter activity by Pax6 in transfection experiments. Pax6 occupies the binding site in the proximal promoter *in vivo* as demonstrated by the chromatin immunoprecipitation assay. Altogether these results identify the *Optimedin* gene as a downstream target regulated by Pax6. Although the function of optimedin is still not clear, it is suggested to be involved in cell-cell adhesion and cell attachment to the extracellular matrix. Pax6 regulation of *Optimedin* in the eye and brain may directly affect multiple developmental processes, including cell migration and axon growth.

The *Optimedin* gene, also known as *Olfactomedin 3*, encodes a secreted protein belonging to a family of olfactomedin domain-containing proteins (1). Olfactomedin was originally identified as a glycoprotein exclusively present in the bullfrog olfactory neuroepithelium (2). Homologues of this protein were subsequently found in a variety of tissues from different species ranging from sea urchin to human (3–9). Most of the olfactomedin-related proteins have a variable N terminus and a more conserved C terminus, which is called the olfactomedin domain. There is also a small family of calcium-independent seven-transmembrane receptors for latrotoxin (CIRL1-CIRL3) with a large N-terminal extracellular part containing an olfactomedin domain (10–14).

Olfactomedin domain-encoding genes show tissue-specific expression patterns. In adult mammals, *Olfactomedin 1*, also known as *Noelin-1* and *Pancortin*, is expressed in brain, lung, kidney, and retina (1, 3, 15). *Optimedin* is expressed in the retina and brain (1, 16). Human *hOLF44*, also known as *OLFACTOMEDIN-LIKE 3* and *HNOEL-ISO*, is expressed in various tissues and is particularly abundant in placenta (17). In the rat eye, the *Hnoel-iso* gene was more actively expressed in the iris and sclera than in other eye structures (18). The human *GW122/hGC-1* gene, which is also named *pDP4* in mice (19) and *tiarin* in *Xenopus* (7), is expressed in bone marrow, small intestine, colon, and prostate (20). The *myocilin* gene is highly expressed in the eye trabecular meshwork and sclera and less actively expressed in several other ocular and non-ocular tissues (1, 21–24). *CIRL1* and *CIRL3* genes, also known as *Latrophilin-1* and *Latrophilin-3*, respectively, are expressed in the brain and eye (12, 18), whereas the *CIRL2* gene is expressed in the brain, lung, liver, and eye (12, 18). It has been reported that expression patterns of individual olfactomedin domain encoding genes may differ between different vertebrates (25).

Although the exact functions of different olfactomedin domain-containing proteins as well as the molecular mechanisms of their action are still not known, a growing amount of evidence indicates that proteins belonging to this family may play important roles in the normal development of different organs. Noelin-1 is involved in the regulation of the production of neural crest cells by the neural tube in chicken (5) and promotes neurogenesis in *Xenopus* (26). *Xenopus* Tiarin may participate in the specification of the dorsal neural tube (7). Sea urchin amassin mediates the massive intercellular adhesion of coelomocytes, the immune cells contained in the coelomic cavity (4).

Mutations in the olfactomedin domain may be deleterious for the functions of these proteins. For example, mutations in the olfactomedin domain of the human *MYOCILIN* gene may lead to juvenile open-angle glaucoma and in some cases to adult onset glaucoma (27, 28). Unlike wild-type myocilin, mutated myocilin does not move properly through the secretory pathway and is not secreted from cells (29–31). In the presence of mutated myocilin, secretion of wild-type myocilin is also inhibited. It has been suggested that chronic expression of mutated, non-secreted myocilin leads to trabecular meshwork cell death and, ultimately, a dominant glaucoma phenotype (32, 33).

Because olfactomedin-related proteins appear to play significant roles in normal development and pathology, regulation of their activity represents an important area of research. Not much is known about regulation of these genes. It has been demonstrated that the human *MYOCILIN* gene is activated by glucocorticoids with a delayed kinetics (34–36). It has been suggested that the upstream regulatory factor may be essential for the regulation of the *MYOCILIN* gene (37). The ETS-family transcription factor PU.1 (Spi-1) may be involved in the regulation of the *pDP4* gene in mature mouse granulocytes (19).

In the present work, we investigated the regulation of the mouse *Optimedin* gene. In the mouse brain and retina, only the *Optimedin A* promoter is actively used. The proximal promoter of the *Optimedin A* gene contains putative binding sites for several transcription factors, including a homeo- and paired domain protein Pax6. Pax6 is a transcription factor that is critical for development of several tissues including eyes, central nervous system, and endocrine glands (see Ref. 38 for review). Several downstream targets of Pax6 have been identified and they include transcription factors, cell adhesion molecules, hormones, and structural proteins (38, 39). Results presented in this article indicate that the *Optimedin* gene is another downstream target gene for Pax6.

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MATERIALS AND METHODS

Cell Cultures and Plasmid Construction—Monkey COS7 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Rat retinal ganglion RGC-5 cells (40) were kindly provided by Dr. N. Agarwal (University of North Texas Health Science Center). RGC-5 cells were incubated in F-12/Dulbecco's modified Eagle's medium (1:1), containing 5% fetal bovine serum, 1.5 mM L-glutamine, 7.5 mM Na-pyruvate, 0.1 mM nonessential amino acids. Murine βTC3 cells were kindly provided by Dr. S. Efrat (Albert Einstein College of Medicine, New York). βTC3 cells were incubated in Dulbecco's modified Eagle's media, containing 15% horse serum and 2.5% bovine serum. All cell lines were incubated at 37 °C in an atmosphere of 5% CO_2.

Different regions of the 5′-flanking region of the mouse *Optimedin A* promoter were amplified by PCR and cloned into the promoterless, enhancerless expression vector *pGL3-Basic* (Promega) upstream of the *Luciferase* reporter gene. KpnI/SmaI restriction sites were used for cloning of the −3153/+98 and −2829/+98 promoter fragments, whereas XhoI/HindII restriction sites were used for cloning of the −136/+24 promoter fragment. These promoter constructs were designated p3153OPT, p2829OPT, and p136OPT, respectively. Constructs containing mutations in the putative Pax6 and Sox binding sites were produced using a PCR-based site-directed mutagenesis kit (Stratagene, La Jolla, CA). A putative Pax6 binding site, 5′-CTGAACTGGAGCGTAAG-3′ (position −86/−70), was changed to 5′-CTGAACTGGAtctTAAG-3′. A putative Sox binding site, 5′-TAACAAAT-3′ (position −60/−52), was changed to 5′-gggCAAT-3′. The resulting mutated plasmids (see Fig. 1, *B–C*) were named p2829MUTp, p136MUTp (Pax6 mutants), p2829MUTs and p136MUTs (Sox mutants). The identity of all constructs was confirmed by sequencing.

Sox2, *Sox11*, and *Brn1* cDNAs were cloned into the *pCMV5* expression vector (41, 42) and were kindly provided by Drs. A. Rizzino (University of Nebraska) and D. Robbins (University of Michigan), respectively. *Sox8* cDNA was cloned into the *pcDNA3* expression vector (43) and was kindly provided by Dr. P. Koopman (The University of Queensland, Australia). *Brn3b*, *NeuroD*, and *Math5* cDNAs were cloned into the *pRK5* expression vector and were kindly provided by Dr. L. Gan (University of Rochester). *Six5* cDNA was cloned into the *pFLAG-CMV-2* vector (44), kindly provided by Dr. K. Kawakami (Jichi Medical School, Japan). cDNAs encoding Pax6 and Pax6(5a) were cloned into the *pKW10* expression vector; the paired domain of Pax6, and the paired domain of Pax6(5A) were cloned into the vector *pETH2a* (45).

Luciferase Assay—For luciferase assay, COS7 or RGC-5 cells were plated on the 24-well plates at a density of 5×10^4 cells/well for COS7 cells and $1.5–2 \times 10^4$ cells/well for RGC-5 cells. Cells were transfected 24 h after plating using FuGENE 6 (Roche Applied Science) and following the protocol recommended by the manufacturer. *pRL-CMV* or *pRL-null* plasmids provided an internal standard of luciferase activity to which all the measurements were normalized. Three independent transfection reactions were performed for each combination. Dual luciferase assay was performed 48 h after transfection using the Dual Luciferase Reporter® Assay 1000 System (Promega). All experiments were repeated at least twice.

Western Blotting—To estimate amounts of Sox2 and Sox11 proteins in the nuclear extracts, different amounts of these extracts were separated by SDS-PAGE and transferred onto nitrocellulose membrane. The membranes were blocked in the blocking solution (Roche Applied Science) at 4 °C overnight. Primary monoclonal mouse anti-FLAG antibodies (Sigma) were used in 1:4000 dilution. Secondary anti-mouse antibodies conjugated with horseradish peroxidase (Amersham Biosciences) were used in 1:10000 dilution. To estimate the content of Pax6 in COS-7, RGC-5, and βTC3 cells, cells were lysed in the lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 20 mM dithiothreitol, 1% Nonidet P-40, 0.2% SDS), sonicated, centrifuged at 14,000 × *g*, and the supernatant was used for Western blotting. Rabbit anti-Pax6 polyclonal IgG (Covance, Richmond, CA) were used in 1:200 dilution as a primary antibody. Anti-rabbit horseradish peroxidase-conjugated antibodies (Amersham Biosciences) were used as a secondary antibody in 1:4000 dilution. All blots were visualized by SuperSignal Chemiluminescent Detection kit (Pierce Biotechnology).

Electrophoretic Mobility Shift Assays (EMSA)[2]—COP8 cells were transfected with different Pax6-expressing constructs. Nuclear extracts were prepared as described (46). The levels of Pax6 and Pax6(5a) expression were evaluated by Western blotting as described above. EMSA with *Escherichia coli* expressed and affinity purified paired domains was performed as described (45). Final concentration of the binding reaction (1× EMSA buffer) was 4% Ficoll, 10 mM Tris, pH 8, 1 mM dithiothreitol, 1 mM EDTA, 100 mM KCl. Anti-Pax6 antibodies used for EMSA were rabbit polyclonal antibodies directed against the Pax6 paired domain (47) or the C terminus (48). Anti-Pax2 rabbit polyclonal antibody raised against the C terminus of Pax2 was purchased from Covance Research Product (Berkley, CA).

COS7 cells were transfected with expression constructs *pCMVSox2FLAG* or *pCMVFLAGSox11*. Nuclear extracts were prepared 48 h after transfection using NE-PER® Reagents (Pierce Biotechnology) according to the manufacturer's instructions. The binding reaction contained 17–18 μl of 1× binding buffer (20 mM HEPES, pH 7.8, 4% Ficoll, 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol, 25 μg/ml poly(dG-dC)) and corresponding oligonucleotides. Oligonucleotides were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Labeled, double-stranded oligonucleotides were used at ~0.1 ng/μl and 20,000 cpm/μl per reaction. In competition experiments, 50- or 500-fold excess of unlabeled double-stranded oligonucleotides were added to binding reactions.

Chromatin Immunoprecipitation (ChIP) Assay—The whole mouse embryonic brain (E17) was used as a tissue source of chromatin. Brain tissues were homogenized in 1× phosphate-buffered saline (900 μl/brain) with protease inhibitors (Roche Applied Science). Proteins were cross-linked in 1% formaldehyde for 15 min at room temperature with gentle shaking. Cross-linking was terminated by treatment with 125 mM glycine for 5 min and two washes in 1× phosphate-buffered saline. Samples were then processed using a ChIP assay kit, essentially as described by the manufacturer (Upstate Biotechnology, Lake Placid, NY). In brief, the cells were lysed in SDS lysis buffer with protease inhibitors, then sonicated using a model W-220F Heat Systems Ultrasonics (Farmingdale, NY) to shear DNA to fragments with a length of 100–1000 bp. 1% of the cell lysate was saved as input chromatin for PCR analysis. To reduce nonspecific background, the rest of the cell lysate was precleared by incubation with salmon sperm DNA/protein A-agarose slurry. Supernatants from three equal reactions were incubated with 10 μg of normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA), 5 μg of rabbit anti-Pax6 polyclonal IgG (Covance), or 5 μg of goat anti-Sox11 polyclonal IgG (Santa Cruz Biotechnology) at 4 °C overnight. One additional reaction without any antibodies served as a negative control. Chromatin-antibody complexes were precipitated by incubation with Protein A-agarose beads. Chromatin was eluted from the beads after washes in several buffers provided with the kit. The DNA-protein cross-links in all samples including the input were reversed by incubation for 4 h at 65 °C followed by incubation with

[2] The abbreviations used are: EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation.

proteinase K for 1 h at 45 °C. DNA was isolated by phenol/chloroform extraction and ethanol precipitation. The PCR primer pairs used for ChIP assay were as follows. The first pair spanning the −235/+104 region was 5′-ATAACTCAATGTCAGTTCACTT-3′ (forward) and 5′-TCCCCTTCTGTTAGACAGCC-3′ (reverse); the second pair in the region −3719/−3421 was 5′-GAAACGCTATATGACAGCCTCT −3′ (forward) and 5′-CTTCAATTTTTAGTGAATGCATG −3′ (reverse); the third pair located in the 6th intron of the *Optimedin* gene was 5′-GGTAAGTTTAGATGATTGATGAT-3′ (forward) and 5′-GTACACATCATGAAACACAATGG-3′ (reverse). The 2nd and 3rd pairs served as negative controls. PCR conditions were as follows: 3 min at 94 °C followed by 29 cycles of 30 s at 94 °C, 40 s at 55 °C, 1 min at 72 °C, and a final extension for 10 min at 72 °C. PCR products were separated by 1% agarose gel electrophoresis and visualized by staining with ethidium bromide.

RESULTS

Analysis of the Proximal Promoter Sequence of the Optimedin Gene—Two *Optimedin* gene transcripts transcribed from different promoters were identified in rat tissues (1). *Optimedin A* was preferentially expressed in the retina and brain, whereas *Optimedin B* was preferentially expressed in the combined tissues of the eye angle (trabecular meshwork, iris, and ciliary body). In the adult rat retina, *Optimedin A* was expressed mainly in the ganglion and inner nuclear layers (1). A similar pattern of *Optimedin* gene expression was observed in mouse tissues, although the level of its expression in the mouse eye angle tissues was lower than in rats (not shown).

Because we were interested in the *Optimedin* gene expression in the retina and brain, we compared the *Optimedin A* promoter sequences from mouse, rat, and human. The *Optimedin A* promoter will be called the *Optimedin* promoter throughout the paper. Two conserved regions were identified that were located in the positions (−211/−1) and (−3070/−2759) of the mouse promoter (Fig. 1*A*). The mouse proximal promoter region (positions (−211/−1) did not contain a consensus TATA box sequence. It contained several closely spaced putative binding sites for Pax6, Sox, Six, and USF transcription factors (Fig. 1*A*). Some of these sites were well conserved in the rat and human promoters as well.

Activity of the Optimedin Promoter in Vitro—The basal activity of the *Optimedin* promoter and possible involvement of several transcription factors in regulation of its activity was first tested in COS7 cells. Several promoter deletion constructs were prepared. The longest p3153OPT construct included both conserved elements identified in the *Optimedin* promoter, whereas the shortest p136OPT construct included only part of the proximal conserved element (Fig. 1, *A and B*). p3153OPT, p2829OPT, and p136OPT gave 10.3 ± 2.6-, 6.0 ± 1.2-, and 8.3 ± 2.1-fold stimulation over the pGL3 vector in COS7 cells (not shown). p136OPT will be called a proximal promoter throughout the paper.

The effects of a number of transcription factors on *Optimedin* promoter activity were tested in transfection experiments. Transcription factors that were used in these experiments are expressed in the retina and brain and belong to different classes. Brn1 and Brn3B are the POU-domain transcription factors. *Brn1* gene is prominently expressed in the embryonic brain (49), whereas *Brn3b* is specifically expressed in the retinal ganglion cells and regulates the expression of genes critical for axon formation (50, 51). Brn3b did not show significant stimulation of the p2829OPT promoter activity in co-transfection experiments when compared with pGL3 vector (Fig. 2*D*). Brn1 showed 4.8-fold stimulation of the p2829OPT in comparison with pGL3 (2.0-fold). Brn1 showed a similar 4.8-fold stimulation of the p136OPT (not shown). However, we were unable to identify a putative Brn1 binding site in the *Optimedin* proximal promoter.

Math5 and NeuroD belong to a family of basic helix loop helix transcription factors and are required for retinal ganglion (Math5) and amacrine (NeuroD) cell development (see Ref. 52 for review). Neither Math5 nor NeuroD stimulated the p2829OPT promoter (Fig. 2*D*).

Six5 is a member of the Six family of transcription factors. It is expressed in the ganglion and inner nuclear layers of the retina (53). A putative Six5 binding site, 5′-TCAAGTTGC-3′, was identified in the *Optimedin* proximal promoter at the −104/−95 position. It is a perfect match to the consensus Six5 binding site, TCARRTTKC, where R is G or A and K is G or T (53). However, Six5 did not show significant stimulation of the p2829 promoter (Fig. 2*D*).

Two putative Sox binding sites, showing similarity to the Sox HMG-domain binding consensus sequence 5′-(A/T)(A/T)CAA(A/T)G-3′ (54), were identified in the optimedin proximal promoter. One, located at −60/−52 (5′-TAACAAAT-3′), was conserved in the mouse, rat, and human *OPTIMEDIN* promoters, whereas the second site at −94/−87 (5′-CAGCAAAG-3′) contained two substitutions in the human promoter (Fig. 1*A*). Several *Sox* genes are expressed in the retina and brain. Three Sox transcription factors, Sox2, Sox8, and Sox11, belonging to groups B, E, and C of Sox proteins (44, 45), respectively, were tested in co-transfection experiments. Among the Sox proteins tested, Sox11 produced a 16.9-fold activation of the p2890OPT in COS7 cells (Fig. 2*A*). We concentrated on the Sox11 transcription factor that provided the highest level of stimulation in COS7 cells.

When the proximal p136OPT promoter was used, Sox11 stimulated its activity by 11.2-fold (Fig. 2*B*). These data indicate that the two putative Sox binding sites at positions −60/−52 and −94/−87 may play a key role in the activation of the *Optimedin* promoter by Sox11. To confirm a functional importance of these sites, the conserved −60/−52 site was mutated (5′-TAACAAAT-3′ to 5′-gggCAAAT-3′; see Fig. 1, *B* and *C*). Although p136MUTs and p136OPT constructs had similar basal promoter activities, Sox11 stimulation of the mutated construct was significantly reduced when compared with the wild-type construct (4.3- *versus* 11.2-fold; see Fig. 2*B*). This provides additional support to the suggestion that the −60/−52 Sox binding site may be essential for Sox11 stimulation. At the same time, because the stimulation of the p136OPT was about 35% lower than stimulation of the p2890OPT, additional Sox binding sites may be present upstream of the −136 position. As an example, two additional putative Sox binding sites are marked in Fig. 1*A* in positions −208/−201 and −184/−177.

Binding of Sox proteins to the putative Sox binding site in the proximal *Optimedin* promoter was tested by EMSA. Equal amounts of Sox2 or Sox11 proteins, as judged by Western blot experiment with FLAG antibodies (not shown), were used in the EMSA reactions. Control experiments demonstrated that oligonucleotide FX−, which was shown to bind both Sox2 and Sox11 proteins (55), interacted with Sox2 and Sox11 in nuclear extracts under the conditions used (Fig. 3*A*). Oligonucleotide OPTs2 (less conserved site −94/−87, see Fig. 1*C* for sequences of oligonucleotides) did not bind Sox11 and only weakly bound Sox2 (Fig. 3*A*). Oligonucleotide OPTs1 (more conserved site −60/−52) did not bind Sox11 but bound Sox2 (Fig. 3*A*). Binding to this site was less efficient than binding to the FX− sequence. Two mutations were introduced into the OPTs1 site. In the OPTs1C oligonucleotide, a natural putative binding site was changed to the perfect consensus 5′-AAACAAAG-3′. In the OPTs1M oligonucleotide, a mutation that reduced the stimulatory activity of Sox11 (Fig. 2*B*) was introduced (see Fig. 1*C*). Both Sox2 and Sox11 bound the OPTs1C sequence but did not bind the OPTs1M sequence (Fig. 3*B*). Finally, competition experiments

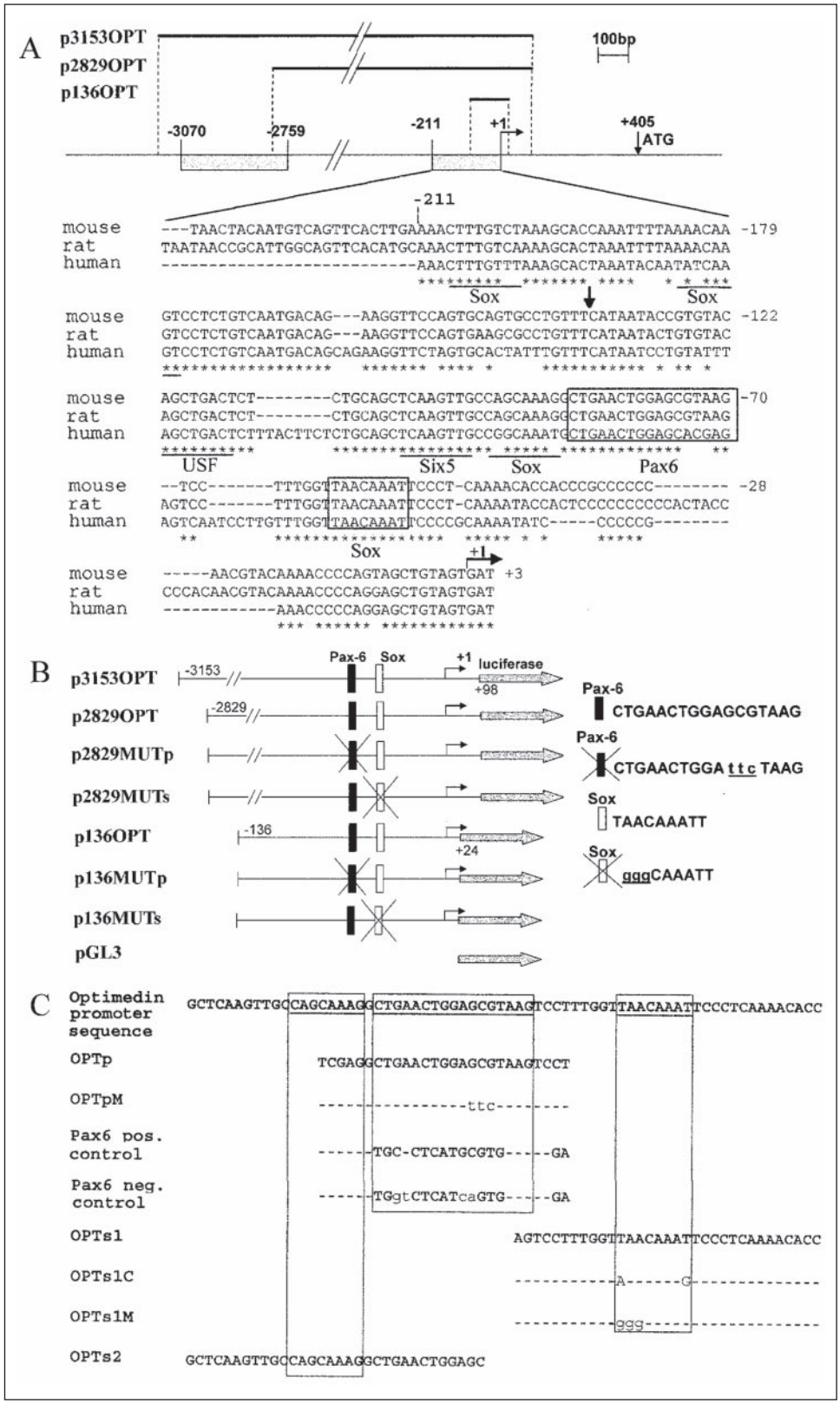

FIGURE 1. **Diagram of the mouse *Optimedin A* promoter and the constructs and oligonucleotides used in transfection experiments and EMSA.** *A*, comparison of the mouse, rat, and human *OPTIMEDIN* proximal promoters. Position +1 in the mouse *Optimedin A* gene corresponds to a putative transcription initiation site. Putative binding sites for different transcription factors are *underlined*. Pax6 and Sox binding sites that were analyzed in more details are *boxed*. *Asterisk* (*) marks the residues that are conserved in all three promoter sequences. The *vertical arrow* marks the 5′-end of the shortest p136OPT construct used in this work. *B*, diagrams of *Optimedin* promoter constructs that were used for transfection experiments. *Crossed boxes* correspond to the mutated putative binding sites for Pax6 (−86/−70) and Sox (−60/−52). *C*, oligonucleotides that were used in EMSA. Regions corresponding to the putative binding sites for Pax6 and Sox are *underlined* in the *Optimedin* promoter sequence.

demonstrated that while the unlabeled OPTs1 oligonucleotide competed for binding with labeled OPTs1 oligonucleotide, mutated OPTs1M oligonucleotide did not compete at all (Fig. 3*C*). The OPTs1C sequence was the most efficient competitor. We conclude that under the conditions used for the EMSA reactions, Sox11, unlike Sox2, does not bind to the putative Sox binding sites in the *Optimedin* proximal promoter.

Indirect support for the importance of putative Sox binding sites for the stimulatory activity of Sox11 came from a transfection experiment in which stimulatory activity of Sox11 was detected in the presence of

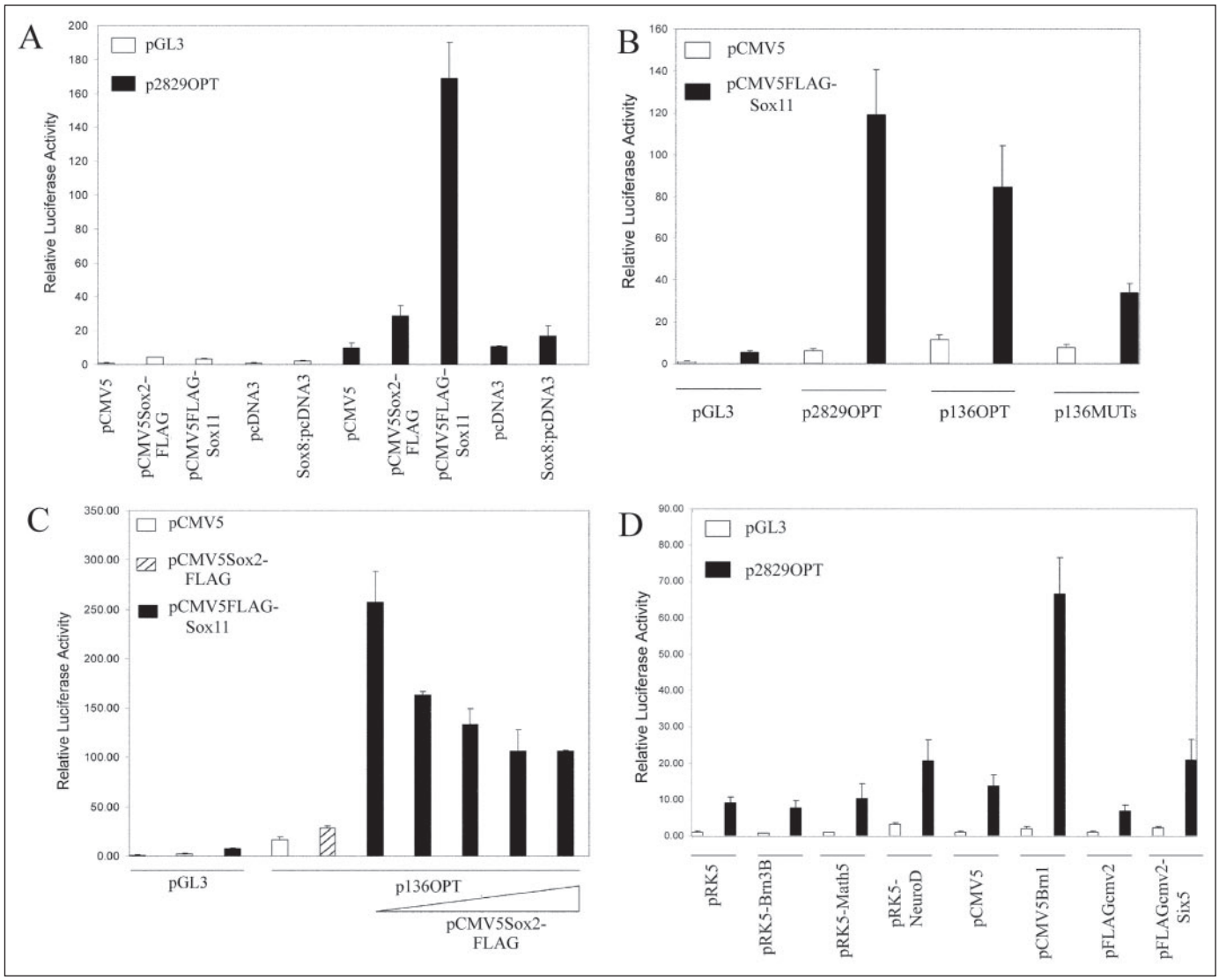

FIGURE 2. **Effects of different transcription factors on the *Optimedin* gene promoter activity in COS7 cells.** COS7 cells were co-transfected with different *Optimedin* promoter constructs and vectors or plasmids encoding the indicated transcription factors. Luciferase activity is normalized relative to the activity of pRL-null or pRL-TK vectors. Activity of the pGL3 vector co-transfected with pCMV5, pcDNA3, pRK5, or pFLAGcmv2 (depending on experiment) was arbitrarily set at 1. *A*, 50 ng of pGL3 or p2829OPT were co-transfected with 20 ng of pCMV5, pcDNA3, pCMV5Sox2FLAG, pCMV5-FLAGSox11, or Sox8pcDNA. *B*, 50 ng of pGL3, p2829OPT, p136OPT, or p136MUTs were co-transfected with 20 ng of pCMV5 or pCMV5FLAGSox11. *C*, 50 ng of pGL3 or p136OPT were co-transfected with 20 ng of pCMV5, pCMV5Sox2FLAG, or pCMV5FLAGSox11 or 20 ng of pCMV5FLAGSox11 together with increasing amounts (1, 5, 10, and 20 ng) of pCMV5Sox2FLAG. *D*, 50 ng of pGL3 or p2829OPT were co-transfected with 20 ng of pRK5, pCMV5, pFLAGcmv2, pRK5-Brn3B, pRK5-Math5, pRK5-NeuroD, pCMV5Brn1, or pFLAGcmv2-Six5. pBluescript KS(+/−) DNA was added to have equal amounts of DNA in all transfection experiments.

increasing amounts of Sox2 protein. We suggested that increasing amounts of the Sox2 protein, which does not stimulate the *Optimedin* promoter, will occupy these sites and reduce stimulatory activity of Sox11. Indeed, we observed reduced levels of Sox11 stimulatory activity in the presence of increasing amounts of Sox2 (Fig. 2*C*).

Pax6 Binds a Putative Binding Site in the Optimedin Proximal Promoter—The expression pattern of the *Optimedin* gene in the retina overlaps well with that for the *Pax6* gene (1, 56, 57). Therefore we tested possible involvement of Pax6 in the regulation of the *Optimedin* promoter. The ability of the putative Pax6 binding site located at −86/−70 in the proximal *Optimedin* promoter to bind Pax6 was tested by EMSA. This site (*OPTp* in Fig. 4; see Fig. 1*C* for sequences of oligonucleotides) bound Pax6 well. Replacement of three nucleotides in the binding site (GCG to ttc replacement) completely eliminated Pax6 binding to the mutated oligonucleotide. Pax6 consensus and mutated consensus oligonucleotides (58) were used in competition experiments. Consensus competitor completely eliminated binding of Pax6 to the putative binding site, whereas mutated consensus competitor did not compete at all (Fig. 4*A*). Addition of antibodies against the C-terminal part of Pax6 produced supershift bands, whereas addition of antibodies against the paired domain of Pax6 completely eliminated binding of Pax6. Addition of antibodies against the C-terminal part of Pax2 did not affect the binding of Pax6. We concluded that Pax6 interacts with the proximal *Optimedin* promoter through the paired domain.

There are two alternatively spliced forms of Pax6 mRNA in vertebrates, Pax6 and Pax6(5A). Pax6(5a) contains a 14-amino acid insertion in the paired domain encoded by a separate exon (56, 59). Recent data suggest that the Pax6(5a) isoform promotes the development of the neural retinal structures (60). It has been shown that Pax6 and Pax6(5a) forms are present in the adult retina in equal amounts (61). EMSA demonstrated that Pax6(5a), unlike Pax6, does not bind to the putative binding site at −86/−70 (Fig. 4*B*, *upper panel*), although equal amounts of Pax6 and Pax6(5a) proteins were present in nuclear extracts (Fig. 4*B*, *lower panel*).

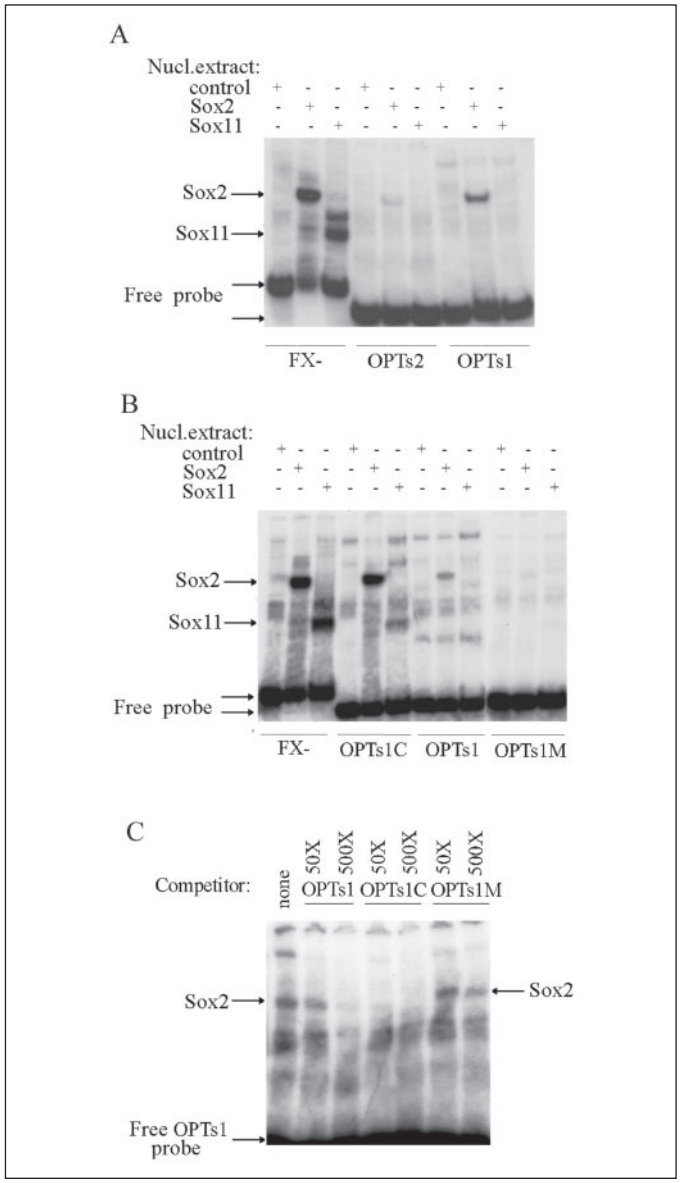

FIGURE 3. **Binding of Sox2 and Sox11 to the putative binding sites in the *Optimedin* proximal promoter.** *A*, crude nuclear extracts prepared from COS7 cells transfected with either Sox2 or Sox11 expression constructs were incubated with labeled OPTs2 or Ops1 probes. The FX− probe has been previously shown to bind Sox11 (55) and served as a positive control. *B*, the same as in *A* but with two additional mutated probes, OPTs1C and OPTs1M. In the OPTs1C oligonucleotide, a natural putative binding site was changed to the perfect consensus 5′-AAACAAAG-3′. OPTs1M contained a mutation TAA→ggg in the putative Sox binding site −60/−52. *C*, competition of different oligonucleotides for binding to the OPTs1 probe. 50 and 500× molar excesses of competitors were used.

To confirm that the paired domain of Pax6 is critical for the interaction with the *Optimedin* proximal promoter, EMSA was performed using the paired domain of Pax6 or Pax6(5a). The results of these experiments demonstrated that the paired domain of Pax6, but not Pax6(5a), is able to bind to OPTp (Fig. 4*C*, *upper panel*). Paired domains of Pax6 and Pax6(5A) did not bind to mutated OPTpM.

Pax6 Stimulation of Optimedin Promoter Activity—The *Optimedin* promoter constructs were tested in cells that express (RGC-5) and do not express (COS7) Pax6 (Fig. 5*A*). p3153OPT, p2829OPT, and p136OPT gave similar levels of stimulation over the pGL3 vector in COS7 cells. Co-transfection with Pax6 resulted in 2.1-, 7.6-, 10.2-, and 8.2-fold stimulation of activity for pGL3, p3153OPT, and p2829OPT, and p136OPT, respectively (Fig. 5*B*). Because we did not detect significant differences in the activity of p3153OPT, p2829OPT, and p136OPT constructs, subsequent experiments were conducted with p2829OPT as the longest and p136OPT as the shortest promoter constructs. To determine whether the Pax6 site identified by EMSA is important in promoter activation, a triple mutation GCG to ttc that eliminated Pax6 binding was introduced into the p2829OPT and p136OPT constructs (see Fig. 1*B*). These constructs were named p2829MUTp and p136MUTp, respectively. The basal activity of the mutated promoter constructs did not differ significantly from their wild-type counterparts in COS7 cells. However, the stimulatory effect of Pax6 was significantly reduced when mutated instead of wild-type constructs were used in co-transfection experiments (Fig. 5*B*). We conclude that −86/−70 plays a critical role for the stimulation of the *Optimedin* promoter.

Co-transfection of p2829OPT and p136OPT with Pax6 resulted in less than 2-fold stimulation in RGC-5 cells (Fig. 5*C*). When mutated p2829MUTp or p136MUTp were transfected into RGC-5 cells, their basal promoter activity was reduced as compared with wild-type counterparts. Pax6 did not provide any stimulation when co-transfected with the mutant constructs (Fig. 5*C*). These results are consistent with an observation that RGC-5 cells, unlike COS7 cells, contain the endogeneous Pax6 protein. Similar results were obtained with βTC3 cells, which also contain the endogeneous Pax6 protein (not shown).

It has been shown that Pax6 and Pax6(5a) may synergistically activate transcription from some promoters (62). Therefore, we tested possible co-activation mechanisms by Pax6 and Pax6(5a). Pax6(5a) did not activate the proximal *Optimedin* promoter supporting our data that Pax6(5a) does not bind to OPTp (see Fig. 4, *B* and *C*). The activation of the *Optimedin* promoter by Pax6 was slightly suppressed when equal amounts of Pax6 and Pax6(5a) expression plasmids were used in co-transfection experiments (Fig. 5*D*). Mixing of Pax6 and Pax6(5a) in different proportions (5:1, 8:1) did not lead to the increased activation of the *Optimedin* promoter as compared with Pax6 alone (not shown).

It has been shown that Sox2 may form a complex with Pax6 and these two proteins synergistically activate chicken δ-*crystallin* gene enhancer (63). To test whether Sox2 and Pax6 may synergistically activate the *Optimedin* promoter, p2890OPT promoter was cotransfected together with Pax6 and Sox2 expression constructs. Sox2 and Pax6 did not provide synergistic activation with the *Optimedin* promoter (not shown).

Pax6 Occupies the Proximal Optimedin Promoter in Vivo—To determine whether Pax6 binds directly to the proximal *Optimedin* promoter *in vivo*, we performed a ChIP assay using a specific antibody against Pax6 to immunoprecipitate formaldehyde-fixed chromatin from embryonic mouse brain. Endogenous *Optimedin* promoter DNA was amplified by PCR from immunoprecipitated chromatin. In this experiment, we used the upstream region −3719/−3421 and a region of intron 6 of the *Optimedin* gene as the negative control. As shown in Fig. 6, in 17-day embryonic mouse brain, Pax6 binds to the *Optimedin* proximal promoter but not to the upstream region or the intron 6 sequence. When normal rabbit serum IgG fraction or Sox11 antibodies were used for ChIP, no amplification was observed with any tested region. We conclude that Pax6 directly interacts with the *Optimedin* proximal promoter region in the embryonic mouse brain.

DISCUSSION

Pax6 is a highly conserved transcription factor containing the paired and homeobox DNA-binding domains. It has been identified as a key regulator gene of eye development in both vertebrates and invertebrates (64–66). It is also critical for the development of the central nervous system and endocrine glands. Pax6 fulfills its functions through the regulation of genes encoding other transcription factors, receptors, cell

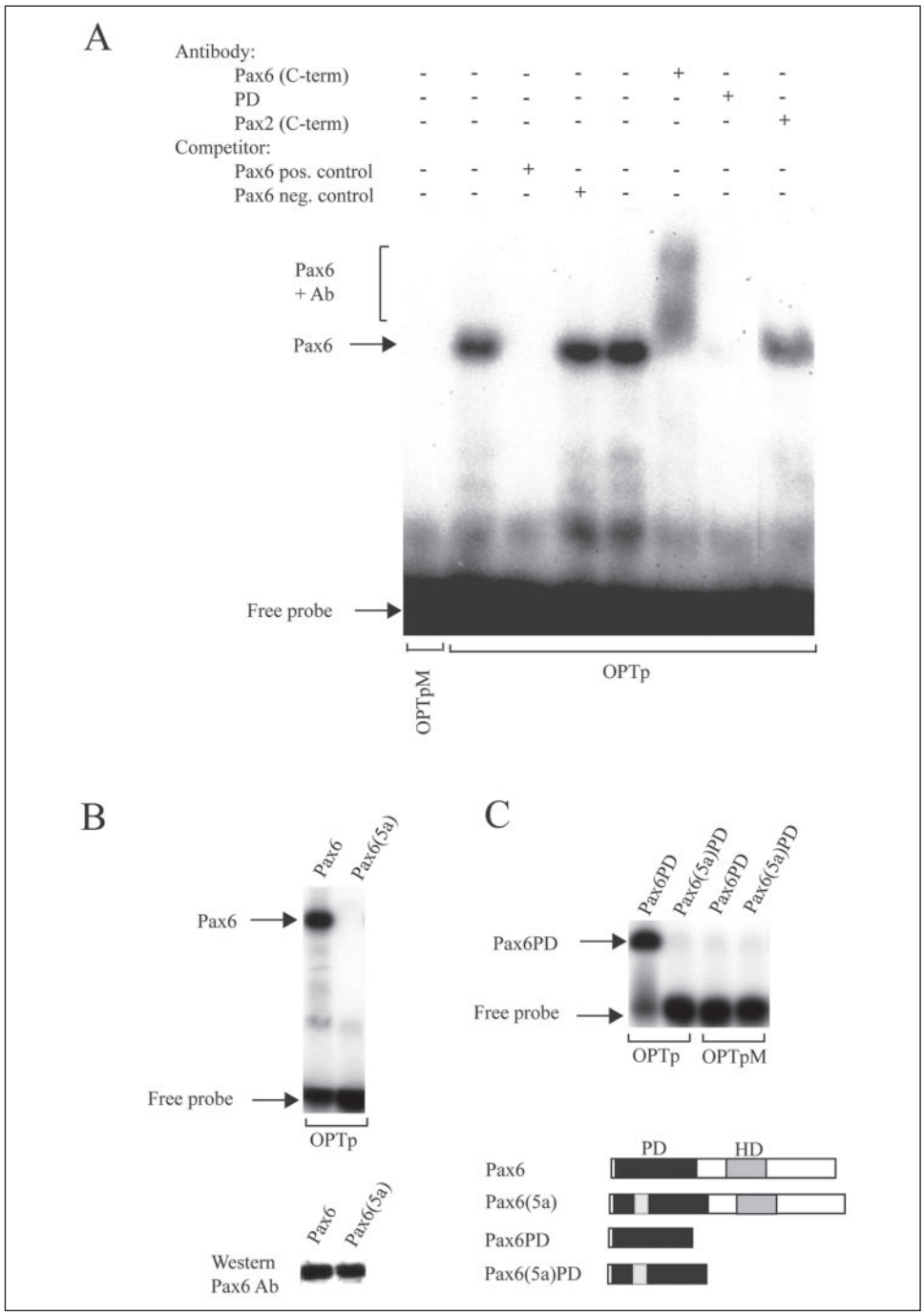

FIGURE 4. **Binding of Pax6 to the putative binding site in the *Optimedin* proximal promoter.** *A*, crude nuclear extracts prepared from COP8 cells transfected with the Pax6 expression construct were incubated with labeled optimedin OPTp or OPTpM probes. Formation of DNA-protein complexes was revealed through EMSA. DNA-protein complex and free probe are marked by *arrows*. Competitor oligonucleotides and antibodies were added as shown. Nucleotide sequences of all oligonucleotides used in these experiments are shown in Fig. 1*C*. *B*, equal amounts of nuclear extracts transfected with Pax6 or Pax6(5a) were analyzed by SDS-PAGE (10% gel) followed by immunoblotting with Pax6 anti-paired domain antibodies (*lower panel*). Formation of DNA-protein complexes was revealed through EMSA (*upper panel*). Positions of DNA-protein complex and free probe are marked on the *left side*. *C*, EMSA (*upper panel*) with *E. coli* expressed and affinity purified paired domain of Pax6 or Pax6(5a). A *lower panel* shows a diagram of all constructs used in these experiments.

adhesion, cell-cell signaling molecules, hormones, and structural proteins (38). It has been shown that gene regulation by Pax6 may involve its interaction with other transcription factors, including Mitf (67), Engrailed (68), Sox2 (63), TATA-binding, and retinoblastoma proteins (69). Eye development is very sensitive to the Pax6 dosage and both the loss and the overexpression of Pax6 may lead to similar defects (70).

The expression pattern of the *Pax6* gene was studied in several species. In mammals, *Pax6* is expressed in different brain regions, including the forebrain, hindbrain, and cerebellum, spinal cord, nasal structures, pancreas, gut, pituitary gland, and eyes from early stages of embryonic development (56, 71). In the developing eye, *Pax6* is expressed in the early optic vesicle and the surface ectoderm. At the optic cup stage, it is expressed in different eye components, including the lens vesicle, the outer and inner optic cup layers, and the optic stalk (56, 72). In the adult eye, *Pax6* is expressed in the lens, the cornea and conjunctiva epithelia, the iris, as well as in the ganglion and inner nuclear cell layers of the retina (56, 57).

The *Optimedin* gene is expressed in the ganglion and inner nuclear layers of the retina, epithelial cells of the iris and ciliary body, and different brain regions (1, 16). Our preliminary *in situ* hybridization results indicated that the *Optimedin* gene is also expressed in the developing trigmental ganglia, pituitary gland, ventral telencephalon and thalamus, and spinal cord.[3] Thus, *Pax6* and *Optimedin* demonstrate the overlap-

[3] O. V. Grinchuk and S. I. Tomarev, unpublished observations.

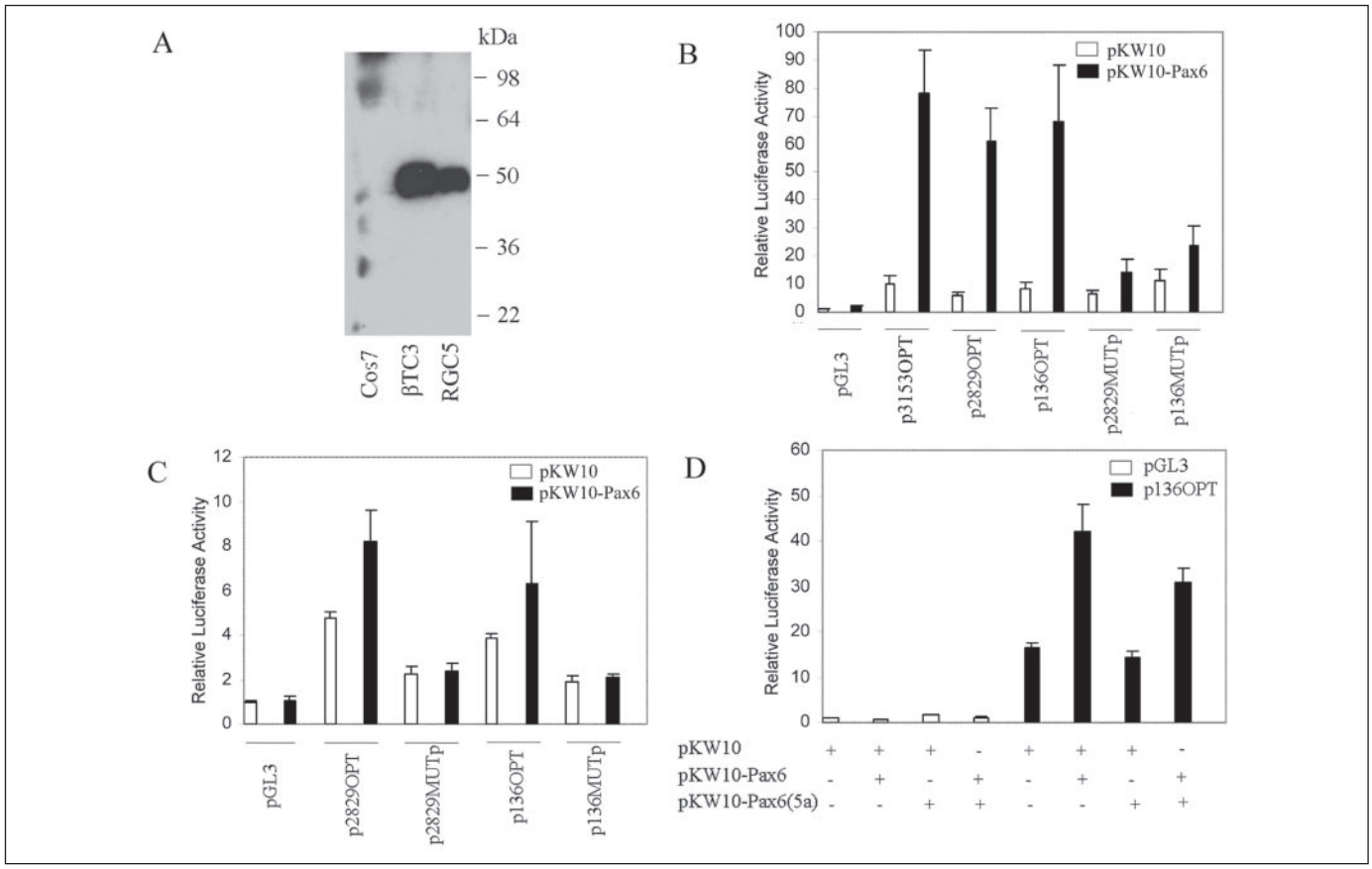

FIGURE 5. **Pax6 regulates the mouse *Optimedin* promoter.** *A*, Western blot analysis of Pax6 presence in COS7, βTC3, and RGC-5 cells. 10 μg of total cell extracts were separated using 10% SDS-PAGE, transferred to a nitrocellulose membrane, and stained with Pax6 antibodies as described under "Materials and Methods." *B–D*, COS7 (*B* and *D*) and RGC-5 (*C*) cells were transfected with the indicated promoter constructs and the *pKW10* vector or plasmids encoding Pax6 (*B–D*) or Pax6(5a) (*D*). Luciferase activity is normalized relative to the activity of *pRL-null* vector. Activity *of pGL3* vector co-transfected with *pKW10* was arbitrarily set at 1. The results of the typical experiments are shown.

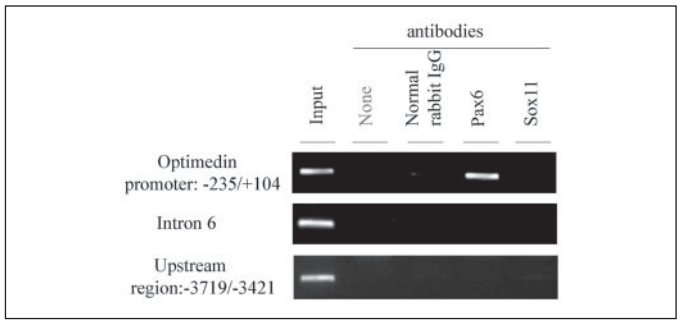

FIGURE 6. **Binding of Pax6 to the proximal *Optimedin* promoter *in vivo*.** E17 embryonic mouse brain was treated with formaldehyde to cross-link endogenous protein and chromatin DNA. Samples of sonicated and purified chromatin were immunoprecipitated with normal rabbit IgG, Pax6, or Sox11 antibodies as indicated, and DNA isolated from immunoprecipitated material was amplified by PCR with primers specific for the *Optimedin* proximal promoter, −3719/−3421 upstream region, and intron 6 sequence of the *Optimedin* gene. Aliquots of chromatin samples prior to immunoprecipitation were also amplified (*left input lanes*).

ping expression patterns of both adult and embryonic brain, retina, and spinal cord. The overlapping expression pattern of *Pax6* and *Optimedin* is consistent with our conclusion that the *Optimedin* gene may be a downstream target of Pax6. This conclusion is based on several observations: 1) the presence of a putative Pax6 binding site in the *Optimedin* proximal promoter; 2) the binding of Pax6 to this site as judged by the EMSA assay; 3) the stimulation of the *Optimedin* promoter *in vitro* by Pax6; 4) the reduction of stimulation after mutation in the putative Pax6 binding site; 5) the interaction of Pax6 with the putative binding site *in vivo* as judged by ChIP assay. Although Pax6 regulates the *Optimedin* gene, we do not know at present whether Pax6 is absolutely essential for the *Optimedin* gene expression *in vivo*. Further experiments with Pax6-deficient tissues may help to answer this question.

The proximal *Optimedin* promoter also contained several putative Sox binding sites. Several *Sox* genes are expressed in the developing and adult eye and brain (73, 74) where their expression overlaps with the expression of the *Optimedin* gene. It is interesting to note that Sox2 and Pax6 may form a molecular complex on the lens-specific enhancer elements (*e.g.* the δ-*crystallin* minimal enhancer) and synergistically activate this enhancer (63). Although Sox2 binds to a putative binding site in the *Optimedin* promoter, it did not stimulate this promoter by itself or in combination with Pax6. At the same time, Sox11 provided high stimulation of the proximal *Optimedin* promoter *in vitro* but did not bind to the putative binding sites as judged by the EMSA or ChIP assays. Similar results were obtained by the laboratory of Rizzino (41) with the fibroblast growth factor-4 enhancer. Sox11 but not Sox2 stimulated the reporter construct in transfection experiments, but only Sox2 was capable of binding to the putative binding site as judged by the EMSA. The authors suggested that there is a domain in the Sox11 protein that has a capability of autoinhibiting its ability to bind DNA *in vitro* and to activate gene expression *in vivo* (41). This suggestion may also explain the results we obtained with the *Optimedin* promoter.

The function of optimedin is still not clear. Our preliminary data indicate that optimedin, similar to amassin, the sea urchin olfactomedin domain-containing protein, may be involved in cell-cell adhesion and

cell attachment to the extracellular matrix.[4] The role of Pax6 in the regulation of several genes encoding cell-adhesion molecules has been previously demonstrated (75, 76). Pax6 regulation of optimedin, a protein contributing to the cell adhesive properties, may directly affect multiple developmental processes including cell migration and axon growth.

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REFERENCES

1. Torrado, M., Trivedi, R., Zinovieva, R., Karavanova, I., and Tomarev, S. I. (2002) *Hum. Mol. Genet.* **11,** 1291–1301
2. Snyder, D. A., Rivers, A. M., Yokoe, H., Menco, B. P., and Anholt, R. R. (1991) *Biochemistry* **30,** 9143–9153
3. Danielson, P. E., Forss-Petter, S., Battenberg, E. L., deLecea, L., Bloom, F. E., and Sutcliffe, J. G. (1994) *J. Neurosci. Res.* **38,** 468–478
4. Hillier, B. J., and Vacquier, V. D. (2003) *J. Cell Biol.* **160,** 597–604
5. Barembaum, M., Moreno, T. A., LaBonne, C., Sechrist, J., and Bronner-Fraser, M. (2000) *Nat. Cell Biol.* **2,** 219–225
6. Kulkarni, N. H., Karavanich, C. A., Atchley, W. R., and Anholt, R. R. (2000) *Genet. Res.* **76,** 41–50
7. Tsuda, H., Sasai, N., Matsuo-Takasaki, M., Sakuragi, M., Murakami, Y., and Sasai, Y. (2002) *Neuron* **33,** 515–528
8. Graveel, C. R., Harkins-Perry, S. R., Acevedo, L. G., and Farnham, P. J. (2003) *Oncogene* **22,** 1730–1736
9. Mukhopadhyay, A., Talukdar, S., Bhattacharjee, A., and Ray, K. (2004) *Mol. Vis.* **10,** 304–314
10. Lelianova, V. G., Davletov, B. A., Sterling, A., Rahman, M. A., Grishin, E. V., Totty, N. F., and Ushkaryov, Y. A. (1997) *J. Biol. Chem.* **272,** 21504–21508
11. Davletov, B. A., Shamotienko, O. G., Lelianova, V. G., Grishin, E. V., and Ushkaryov, Y. A. (1996) *J. Biol. Chem.* **271,** 23239–23245
12. Matsushita, H., Lelianova, V. G., and Ushkaryov, Y. A. (1999) *FEBS Lett.* **443,** 348–352
13. Sudhof, T. C. (2001) *Annu. Rev. Neurosci.* **24,** 933–962
14. Krasnoperov, V. G., Bittner, M. A., Beavis, R., Kuang, Y., Salnikow, K. V., Chepurny, O. G., Little, A. R., Plotnikov, A. N., Wu, D., Holz, R. W., and Petrenko, A. G. (1997) *Neuron* **18,** 925–937
15. Kondo, D., Yamamoto, T., Yaoita, E., Danielson, P. E., Kobayashi, H., Ohshiro, K., Funaki, H., Koyama, Y., Fujinaka, H., Kawasaki, K., Sutcliffe, J. G., Arakawa, M., and Kihara, I. (2000) *J. Am. Soc. Nephrol.* **11,** 803–813
16. Sato, M., Suzuki, K., Yamazaki, H., and Nakanishi, S. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 5874–5879
17. Zeng, L. C., Liu, F., Zhang, X., Zhu, Z. D., Wang, Z. Q., Han, Z. G., and Ma, W. J. (2004) *FEBS Lett.* **571,** 74–80
18. Ahmed, F., Torrado, M., Zinovieva, R. D., Senatorov, V. V., Wistow, G., and Tomarev, S. I. (2004) *Investig. Ophthalmol. Vis. Sci.* **45,** 3081–3090
19. Rosenbauer, F., Wagner, K., Zhang, P., Knobeloch, K. P., Iwama, A., and Tenen, D. G. (2004) *Blood* **103,** 4294–4301
20. Zhang, J., Liu, W. L., Tang, D. C., Chen, L., Wang, M., Pack, S. D., Zhuang, Z., and Rodgers, G. P. (2002) *Gene* (*Amst.*) **283,** 83–93
21. Adam, M. F., Belmouden, A., Binisti, P., Brezin, A. P., Valtot, F., Bevhetoille, A., Dascotte, J.-C., Copin, B., Gomez, L., Chaventre, A., Bach, J.-F., and Garchon, H.-J. (1997) *Hum. Mol. Genet.* **6,** 2091–2097
22. Tomarev, S. I., Wistow, G., Raymond, V., Dubois, S., and Malyukova, I. (2003) *Investig. Ophthalmol. Vis. Sci.* **44,** 2588–2596
23. Escribano, J., Ortego, J., and Coca-Prados, M. (1995) *J. Biochem.* (*Tokyo*) **118,** 921–931
24. Swiderski, R. E., Ross, J. L., Fingert, J. H., Clark, A. F., Alward, W. L., Stone, E. M., and Sheffield, V. C. (2000) *Investig. Ophthalmol. Vis. Sci.* **41,** 3420–3428
25. Moreno, T. A., and Bronner-Fraser, M. (2002) *Mech. Dev.* **119,** 121–125
26. Moreno, T. A., and Bronner-Fraser, M. (2001) *Dev. Biol.* **240,** 340–360
27. Stone, E. M., Fingert, J. H., Alward, W. M., Nguyen, T. D., Polansky, J. R., Sunden, S. F., Nishimura, D., Clark, A. F., Nystuen, A., Nichols, B. E., Mackey, D. A., Ritch, R., Kalenak, J. W., Craven, E. R., and Sheffield, V. C. (1997) *Science* **275,** 668–670
28. Gong, G., Kosoko-Lasaki, O., Haynatzki, G. R., and Wilson, M. R. (2004) *Hum. Mol. Genet.* **13,** R91–R102
29. Jacobson, N., Andrews, M., Shepard, A. R., Nishimura, D., Searby, C., Fingert, J. H., Hageman, G., Mullins, R., Davidson, B. L., Kwon, Y. H., Alward, W. L., Stone, E. M., Clark, A. F., and Sheffield, V. C. (2001) *Hum. Mol. Genet.* **10,** 117–125
30. Caballero, M., Rowlette, L. L., and Borras, T. (2000) *Biochim. Biophys. Acta* **1502,** 447–460
31. Gobeil, S., Rodrigue, M. A., Moisan, S., Nguyen, T. D., Polansky, J. R., Morissette, J., and Raymond, V. (2004) *Investig. Ophthalmol. Vis. Sci.* **45,** 3560–3567
32. Liu, Y., and Vollrath, D. (2004) *Hum. Mol. Genet.* **13,** 1193–1204
33. Joe, M. K., Sohn, S., Hur, W., Moon, Y., Choi, Y. R., and Kee, C. (2003) *Biochem. Biophys. Res. Commun.* **312,** 592–600
34. Polansky, J. R., Fauss, D. J., Chen, P., Chen, H., Lutjen-Drecoll, E., Johnson, D., Kurtz, R. M., Ma, Z. D., Bloom, E., and Nguyen, T. D. (1997) *Ophthalmologica* **211,** 126–139
35. Polansky, J. R., Fauss, D. J., and Zimmerman, C. C. (2000) *Eye* **14,** 503–514
36. Shepard, A. R., Jacobson, N., Fingert, J. H., Stone, E. M., Sheffield, V. C., and Clark, A. F. (2001) *Investig. Ophthalmol. Vis. Sci.* **42,** 3173–3181
37. Kirstein, L., Cvekl, A., Chauhan, B. K., and Tamm, E. R. (2000) *Genes Cells* **5,** 661–676
38. Simpson, T. I., and Price, D. J. (2002) *BioEssays* **24,** 1041–1051
39. Cvekl, A., and Piatigorsky, J. (1996) *BioEssays* **18,** 621–630
40. Krishnamoorthy, R. R., Agarwal, P., Prasanna, G., Vopat, K., Lambert, W., Sheedlo, H. J., Pang, I. H., Shade, D., Wordinger, R. J., Yorio, T., Clark, A. F., and Agarwal, N. (2001) *Brain Res. Mol. Brain Res.* **86,** 1–12
41. Wiebe, M. S., Nowling, T. K., and Rizzino, A. (2003) *J. Biol. Chem.* **278,** 17901–17911
42. Gonzalez, M. I., Tovaglieri, A., and Robins, D. M. (2002) *Mol. Cell. Endocrinol.* **190,** 39–49
43. Schepers, G. E., Bullejos, M., Hosking, B. M., and Koopman, P. (2000) *Nucleic Acids Res.* **28,** 1473–1480
44. Ohto, H., Kamada, S., Tago, K., Tominaga, S. I., Ozaki, H., Sato, S., and Kawakami, K. (1999) *Mol. Cell. Biol.* **19,** 6815–6824
45. Kozmik, Z., Czerny, T., and Busslinger, M. (1997) *EMBO J.* **16,** 6793–6803
46. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17,** 6419
47. Carriere, C., Plaza, S., Martin, P., Quatannens, B., Bailly, M., Stehelin, D., and Saule, S. (1993) *Mol. Cell. Biol.* **13,** 7257–7266
48. Davis, J. A., and Reed, R. R. (1996) *J. Neurosci.* **16,** 5082–5094
49. He, X., Treacy, M. N., Simmons, D. M., Ingraham, H. A., Swanson, L. W., and Rosenfeld, M. G. (1989) *Nature* **340,** 35–41
50. Xiang, M., Zhou, L., Peng, Y. W., Eddy, R. L., Shows, T. B., and Nathans, J. (1993) *Neuron* **11,** 689–701
51. Gan, L., Wang, S. W., Huang, Z., and Klein, W. H. (1999) *Dev. Biol.* **210,** 469–480
52. Hatakeyama, J., and Kageyama, R. (2004) *Semin. Cell. Dev. Biol.* **15,** 83–89
53. Sato, S., Nakamura, M., Cho, D. H., Tapscott, S. J., Ozaki, H., and Kawakami, K. (2002) *Hum. Mol. Genet.* **11,** 1045–1058
54. Harley, V. R., Lovell-Badge, R., and Goodfellow, P. N. (1994) *Nucleic Acids Res.* **22,** 1500–1501
55. Kuhlbrodt, K., Herbarth, B., Sock, E., Enderich, J., Hermans-Borgmeyer, I., and Wegner, M. (1998) *J. Biol. Chem.* **273,** 16050–16057
56. Walther, C., and Gruss, P. (1991) *Development* **113,** 1435–1449
57. Koroma, B. M., Yang, J. M., and Sundin, O. H. (1997) *Investig. Ophthalmol. Vis. Sci.* **38,** 108–120
58. Epstein, J., Cai, J., Glaser, T., Jepeal, L., and Maas, R. (1994) *J. Biol. Chem.* **269,** 8355–8361
59. Ton, C. C. T., Hirvonen, H., Miwa, H., Weil, M. M., Monaghan, P., Jordan, T., van Heyningen, V., Hastie, N. D., Meijers-Heijboer, H., Drechsler, M., Royer-Pokora, B., Collins, F., Swaroop, A., Strong, L. C., and Saunders, G. F. (1991) *Cell* **67,** 1059–1074
60. Azuma, N., Tadokoro, K., Asaka, A., Yamada, M., Yamaguchi, Y., Handa, H., Matsushima, S., Watanabe, T., Kohsaka, S., Kida, Y., Shiraishi, T., Ogura, T., Shimamura, K., and Nakafuku, M. (2005) *Hum. Mol. Genet.* **14,** 735–745
61. Zhang, W., Cveklova, K., Oppermann, B., Kantorow, M., and Cvekl, A. (2001) *Mol. Vis.* **7,** 1–5
62. Chauhan, B. K., Yang, Y., Cveklova, K., and Cvekl, A. (2004) *Nucleic Acids Res.* **32,** 1696–1709
63. Kamachi, Y., Uchikawa, M., Tanouchi, A., Sekido, R., and Kondoh, H. (2001) *Genes Dev.* **15,** 1272–1286
64. Halder, G., Callaerts, P., and Gehring, W. J. (1995) *Science* **267,** 1788–1792
65. Tomarev, S. I., Callaerts, P., Kos, L., Zinovieva, R., Halder, G., Gehring, W., and Piatigorsky, J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 2421–2426
66. Hill, R. E., Favor, J., Hogan, B. L., Ton, C. C., Saunders, G. F., Hanson, I. M., Prosser, J., Jordan, T., Hastie, N. D., and van Heyningen, V. (1991) *Nature* **354,** 522–525
67. Planque, N., Leconte, L., Coquelle, F. M., Martin, P., and Saule, S. (2001) *J. Biol. Chem.* **276,** 29330–29337
68. Plaza, S., Langlois, M. C., Turque, N., LeCornet, S., Bailly, M., Begue, A., Quatannens, B., Dozier, C., and Saule, S. (1997) *Cell Growth & Differ.* **8,** 1115–1125
69. Cvekl, A., Kasanchi, F., Brady, J. N., and Piatigorsky, J. (1999) *Investig. Ophthalmol. Vis. Sci.* **40,** 1343–1350

[4] H.-S. Lee and S. I. Tomarev, manuscript in preparation.

70. Schedl, A., Ross, A., Lee, M., Engelkamp, D., Rashbass, P., van Heyningen, V., and Hastie, N. D. (1996) *Cell* **86,** 71–82
71. Stoykova, A., and Gruss, P. (1994) *J. Neurosci.* **14,** 1395–1412
72. Li, H. S., Yang, J. M., Jacobson, R. D., Pasko, D., and Sundin, O. (1994) *Dev. Biol.* **162,** 181–194
73. Cheung, M., Abu-Elmagd, M., Clevers, H., and Scotting, P. J. (2000) *Brain Res. Mol. Brain Res.* **79,** 180–191
74. Uwanogho, D., Rex, M., Cartwright, E. J., Pearl, G., Healy, C., Scotting, P. J., and Sharpe, P. T. (1995) *Mech. Dev.* **49,** 23–36
75. Meech, R., Kallunki, P., Edelman, G. M., and Jones, F. S. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 2420–2425
76. Edelman, G. M., and Jones, F. S. (1998) *Brain Res. Brain Res. Rev.* **26,** 337–352

The *Optimedin* Gene Is a Downstream Target of Pax6

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