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 OLFACTO MEDIN 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.
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% CO₂.

Different regions of the 5′-flanking region of the mouse Optimedina promoter were amplified by PCR and cloned into the promoterless, luciferase reporter gene. KpnI/SmaI restriction sites were used for cloning of the 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’-CTGAACTGGAGGCTAAG-3’ (position −86/−70), was changed to 5’-CTGAAACTGGATctTAAG-3’. A putative Sox binding site, 5’-TAACAAAT-3’ (position −60/−52), was changed to 5’-gggCAAT-3’.

Luciferase Assay—For luciferase assay, COS7 or RGC-5 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. Western blotting was performed using polyclonal IgG (Covance, Richmond, CA) and monoclonal antibodies against the C terminus of Pax6 were purchased from Covance Research Product (Berkley, CA).

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Electrophoretic Mobility Shift Assays (EMSA)—To estimate amounts of Sox2 and Sox11 proteins Western Blotting—For Western blotting, Rabbit anti-Pax2 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)—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, 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).

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 Ultra-Turrax. Sonicated lysates were precleared by incubation with Protein A-agarose beads. Chromatin was eluted from the agarose beads and cross-linked DNA was released with 200 µl of 1.8 M NaCl. The eluted DNA was precipitated with 50 µl of protein A-agarose beads. The bound DNA was eluted with 200 µl of 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).

2 The abbreviations used are: EMSA, electrophoretic motility shift assay; ChIP, chromatin immunoprecipitation.
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protease 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′-ATAACTCAAGTGCTTACCT-3′ (forward) and 5′-CTCCCTCTGTAGAGCCG-3′ (reverse); the second pair in the region −371/−342 was 5′-GAACGTATACGACGCCTCT-3′ (forward) and 5′-CTTCAATTATCGAGTACGAT-3′ (reverse); the third pair located in the 6th intron of the Optimedin gene was 5′-GGTAATTGAGTATATGAG-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. 1A). 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. 1A). 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, p2890OPT, 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. 2D). 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. 2D).

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′-TCAAGTTCG-3′, was identified in the Optimedin proximal promoter at the −104/−95 position. It is a perfect match to the consensus Six5 binding site, TCARCTTCC, 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. 2D).

Two putative Sox binding sites, showing similarity to the Sox HMG-domain binding consensus sequence 5′-(A/T)(A/T)CACAA(A/T)G-3′ (54), were identified in the optimedin proximal promoter. One, located at −60/−52 (5′-TAAACAAAT-3′), was conserved in the mouse, rat, and human OPTIMedin promoters, whereas the second site at −94/−87 (5′-CAGCAAAAG-3′) contained two substitutions in the human promoter (Fig. 1A). 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. 2A). 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. 2B). 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′-gggCAAAAT-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. 2B). 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. 1A 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. 3A). Oligonucleotide OPTs2 (less conserved site −94/−87, see Fig. 1C for sequences of oligonucleotides) did not bind Sox11 and only weakly bound Sox2 (Fig. 3A). Oligonucleotide OPTs1 (more conserved site −60/−52) did not bind Sox11 but bound Sox2 (Fig. 3A). 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. 2B) was introduced (see Fig. 1C). Both Sox2 and Sox11 bound the OPTs1C sequence but did not bind the OPTs1M sequence (Fig. 3B). Finally, competition experiments
demonstrated that while the unlabeled OPTs1 oligonucleotide competed for binding with labeled OPTs1 oligonucleotide, mutated OPTs1M oligonucleotide did not compete at all (Fig. 3C). 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 OPTs1 oligonucleotides that were used in EMSA. Regions corresponding to the putative binding sites for Pax6 and Sox are underlined in the Optimedin promoter sequence.
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. 2C).

**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. 1C 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. 4D). 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. 4B, upper panel), although equal amounts of Pax6 and Pax6(5a) proteins were present in nuclear extracts (Fig. 4B, lower panel).
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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 Sox2 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...

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. 4C, 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 p3153OPT, p2829OPT, and p136OPT) and do not express (COS7) Pax6 (Fig. 5A). 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 70-fold activation of p3153OPT, p2829OPT, and p136OPT, respectively (Fig. 5B). Because we did not detect significa-

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 GCC to ttc that eliminated Pax6 binding was introduced into the p2829OPT and p136OPT constructs (see Fig. 1B). 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. 5B). We conclude that Pax6 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. 5C). 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. 5C). These results are consistent with an observation that RGC-5 cells, unlike COS7 cells, contain the endogenous Pax6 protein. Similar results were obtained with βTC3 cells, which also contain the endogenous 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. 5D). 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 and Sox3 may form a complex with Pax6 and these two proteins synergistically activate chicken β-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 Sox2 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.

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 OPTs1 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′-AAAACAAAG-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.

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...
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 trigeminal ganglia, pituitary gland, ventral telencephalon and thalamus, and spinal cord. Thus, Pax6 and Optimedin demonstrate the overlap-
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 /H9254-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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