Pax6 Interacts with cVax and Tbx5 to Establish the Dorsoventral Boundary of the Developing Eye*

Laurence Leconte‡, Laure Lecoin‡, Patrick Martins‡, and Simon Saule‡¶

From the ‡CNRS UMR 146, Institut Curie Section de Recherche, Bâtiment 110, Centre Universitaire, 91405 Orsay Cedex, France and §CNRS UMR6548, Transcriptional Regulation and Differentiation, Bâtiment Sciences Naturelles, Parc Valrose, Université de Nice, 06108 Nice cedex, France

Dorsoventral pattern formation of the optic cup is essential for vertebrate eye morphogenesis and retinotectal topographic mapping. Doral and ventral aspects of the eye are distinct at early stages of development; cVax homeodomain protein expression is confined to the ventral optic cup, whereas Tbx5 (T-box transcription factor) expression domain becomes restricted to the dorsal region. Misexpression of cVax or Tbx5 induces profound defects in eye morphology and abnormal visual projections. In the Pax6−/− mutant Tbx5 fails to be expressed, and Vax1 and -2 are abnormally present in the entire optic vesicle. During eye development Pax6 becomes expressed in a gradient at the optic cup stage due to the specific activation of a highly conserved intronic α enhancer in the Pax6 locus. We observed that the highest level of Pax6 in the optic cup corresponds to the boundary between non-overlapping cVax and Tbx5 territories. To further investigate how these transcription factors control the patterning of the eye, we overexpressed Pax6 in the chick optic cup (E2) using in ovo electroporation. We observed that overexpression of Pax6 extends the Tbx5 and Bmp4 domains but reduces the cVax expression domains in the E3 chick eye. This results in an abnormal eye phenotype at E4. In addition, we showed that cVax and Tbx5 interact with Pax6 and modulate in an opposite manner the activity of the Pax6 α enhancer. Moreover, the Pax6/cVax interaction inhibits the transactivation properties of Pax6. These results demonstrate that Pax6 together with cVax and Tbx5 mediate dorsoventral patterning of the eye.

Formation of the vertebrate eye involves a series of morphogenetic and inductive events that begin with the evagination of the optic vesicles from the forebrain. This is followed by invagination of the optic vesicles to create the optic cups. This results in the formation of two layers giving rise to the retina pigment epithelium, the neural retina, parts of the ciliary body, and a portion of the iris (1). An early and key event in eye development is the establishment of the nasal-temporal and dorsal-ventral axes in the optic primordium, which is fundamental for the later formation of the retinotectal projections.

Development of the dorsoventral polarity has already been initiated in the optic vesicle stage and precedes the onset of neuronal differentiation (2, 3). At this stage gene expression is restricted along the dorsoventral axis, thus dividing the retina into several domains (4). Two transcription factors expressed in a mutually exclusive manner specify the dorsal and ventral compartments of the developing retina; Tbx5, a member of the T-box gene family, is expressed in the dorsal part of the retina in mouse and chicken (5, 6). Misexpression of Tbx5 in the developing eye induced dorsalization of the eye and altered projections of retinal ganglion cell axons (7). Pax6 homeodomain protein expression is restricted to the ventral part of the developing eye in mouse (Vax1 and -2) and chicken (cVax) (8). Vax2 inactivation in mouse revealed an important role of this gene in the specification of the ventral optic vesicle (9, 10). Misexpression of cVax leads to ventralization of the retina and abnormal retinotectal projections (8). Therefore, the correct expression of Tbx5 and cVax is critical for the early determination of the dorsal and ventral compartments of the eye and for the proper development of the retinotectal projections.

Pax6 is a highly conserved transcription factor with two DNA binding domains, the paired and the homeodomain, and a transactivation domain (11, 12). Pax6 encodes different proteins through alternative splicing and internal initiations (13). Three proteins of 48, 46, and 43 kDa contain a paired domain, but two proteins of 33 and 32 kDa are devoid of this DNA binding domain. Pax6 is required for the proper development of the pancreas and the nervous system, where it is known to be critical for eye development (14, 15). Pax6 is expressed in presumptive eye tissues. Both the semi-dominant inheritance pattern of the Pax6 mutant phenotype (16, 17) and experimental manipulations using transgenes based on Pax6 locus (18) indicate that achieving the correct level of Pax6 is important for development of a normal eye. Because in Pax6−/− mice formation of the embryonic eye is disrupted early in development when the optic vesicle fails to form an optic cup (19), the function of Pax6 at these early steps is not well understood. Pax6 is first widely expressed in the optic vesicle. Later, in the optic cup, Pax6 becomes expressed in a distal (high)-proximal (low) gradient due to the specific activity in the retina of a highly conserved intronic enhancer named α (20, 21). Interestingly, this gradient initiates at the timing of dorsoventral axis formation, at the onset of cVax expression and when Tbx5 becomes restricted to the dorsal part of the retina. In addition, it was recently reported that in the Pax6−/− mouse mutant small eye (Sey), Tbx5 failed to be expressed, whereas Vax1/Vax2 expression extended over the entire optic vesicle (21). Therefore, it was of particular interest to investigate the relevance of Pax6 for cVax/Tbx5 expression and its potential role in the dorsoventral patterning of the eye.

Overexpression of Pax6 in the chick optic cup using in ovo electroporation extended Tbx5 and Bmp4, reduced cVax expression domains, and caused defects in eye morphology. In addition, we showed that Tbx5 and cVax interact with Pax6

* This work was supported by the Curie Institute, CNRS, Association pour la Recherche Centre le Cancer, and Retina France. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
¶ To whom correspondence should be addressed. Tel.: 33-1-6986-7153; Fax: 33-1-6907-4525; E-mail: Simon.Saule@curie.u-psud.fr.
and that they modulate in an opposite manner the activity of the Pax6 α enhancer. Finally, we observed that in the optic cup the highest level of Pax6 expression corresponds to the boundary between the non-overlapping cVax and Tbx5 territories. Taken together, these data strongly suggest that Pax6, together with cVax and Tbx5, contributes to the establishment of the dorsoventral axis of the retina.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Green fluorescent protein (GFP)1 was expressed from the pVNC3-EGFP vector. Quail cDNA encoding the Pax6 isoforms such as p46, p48, and p30 (12) and the mouse Mitf cDNA were cloned into the pSG5 expression vector (22). The p46Δ170–342 resulted from a PstI fragment deletion, and the p46Δ170–342 resulted from a KpnI fragment deletion of the pSG5p46 (12). Details on the G3–138 glucagon promoter have been already described (23). The Tbx5 expression vector and the GST-Tbx5 plasmids were provided by Dr. Colin Goding. A HindIII-EcoRI fragment from the pMIW-cVax provided by Dr. Constance Cepko (8) was cloned into the pV2NC7-HA. pTKCAT-EP and pTKCAT constructs were previously described (20) as well as GST-p46, paired and homeodomains (24). For the synthesis of GST-cVax, a 983-bp fragment of cVax cDNA, was generated by PCR using the pVNC2-cVaxHA vector as a template and primers containing cloning sites (GGGACGTGCAGCCGAATTCCATGTTTGGGAAACAAG and CGCGCTGAGCTTTATTGTTGGTCCGGGAGTAGGG) and cloned into the EcoRI-XhoI sites of the pGEX-4T-3.

In *Ovo Chick Electroporation*—Fertilized white Leghorn chick embryos were incubated at 38 °C in a humid atmosphere for 2 days. DNA (2 μg/ml) was injected into one optic cup of HH stage 12–13 (E2) chick embryos. An electric current (5 × 20-V pulses for 50 ms, with an interval of 500 ms between each pulse) was then applied over the optic cup using an ECM 830 BTX electroporator. The untreated optic cup served as an internal control. Expression of GFP was detected with a fluorescence stereomicroscope (Leica MZFLIII). GFP-positive embryos were processed for *in situ* hybridization or incubated for a longer period of time.

**In Situ Hybridization**—*In situ* hybridization was performed as previously described (25). A plasmid containing the Pax6 cDNA was linearized with XhoI and transcribed with T3 RNA polymerase to generate a RNA probe. Probes for the chick cVax, Tbx5, and Bmp4 genes were kind gifts from Drs. Paola Bovolenta and Maria Marx and were prepared as described (26, 27). Simultaneous detection of cVax and Tbx5 transcripts using two-color *in situ* hybridization was performed by sequential detection of digoxigenin- and fluorescein-labeled probes with alkaline phosphatase-conjugated antibodies (28). After in toto hybridization treatment, embryos were rinsed with phosphate-buffered saline, 15% saccharose, and 7.5% gelatin, frozen, and cryostat-sectioned. Following overnight fixation in 4% paraformaldehyde, cryosections were washed three times in 50 mM sodium phosphate buffer (pH 7.2) for 30 min and incubated with a 1:3000 dilution of the rabbit anti-GFP antibody (A-11122) for 16 h in the same buffer. The sections were then washed, treated with a 1:2000 dilution of the rabbit anti-β-galactosidase antibody (5B11) in 50 mM sodium phosphate buffer (pH 7.2) for 2 h, washed, and incubated with rabbit anti-rabbit biotin (Vector Laboratories) and streptavidin extra-link (Vector Laboratories) for 1 h each. Sections were washed and incubated with a 1:3000 dilution of the goat anti-rabbit biotin (Vector Laboratories) for 1 h. Sections were then washed and incubated with a 1:3000 dilution of the goat anti-biotin (Vector Laboratories) for 1 h. The sections were washed and mounted on slides coated with gelatin (1% wt/vol) and coverslipped with DAPI mounting medium (Vector Laboratories).

**RESULTS**

Overexpression of Pax6 in the Optic Cup Leads to an Abnormal Eye Phenotype—In the Pax6 mutant mouse (Sey), dorsal characteristics of the optic cup are lost at the expense of ventral ones, suggesting that Pax6 may play a role in regulating retinal dorsoventral polarity. To test this possibility we used *in ovo* electroporation to overexpress different Pax6 constructs in the chick optic cup. These constructs are depicted in Fig. 1A. First, a plasmid carrying the full-length cDNA coding for p46 or p48 and a plasmid carrying the green fluorescent protein (EGFP) were co-electroporated into one optic cup of E2 chick embryos (HH 12–13); the other eye of the same embryo served as a control. This enabled visualization of the electroporation target site by virtue of GFP expression. As a negative control embryos were electroporated with the plasmid carrying GFP alone. 48 h later eyes electroporated with p46 or p48 showed an abnormal phenotype (Fig. 1, B and D); oval-shaped eyes were formed instead of normal round eyes, and Pax6-electroporated eyes

---

1 The abbreviations used are: GFP, green fluorescent protein; BHK, baby hamster kidney; TK, thymidine kinase; GST, glutathione S-transferase; CMV, cytomegalovirus; CAT, chloramphenicol acetyltransferase.


TABLE I

Pax6 overexpression caused an abnormal eye phenotype

| Transgenes | No. of electroporated chick embryos | No. of live embryos after electroporation | No. of positive in optic cup | No. of rotated eye phenotype |
|------------|------------------------------------|------------------------------------------|-----------------------------|-----------------------------|
| GFP        | 116                                | 46                                       | 35 (76%)                    | 0                           |
| p46        | 164                                | 78                                       | 48 (62%)                    | 28 (58%)                    |
| p48        | 25                                 | 15                                       | 8 (54%)                     | 4 (30%)                     |
| p30        | 61                                 | 36                                       | 17 (27%)                    | 5 (30%)                     |
| p46 Δ170–342| 39                                 | 17                                       | 12 (70%)                    | 4 (33%)                     |
| p46 Δ48–211| 49                                 | 28                                       | 23 (82%)                    | 0                           |
| Mitf       | 30                                 | 13                                       | ND                          | 0                           |

were rotated toward the ventral midline and sometimes presented a widened optic fissure (Fig. 1B). This phenotype was not observed after electroporation of GFP alone (Fig. 1C) or microphthalmia (Mitf), another transcription factor important for eye development (Ref. 22; Table I).

Pax6 is a transcription factor with three functional domains, two DNA binding domains (paired and homeo) and a transactivation domain (Fig. 1A). To investigate which domain of Pax6 was involved in the eye phenotype, we electroporated different constructs including the p30 Pax6 isoform and two truncated forms of Pax6 (p46Δ48–211 and p46Δ170–342). The same abnormal eye phenotype was observed after electroporation of p30, which is devoid of the paired domain (Fig. 1E). The p46Δ170–342 contains an intact paired domain but is devoid of the homeodomain and part of the transactivation domain, which abolishes transactivation activity (Fig. 1A) (12). Surprisingly, the same abnormal eye morphology was observed after electroporation of the p46Δ170–342 (Fig. 1F), indicating that the eye phenotype we observed was independent of the transactivation properties of Pax6. Electroporation of the p46Δ48–211, which lacks both in the paired and homeodomain but contains the entire transactivation domain, did not alter eye morphology (Fig. 1G). Taken together these results suggest that, in contrast to the transactivation domain, both of the DNA binding domains in Pax6, namely the paired and the homeodomains, were involved in causing the observed abnormal eye morphology.

Overexpression of Pax6 Reduces cVax and Extends Tbx5 and Bmp4 Expression Domains—To address the relationship between this abnormal eye phenotype and the expression profiles of known early markers of dorsoventral polarity, we examined the consequence of overexpressing Pax6 in the optic cup on cVax, Tbx5, and Bmp4 expression. Embryos were sacrificed 24 h after electroporation and analyzed for gene expression by whole-mount in situ hybridization. Electroporation of GFP alone or p46Δ48–211 did not alter the expression domains of cVax, Tbx5, or Bmp4 (data not shown). However, overexpression of the different Pax6 constructs (p30, p46, p48, and p46Δ170–342) caused an expansion of the dorsal markers (Tbx5 and Bmp4) and a reduction of the ventral marker cVax (Fig. 2 and data not shown). Any modification in the gene expression territory was measured using metamorph software. For example, we observed an extension of the Tbx3 territory after overexpression of p46 (compare Figs. 2, A and B) or p30 (compare Figs. 2, C and D). Extension of the Bmp4 expression domain was also observed after p46 overexpression (compare in situ staining in Figs. 2, F and J with E). These extensions correlated with the electroporated retinal domain as indicated by GFP expression (Fig. 2, G and J). Analyses of eye sections confirmed the extension of the Bmp4 expression domain (Fig. 2H). The cVax expression domain was reduced after misexpression of Pax6. Fig. 2, K–P, shows two examples of embryos electroporated with the p46 (Fig. 2, K–M) and the p48 (Fig. 2, N–P) isoforms. Compared with the control eyes, the injected eyes showed a reduction of the cVax expression domain (compare Fig. 2, K and N with L and O). Analyses using serial sections confirmed that cVax expression was reduced and limited to the ventral most region of the retina (Fig. 2M). In the ventral view, intensity of the cVax staining in the optic stalk was reduced in the electroporated side compared with the control side (Fig. 2P).

cVax and Tbx5 Proteins Physically Interact with Pax6 Paired and Homeodomains—Because both the Pax-6 paired domain and homeodomain were independently able to alter cVax and Tbx5 expression patterns, we tested whether Pax6 interacts physically with cVax and Tbx5. To this end we prepared glutathione-Sepharose beads coupled with a GST fusion protein containing the full-length Pax6 protein (GST-p46; amino acids...
Both Tbx5 and cVax proteins interact with Pax6. A, both Tbx5 and cVax interact with the full-length Pax6 protein (p46). 35S-Labeled GFP (lanes 1–4), cVax (lanes 5–7), p46 (lanes 8–10), and Tbx5 (lanes 11–13) proteins were incubated with GST fusion proteins as indicated. GST alone showed no interaction with GFP (lane 1), cVax (lane 5), p46 (lane 8), or Tbx5 (lane 11) proteins. No interaction was detected between the GFP protein and p46 or Tbx5 (lanes 2 and 3). However, strong interactions were observed between cVax and p46 (lane 6) or between Tbx5 and p46 (lanes 9 and 12). Lanes 4 and 7, 10 and 11, 20% input. B, Tbx5 and cVax interact with both p46 paired and homeo DNA-binding domains. No retention was observed with GST alone (lanes 1 and 4). Both Tbx5 and cVax were able to bind the p46 paired domain (prd, lanes 2 and 5) and homeodomain (Hom, lanes 3 and 6). C, the Pax6 transactivation domain is not involved in Pax6/Tbx5 or Pax6/cVax interactions. The 35S-labeled p46 (lanes 1–4) or p46ΔG48–211 (lanes 5–8) proteins were incubated with GST proteins as indicated. GST alone showed no interaction with p46ΔG48–211 (lane 3) or p46ΔG48–211 (lane 5). Although cVax and Tbx5 interact with the full-length protein p46 (lanes 2 and 3), they were not able to interact with p46ΔG48–211, indicating that the p46 transactivation domain is not required for these interactions. It is interesting to note that the p30 paired-less protein (indicated by an arrowhead), which is synthesized together with the p46 product because of an internal AUG initiation codon (12, 22), also binds to the GST-cVax or to the GST-Tbx5.

1–416), the full-length Tbx5 protein (GST-Tbx5), or the full-length cVax protein (GST-cVax). Glutathione-Sepharose beads coupled to GST alone served as a negative control. No interaction was detected with the radiolabeled GFP protein (Fig. 3A, lanes 1–3) or with the GST alone (Fig. 3A, lanes 1, 5, 8, 11; B, lanes 1 and 4; C, lanes 1 and 5). However, strong protein-protein interactions were observed between Pax6 and cVax (Fig. 3A, lane 6) as well as between Pax6 and Tbx5 (Fig. 3A, lanes 9 and 12).

To identify which domains of Pax6 are required for physical interaction with Tbx5 and cVax, we used GST fusion proteins containing either the paired domain from the p46 (GST-paired; amino acids 3–131) or the p46 homeodomain (GST-homeo; amino acids 224–284). Both the Pax6 paired and homeodomain were independently able to bind the radiolabeled Tbx5 (Fig. 3B, lanes 2 and 3) or cVax (Fig. 3B, lanes 5 and 6), indicating that both domains are involved in Pax6/Tbx5 and Pax6/cVax interactions. Finally, we tested these interactions with the truncated form p46ΔG48–211, which did not alter eye morphology and did not change dorsoventral marker expression patterns. Fig. 3C shows that the interactions observed with the p46 wild type protein (Fig. 3C, lanes 2 and 3) as well as with the paired-less proteins (arrowhead) produced by internal initiation (13) were almost completely lost with the p46ΔG48–211 protein (Fig. 3C, lanes 6 and 7), indicating that cVax/Pax6 or cVax/Tbx5 protein-protein interactions do not involve the transactivation domain but require the paired and/or the homeodomains.

The Highest Level of Pax6 Expression in the Chick Optic Cup Corresponds to the Boundary between cVax and Tbx5 Territories—It has been previously shown that Pax6 is expressed in a distal (high) proximal (low) gradient in the developing mouse eye. This pattern of expression appears at the optic cup stage and results from the activity of the conserved α enhancer in intron 4 of the Pax6 genomic locus (21). To compare the spatial expression profiles of the early known markers of dorsoventral polarity cVax and Tbx5 with Pax6 in the chick optic cup, we performed whole-mount in situ hybridization of E3 chick embryos. As shown in Fig. 4A, Tbx5 and cVax were expressed in non-overlapping domains in the optic cup; Tbx5 was restricted to the dorsal part of the retina, whereas cVax was present in the ventral retina and the optic stalk. In addition, there was a white narrow band at the nasal/temporal level of the retina separating cVax and Tbx5 expression domains (Fig. 4A, arrows). Interestingly, this cVax/Tbx5 boundary corresponded to the highest activity of Pax6 expression in the optic cup (Fig. 4B, arrows). In summary, at the early optic cup stage (HH18–19), the retina is divided into four domains of restricted gene expression along the dorsoventral axis; they are a first dorsal domain where Pax6 and Tbx5 are coexpressed, a second domain where only Pax6 is strongly expressed, a third domain where Pax6 and cVax are coexpressed, and finally, a ventral domain where only cVax is present (Fig. 4C).

cVax Represses Whereas Tbx5 Increases Pax6 α Enhancer Activity in Vitro—Because Pax6 activity via the intronic α enhancer contributes to the establishment of Pax6 gradient expression in the mouse retina (21), we next asked whether cVax or Tbx5 could directly modulate Pax6 regulatory sequences. We used two constructs containing the thymidine kinase (TK) promoter driving the CAT reporter gene alone (pTKCAT) or linked to the Pax6 α enhancer (pTKCAT-EFP). E8 quail neuroretina cells were transfected with cVax or Tbx5 together with the CAT constructs. Compared with the basal activity observed with the TK promoter alone, transfection of the α enhancer alone resulted in a 4-fold increase in CAT activity (Fig. 5A). The CAT activity of the α enhancer was reduced in presence of cVax in a dose-dependent manner (Fig. 5A). By contrast, Tbx5 expression increased the activity of the α enhancer (Fig. 5A). These data show that cVax and Tbx5 were able to modulate Pax6 α enhancer activity but in an opposite way, suggesting that they might contribute to the establishment of the Pax6 gradient.

Because cVax and Tbx5 proteins were able to physically interact with Pax6, we then asked whether these interactions could modulate the Pax6 transcriptional activity in vitro. We used as a Pax6 target gene the glucagon promoter linked to the CAT reporter gene (23). cVax and Tbx5 were cotransfected with Pax6 in BK21 cells together with the glucagon promoter. Compared with the vector control, Pax6 expression resulted in a 35-fold increase of CAT activity (Fig. 5B). Co-transfection of cVax with Pax6 resulted in a reduction of the Pax6 transcriptional effect in a dose-dependent manner (Fig. 5B). Co-transfection of Pax6 with increasing amounts of Tbx5 did not change...
Coexpression (red light purple), cVax only (of restricted gene expression along the dorsoventral axis; Pax6 and Tbx5 coexpressed (blue summary model of optic cup showing the gradient expression of Pax6. Pax6 is totally absent from the most ventral retina and expressed in the far dorsal part. Note that the highest level of Pax6 expression corresponds to the region where neither Tbx5 nor cVax is expressed (arrows). Scale bar, 0.1 mm. C, summary model of Pax6, Tbx5, and cVax gene expression domains in the early optic cup (HH18–19). Note that the retina is divided in four domains of restricted gene expression along the dorsoventral axis; Pax6 and Tbx5 coexpressed (blue in dorsal), Pax6 only (dark purple), Pax6 and cVax coexpressed (light purple), cVax only (red in ventral).

**FIG. 4.** The highest level of Pax6 expression at the optic cup stage corresponds to the boundary between Tbx5 and cVax expression territories. A, simultaneous detection of Tbx5 and cVax RNA in the E3 chick optic cup using two-color *in situ* hybridization. Tbx5 (blue) is expressed in the dorsal part of the optic cup, whereas cVax (red) is present in the ventral part. Note that at the boundary between Tbx5 and cVax expression domains, there is a narrow white band where neither gene is expressed (arrowheads). B, *in situ* detection of Pax6 RNA in the E3 chick optic cup showing the gradient expression of Pax6. Expression domains, there is a narrow white band where neither gene is expressed (arrowheads).

**FIG. 5.** Effect of cVax and Tbx5 on Pax6-transactivating properties. A, effect of cVax and Tbx5 on Pax6 α enhancer activity. E8 quail neuroretina cells were transiently transfected with increasing amounts of a plasmid encoding cVax or Tbx5 together with the TK promoter driving the CAT reporter gene alone (pTKCAT construct) or linked to the Pax6 α enhancer (pTKCAT-EP construct). As a control, open columns show the basal CAT activity of the pTKCAT construct. Filled columns show the CAT activity of the construct containing the Pax6 α enhancer. This CAT activity is progressively reduced in the presence of cVax in a dose-dependent manner and is rather increased in the presence of Tbx5. B, effect of cVax/Pax6 and Tbx5/Pax6 interactions on Pax6 transcriptional activity. BHK21 cells were transiently transfected with 200 ng of a CAT reporter glucagon promoter construct (pG3–138). CAT activities were expressed relative to the value for the pG3–138 with the empty vector (set at a value of 1). Expression of Pax6 alone (p46) resulted in a 35-fold increase of CAT activity. Expression of either cVax or Tbx5 alone did not show significant CAT activity. Co-transfection of Pax6 with increasing amounts of cVax resulted in a decrease of CAT activity in a dose-dependent manner. In contrast, co-transfection of Tbx5 and Pax6 did not significantly change Pax6 transcriptional level.

**DISCUSSION**

Our data provide evidence that overexpression of Pax6 at the optic cup stage caused dorsalization of the developing eye by up-regulating Tbx5 and down-regulating cVax. Tbx5 and cVax interact with Pax6 and modulate in an opposite manner the activity of the Pax6 α enhancer, which is essential for its gradient expression. Most importantly, the present results suggest that Pax6 is involved in delimiting a boundary between the dorsal and the ventral compartments of the developing eye.

**Molecular Dorsalization of the Developing Eye after Pax6 Overexpression**—In this study we report that overexpression of Pax6 promoted dorsalization of the optic cup; extension of the dorsal markers, Tbx5 and Bmp4, and reduction of the ventral marker cVax were observed in the developing eye after Pax6 overexpression. Consistent with our data, it was previously reported that misexpression of Tbx5 (7) or Bmp4 (7, 29) caused dorsalization of the eye. Conversely, misexpression of cVax has been shown to ventralize the retina (8). Previously, BMP4 and the morphogen sonic hedgehog have been involved in patterning the dorsoventral axis of the eye by antagonistic actions (29–31). Interestingly, we observe that Pax6 electroporation produced similar effects to those found after Bmp4 overexpression (29) or after blocking Sonic hedgehog activity by antibodies (31). In all cases, the expression of dorsal markers was expanded, whereas ventral markers were reduced, and similar morphological defects (eye deformation and rotation toward the ventral midline) were observed. Interestingly, oval-shaped eyes were also observed after overexpression of Tbx5 (7). Because of Tbx5 up-regulation and cVax down-regulation in Pax6 misexpressing eyes, the morphological defects that we observe could result from an abnormal development of the dorsal retina at the expense of the ventral retina. In the Pax6−/− mutant mouse (Sey), Tbx5 is no longer expressed, whereas Vax1/Vax2 extend to the entire optic vesicle (21). Thus, the absence of Pax6 induces ventralization of the eye, whereas overexpression of Pax6 causes dorsalization of the eye, suggesting that, during normal development, Pax6 may activate Tbx5 and repress Vax.

**Pax6 Interacts with Tbx5 and cVax for Dorsoventral Patterning of the Eye**—What are the molecular mechanisms by which Pax6 regulates Tbx5 and cVax activity? Our data suggest that the observed eye phenotypes arise as a result of Pax6 protein interactions with cVax and Tbx5 rather than by a direct effect of Pax6 on cVax or Tbx5 regulatory sequences. First, we observed that all the Pax6 isoforms we electroporated (p46, p48, and p30) were able to dorsalize the eye. The p46 and p48 paired...
domains bind distinct DNA sequences, which are also different from the one recognized by the homeodomain (32). Therefore, it is very unlikely that these Pax6 isoforms share the same target genes. Second, the p46Δ170–342, which is no longer able to transactivate (12), was still able to dorsalize the eye, suggesting that this phenotype was independent from Pax6 transcriptional activation abilities. Thus, we favored the hypothesis that protein-protein interactions are involved in eye dorsalization. Indeed, we demonstrated that Pax6 protein was able to interact with both Tbx5 and cVax proteins. We observed that the two Pax6 DNA binding domains (paired and homeodomain) were also able to bind both Tbx5 and cVax proteins. Consistent with this, both the p30 isoform, which contains only the homeodomain, and the p46Δ170–342 construct, which contains only the paired domain, were able to dorsalize the eye. In addition, the p46Δ48–211 construct, which lacks both DNA binding domains but contains the entire transactivation domain, did not interact with cVax or Tbx5. We did not observe any abnormal eye phenotypes or alterations in cVax/Tbx5 expression patterns after p46Δ48–211 electroporation. This suggests that these protein-protein interactions occur via the paired, the homeodomain, or both. At the functional level, we showed that Pax6/cVax interaction inhibited Pax6 transactivation properties. Because Pax6 is able to transactivate its own promoters (20), inhibition of Pax6 activity through cVax interaction contributes to Pax6 down-regulation, also mediated by the c-Vax repression of the intronic α enhancer in the retina. Pax6/cVax interaction may, therefore, play an important role in the early determination of the dorsal versus ventral retina.

A Gradient of Pax6 Expression May Be Required for Establishing Dorsal-Ventral Boundary in the Developing Eye—Pax6 has been shown to be expressed in a gradient in the developing mouse eye after activation of an intronic p46/H9004 enhancer. This gradient expression corresponds to the boundary between dorsal and ventral cell types (35). Therefore, during pituitary gland differentiation underlines the fact that Pax6 acts not only as a transcription factor but also through interactions with other transcription factors.

Acknowledgments—We thank Paula Bovolenta and Thierry Jaffredo for advice on electroporation, Anne Helene Monsoro-Burq and Keely Bumsted O’Brien for critical comments on the manuscript, Vincent Dolez for help with the transfection experiments, and Oceane Anzeo for technical assistance.

REFERENCES

1. Sivak, B., and Sivak, J. (2000) in Vertebrate Eye Development (Finin, M.E., eds) pp. 1–14, Springer-Verlag, Berlin

2. Dutting, D., and Meyer, S. U. (1995) Int. J. Dev. Biol. 39, 921–931

3. Uemonsa, T., Sakagami, K., Yasuda, K., and Araki, M. (2002) Dev. Biol. 248, 319–330

4. Peters, M. A., and Cepko, C. L. (2002) Dev. Biol. 251, 59–73

5. Gibson-Brown, J. J., Agulnik, S. I., Silver, L. M., and Papaioannou, V. E. (1998) Mech. Dev. 74, 165–169

6. Gibson-Brown, J. J., Agulnik, S. I., Silver, L. M., Niwander, L., and Papaioannou, V. E. (1998) Development 125, 2499–2509

7. Koshita-Takeuchi, K., Takeuchi, J.K., Matsumoto, K., Momose, T., Uno, K., Hohepker, V., Ogura, K., Saito, N., Nakamura, H., Yasuda, K., and Ogura, T. (2000) Science 287, 134–137

8. Schultz, D., Furukawa, T., Peters, M. A., Kozak, C. A., and Cepko, C. L. (1999) Neuron 24, 541–553

9. Barbieri, A. M., Broccoli, V., Bovolenta, P., Alfonso, G., Marchitello, A., Mochetti, C., Crippa, L., Bullone, A., Marigo, V., Ballabio, A., and Banfi, S. (2002) Development 129, 605–613

10. Mui, S. H., Hindges, R., Feinberg, D. D., Lemke, G., and Bertuzzi, S. (2002) Development 129, 797–804

11. Martin, P., Carriere, C., Dozier, C., Quatannens, B., Mirabel, M. A., Vandenbunder, B., Stehelin, D., and Saule S. (1992) Oncogene 7, 1721–1728

12. Carriere, C., Plaza, S., Caboche, J., Dozier, C., Bailly, M., Martin, P., and Saule, S. (1995) Development 120, 1531–1540

13. Carriere, C., Plaza, S., Martin, P., Quatannens, B., Bailly, M., Stehelin, D., and Saule, S. (1993) Mol. Cell. Biol. 13, 7257–7266

14. Chow, R. L., Altmann, C. R., Lang, R. A., and Hemmati-Brivanlou, A. (1999) Development 126, 4213–4222

15. Simpson, T. I., and Price, J. (2002) BioEssays 24, 1041–1051

16. Callaert, P., Halder, G., and Gehring, W. J. (1997) Annu. Rev. Neurosci. 20, 483–532

17. Hill, R. E., Favor, J., Hogan, B. L., Ten, C. C., Saunders, G. F., Hanson, I. M., Prosser, J., Jordan, T., Hastie, N. D., and van Heyningen, V. (1991) Nature 354, 522–525

18. Schedl, A., Ross, A., Lee, M., Engelkamp, D., Rashbass, P., van Heyningen, V., and Hastie, N. D. (1996) Oncogene 12, 71–82

19. Grindley, J. C., Davidson, D. R., and Hill, R. E. (1995) Development 121, 1433–1442

20. Plaza, S., Dozier, C., Langlois, M. C., and Saule, S. (1995) Mol. Cell. Biol. 15, 992–993

21. Baumer, N., Marquardt, T., Stoykova, A., Ashery-Padan, R., Chowdhury, K., and Gruss, P. (2002) Development 129, 4535–4545

22. Planque, N., Leconte, L., Coquelle, P., Martin, P., and Saule S. (2001) J. Biol. Chem. 276, 29330–29337

23. Ritz-Laser, B., Estreicher, A., Klages, N., Saule, S., and Philippe, J. (1999) J. Biol. Chem. 274, 4124–4132

24. Plaza, S., Langlois, M. C., Turque, N., LeCornet S., Bailly, M., Begue, A., Quatannens, B., Dozier, C., and Saule S. (1997) Mol. Cell. Biol. 17, 789–799

25. Suniga, A., Haramis, A. P., McMahon, A. P., and Zeller, R. (1999) Nature 401, 598–602

26. Sosagawa, S., Takahatake, T., Takahatake, Y., Muramatsu, T., and Takashima, K (2002) Genesis 2, 86–96

27. Barbieri, A. M., Lupo, G., Bullone, A., Andreazolli, M., Mariani, M., Fougerousse, F., Consalez, G. G., Borsani, G., Beckmann, J. S., Barssacchi, G., Ballabio, A., and Banfi, S. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10729–10734

28. Zhang, X. M., and Yang, X. J. (2001) Dev. Biol. 233, 271–290

29. Sun, J., and Desplan, C. (1996) Development 122, 2639–2650

30. Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kowakami, A., van Heyningen, V., Jessell, T., and Briscoe, M. (1997) Cell 86, 169–180

31. Stoykova, A., Treichel, D., Hallonet, M., and Gruss, P. (2000) J. Neurosci. 20, 8042–8050

32. Kissi, C., O’Connell, S., St-Onge, L., Treier, M., Gleierman, A. S., Gruss, P., and Rosenberg, M. F. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 14378–14382

47277
Pax6 Interacts with cVax and Tbx5 to Establish the Dorsoventral Boundary of the Developing Eye
Laurence Leconte, Laure Lecoin, Patrick Martin and Simon Saule

J. Biol. Chem. 2004, 279:47272-47277.
doi: 10.1074/jbc.M406624200 originally published online August 18, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M406624200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 33 references, 17 of which can be accessed free at http://www.jbc.org/content/279/45/47272.full.html#ref-list-1