Development 128, 3987-3994 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV2759

Isolation and characterization of a downstream target of *Pax6* in the mammalian retinal primordium

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Accepted 16 July 2001

SUMMARY

The transcription factor Pax6 is required for eye morphogenesis in humans, mice and insects, and can induce ectopic eye formation in vertebrate and invertebrate organisms. Although the role of Pax6 has intensively been studied, only a limited number of genes have been identified that depend on *Pax6* activity for their expression in the mammalian visual system. Using a large-scale in situ hybridization screen approach, we have identified a novel gene expressed in the mouse optic vesicle. This gene, *Necab*, encodes a putative cytoplasmic Ca^{2+}-binding protein and coincides with *Pax6* expression pattern in the neural ectoderm of the optic vesicle and in the forebrain pretectum. Remarkably, *Necab* expression is absent in both structures in *Pax6* mutant embryos. By contrast, the optic vesicle-expressed homeobox genes *Rx*, *Six3*, *Otx2* and *Lhx2* do not exhibit an altered expression pattern. Using gain-of-function experiments, we show that *Pax6* can induce ectopic expression of *Necab*, suggesting that *Necab* is a direct or indirect transcriptional target of *Pax6*. In addition, we have found that *Necab* misexpression can induce ectopic expression of the homeobox gene *Chx10*, a transcription factor implicated in retina development. Taken together, our results provide evidence that *Necab* is genetically downstream of *Pax6* and that it is a part of a signal transduction pathway in retina development.

Key words: Necab, Pax6, Retina, Eye development, Mouse

INTRODUCTION

The eye originates from the diencephalic part of the prospective forebrain in avian and in mouse embryos (Couly and Le Douarin, 1988). At the morphological level, the eye primordium is first revealed by the appearance of the optic sulcus at 8.5 days post coitum (dpc), a neural ectoderm-derived structure that will give rise to the neural retina and to the retinal pigmented epithelium. At the neural plate stage, the presumptive eye field is defined by the expression pattern of a group of homeobox genes that includes *Pax6*, *Rx*, *Otx2*, *Six3* (Walther and Gruss, 1991; Oliver et al., 1995; Furukawa et al., 1997; Mathers et al., 1997) and *Lhx2* (G. B., unpublished). Genetic ablation or spontaneous mutations in the mouse revealed a requirement of these genes in early eye morphogenesis (Hogan et al., 1986; Hill et al., 1991; Acampora et al., 1995; Matsuo et al., 1995; Mathers et al., 1997; Porter et al., 1997), with the exception of *Six3* (for which currently no mouse mutant is available). *Pax6* is a member of the paired-box and homeobox-containing gene family (Pax) of transcription factors and is functionally conserved in both humans and insects (Ton et al., 1991; Quiring et al., 1994). Because misexpression of *eyeless*/*Pax6* is sufficient to induce ectopic eye formation in *Drosophila* imaginal discs, *eyeless* has been proposed to function at the apex of the genetic cascade controlling *Drosophila* eye development (Halder et al., 1995; Halder et al., 1998; Gehring and Ikeo, 1999).

In rodents, in vitro grafting experiments and conditional mutagenesis showed a cell-autonomous requirement for *Pax6* during lens placodes formation (Fujiwara et al., 1994; Ashery-Padan et al., 2000). Chimera aggregation studies also revealed a cell-autonomous function for *Pax6* during neural ectoderm development, although *Pax6* mutants do form an optic vesicle (Hill et al., 1991; Grindley et al., 1995; Quinn et al., 1996; Collinson et al., 2000). In addition, recent experiments using conditional mutagenesis have revealed that *Pax6* is also required for the multipotent state of retinal progenitor cells (Marquardt et al., 2001). Although much is known about the role of *Pax6* during eye development in the mouse, few genes have been shown to be dependent on *Pax6* activity for their expression in the visual system.

We report on the isolation of a novel mouse gene that is genetically downstream of *Pax6* in the early developing retina. This gene has been named Necab (Neuronal Ca^{2+} binding protein; T. C. Sudhof, personal communication). *Necab* expression is detected in the neural ectoderm of the optic vesicle and in the pre-tectum, where its expression is abolished in *Pax6* mutant embryos. Gain-of-function experiments revealed that *Pax6* induces ectopic expression of *Necab*. Remarkably, we also found that *Necab* misexpression induces

ectopic expression of *Chx10*. Our results show that *Necab* is genetically downstream of *Pax6* in the mouse retinal and forebrain primordium and suggest that it has an activity in regulating gene expression.

MATERIALS AND METHODS

Isolation of cDNA and gene localization

The 1.9 kb DNA fragments from clone 9066 was used to screen a 15.5 dpc total mouse embryo random-primed λgt10 cDNA library (GibcoBRL). Hybridization was performed at 65°C in Church's hybridization buffer. Membranes were washed four times with 40 mM sodium phosphate/1% SDS (pH 7.2) solutions at the same temperature. Three independent clones were purified and sub-cloned into pBSK+ (*Eco*RI). The human adult retina cDNA library (Clontech) was hybridized with the same DNA fragment, but with the hybridization and washes at 55°C. Fluorescence in situ hybridization (FISH) on metaphase chromosomes was performed using a 1.2 kb DNA fragment (from the human *NECAB* cDNA) and a 15 kb genomic DNA fragment from the mouse *Necab* locus (SeeDNA, Ontario). Double strand sequencing of clone 9066 was performed using a micro capillary sequencer (Sequence Laboratories, Goettingen).

Antibodies

Three synthetic peptides were generated (Ab1, AHRLLREPPPQGRA (amino acids 13-26); Ab2, KPSHAVNESRYGGPT (amino acids 207-221); Ab3, EGQISRLAELIGR (amino acids 252-269). Coupled peptides were injected into rabbits for immunization (Eurogentech, Belgium). Serum from the final bleeding was purified using affinity resin columns, according to the manufacturer recommendations (Immunotech). Eluates were tested at different concentration on paraffin sections by immunohistochemistry using the ABC amplification kit (Vector). Average working concentration for the primary antibodies was 1/50 (second eluate). For immunofluorescence on sections, 17.5 dpc mouse embryos were immersed in cryomatrix solution (Shandon, Pittsburgh) and snap frozen in liquid nitrogen. Embryos were sectioned at 14 μm with a cryostat (Leica), air dried on slides for 10 minutes, washed twice with phosphate-buffered saline (PBS) and fixed for 10 minutes in 4% paraformaldehyde (PFA)/PBS (pH 7.5). After washes in PBS, sections were pre-incubated in 3% bovine serum albumin/0.1% Tween 20/PBS (1 hour), incubated with the primary antibodies overnight at 4°C in the same solution. After washes with PBS, sections were incubated with a diluted (1/1000) goat anti-rabbit secondary antibody (Mobitec) for 1 hour at room temperature, rinsed with PBS and visualized with a fluorescence microscope (Olympus BX60). For the protein-fusion construct, *Necab* cDNA fragment (clone 9075, amino acids 1-378) was inserted in the pcDNA3.1/V5-His B vector (Invitrogen) in frame with the C-terminal V5/His epitope. COS cells were transfected (FuGENE, Roche) for 24-48 hours with the *Necab*-V5/His construct, fixed with 4% PFA/PBS (10 minutes at room temperature), washed, incubated with the anti-V5 primary antibody (1/500), washed and incubated with the goat anti-mouse (Mobitec) secondary antibody (1/1000).

In situ hybridization

Embryos were dissected in PBS, fixed overnight in 4% paraformaldehyde at 4°C and embedded in Paraplast (Monoject Scientific). Sections (10 μm) were cut and dried onto chromalum-gelatin slides. All the steps of high-stringency hybridization and washing were carried out as described previously (Kessel and Gruss, 1991) ^{35}S-labelled RNA probe using SP6, T3 or T7 RNA polymerase were done with Boehringer enzyme according to the directive of the company. Exposure time for the radioactive RNA in situ hybridization was 15 days. For double whole-mount in situ hybridization, preparation were hybridized with digoxigenin-labelled and fluorescein-labelled RNA probes, first visualized with alkaline phosphatase-coupled anti-digoxigenin antibody (1/2000) (Boehringer) and NBT/BCIP substrate (Boehringer) at pH 9.5. To destroy the first antibody, embryos were treated with 0.1M glycine/HCl pH 2.2 for 15 minutes and fixed overnight in 4% PFA/PBS at 4°C. For the second colour reaction, embryos were incubated with an anti-fluorescein-AP antibody (1/2000) (Roche) and treated with the Fast Red TR/NAPHTHOL AS-MX phosphate substrate (Sigma). The DNA fragments used to generate the riboprobe were as follows: 1.4 kb *Six3* cDNA (Oliver et al., 1995); 1.9 kb *Necab* cDNA (clone 9066); and 800 bp *Pax6* cDNA (Walther and Gruss, 1991). The 1.2 kb *Rx* cDNA and 1.4 kb *Lhx2* cDNA were amplified by RT-PCR based on the available sequences in the database and cloned into the PCRII vector (Clontech). The 800 bp Chx10 cDNA fragment was a gift from Roderick McInnes (Toronto) and the 900 bp Otx2 cDNA was a gift from Thomas Theil (Düsseldorf). For cryosectioning, embryos were cryoprotected in 30% sucrose/PBS overnight at 4°C, embedded in cryomatrix solution and snap-frozen in liquid nitrogen. Specimens were cut using a cryostat (Leica) at 14 μm, air dried, washed twice in PBS and mounted in 80% glycerol/PBS solution.

Embryos culture and electroporation

The full-length cDNA fragment of mouse *Pax6* (Walther and Gruss, 1991), mouse *Six3* (Oliver et al., 1995) and mouse *Necab* (clone 9075) were inserted directly into the eucaryotic expression vector pCS2+. Large DNA preparations were purified using the Maxi-prep filter column system (Qiagen), treated with phenol/chloroform/isoamyl alcohol (pH 8.0), chloroform/isoamyl alcohol, precipitated and washed with 70% ethanol. The DNA was resuspended in PBS at a concentration of 5 μg/μl. Mouse embryos from the NMRI strain were isolated at embryonic stages 8.5, according to the time of the vaginal plug. Dissection was initially performed in PBS to isolate the conceptus. The embryos were transferred in M2 medium (Sigma) supplemented with 10% foetal calf serum (FCS)/10 mM glucose at room temperature. The deciduas and Reichert's membrane were carefully removed, keeping the yolk sac and the ectoplacental cone intact. Embryos were transferred into a microcuvette containing PBS and injected (Eppendorf injector, model 5242) with a capillary needle into the midbrain region. The embryos were electroporated (three pulses at 35 Volts) using a generator power supply (BTX, ECM 8300). Injected embryos were transferred into a 6 wells petri dish for cell culture containing 3 ml/well of 100% FCS/10 mM glucose and cultured for 1 to 6 hours into a 5% CO_2 incubator at 37°C. Cultured embryos were transferred into PBS, dissected out of the yolk sac and fixed overnight in 4% PFA/PBS (pH 7.5) at 4°C. Abnormal embryos (abnormal somitogenesis or no beating heart) were discarded. After fixation, embryos were stored at –20°C in 100% methanol. For the culture of the explants, midbrains from 8.5 dpc embryos were isolated in PBS using sharpened tungsten needles, transferred into a microcuvette containing the DNA construct at 1 μg/μl in PBS and pulsed three times (50 mseconds/pulse) at 70 Volts. Explants were mixed with rat collagen (80% rat collagen, 1× modified Eagle's medium, 5% FCS, 2% L-glutamine, equilibrated with $NaHCO_3$ at pH 7.5) and placed into a tissue culture net in a 24 wells petri dish. The collagen was allowed to polymerize for 10 minutes at 37°C and the explants were incubated for 24 hours (37°C, 5% CO_2) in 1 ml of culture solution (Optimem-Glutamax/F12 (3:1) (Gibco), 10mM glucose, 10% FCS, 1% penicillin/streptavidin). Explants were fixed in 4% PFA/PBS, washed in PBS, dissected out of the collagen polymer and processed for in situ hybridization.

RESULTS

Isolation and characterization of *Necab*

Using a previously described large-scale in situ screen for genes expressed in the developing mouse embryo (Neidhardt

et al., 2000), a novel cDNA of 1.9 kb (clone 9066) showing expression in the optic vesicle was isolated. This cDNA fragment was used to screen a 15.5 dpc mouse embryo cDNA library. Three independent clones were isolated, sequenced and compared to the clone 9066. It was found that clone 9066 had a 200 bp non-coding insertion between exon 1 and 2. In addition, a second independent clone from the in situ screen (clone 9075) was isolated, which turned out to be identical to clone 9066, but without the insertion. The nucleotide sequence indicates the presence of two in-frame putative start codons closely matching the Kozak consensus sequence (Fig. 1A). The 5′ untranslated region is extremely rich in GC nucleotides and is contiguous to a CpG island in the genomic sequence (not shown). Conceptual translation of the nucleotide sequence predicted a 389 amino acid protein of 45 kDa. Using clone 9066, we subsequently also screened an adult human retina cDNA library and recovered two independent clones. Sequence analysis confirmed the homology of the human cDNAs with the mouse 9066 clone. Because the human composite cDNAs were missing the corresponding mouse exon 1 and part of exon 2, we used BLAST search in the Human Genome Project database aiming for an extension of the sequence available. Through the homology with the mouse 9075 clone and human exon 2, we identified the missing 5′ region of the human cDNA, except for a small gap at the end of exon 1. Translation of the nucleotide sequence also predicted a 389 amino acid protein. A homology search using the Advance Blast and PsiBlast programs for the whole sequence database also revealed the existence of a highly related sequence from macacus (Fig. 1B). Alignment of the human and mouse amino acid sequences showed an overall identity of 83% and a similarity of 88% (which is approximate considering the possible mistakes in the N-terminal region of the human sequence). The human and macacus sequences were more related to each other than to the mouse sequence, with an identity of 94.5% (when removing the 10 amino acid gap in the macacus sequence at position 298-307). A homology search in the database for conserved functional domains revealed the existence of a putative EF-hand Ca^{2+}-binding domain (Fig. 1A) between amino acids 71 and 121 (CABP-like domain). The identified gene, *Necab*, is homologous to two other genes in the database (Accession Number AF193755 (rat Stip1), AF193756 (human STIP1) and AF193759 (human STIP3)) and is orthologous to rat Stip2 (Accession Number AF193757). They constitute a novel gene family encoding putative Ca^{2+}-binding proteins. Chromosomal mapping by FISH (see Methods) showed the localization of human *NECAB* on chromosome 16q23 (Fig. 1D). The mouse gene was found to map on chromosome 8E1 (Fig. 1D), a region that is synthenic to human chromosome region 16q22-q24. Based on the predicted translation product of the mouse cDNA, we generated three different peptides (see Materials and Methods) for immunization in rabbits in order to raise antibodies against Necab. The purified sera were first tested by immunohistochemistry on paraffin sections. All three immune sera (Ab1, Ab2, and Ab3) gave immunoreactivity in the retina, retinal pigmented epithelium (Fig. 1 C, part I) and olfactory placodes (not shown). Immunoreactivity was also observed in the Rathke's pouch and in the infundibulum: the future pituitary (Fig. 1C, part II). No immunoreactivity was observed in the lens and in the mesenchyme surrounding the eye. We also observed immunoreactivity in the corneal epithelium, which is likely to be an unspecific signal since *Necab* expression was not detected in this structure by in situ hybridization. By immunofluorescence at 17.5 dpc on eye sections, we observed immunoreactivity with all three antibodies in the inner region of the neural retina, including the ganglion cell layer (Fig. 1C, part III). Fluorescence signal was detected in the cytoplasm. All three antibodies were very sensitive to variations in fixation procedures. Western blot analysis of whole embryo protein extracts with Ab1 and Ab3 showed a main immunoreactive band at 45 kDa, which correlated with the predicted molecular weight of the protein (not shown). Ab2 was not immunoreactive on Western blots. To confirm the observed results with the primary antibodies, we overexpressed a fusion protein with a C-terminal V5/His epitope in COS cells. Immunofluorescence imaging with a confocal microscope revealed a cytoplasmic localization of the fusion protein (Fig. 1C, part IV). Western blot analysis of protein extracts from the transfected cells (using the anti-V5 antibody) revealed a 45 kDa band (not shown). Therefore, using two different approaches, we identified Necab to be predominantly a cytoplasmic protein.

Necab coexpresses with *Pax6* in the developing optic vesicles, pretectum and olfactory placodes

Using in situ hybridization we determined that *Necab* starts to be expressed at 8.5 dpc in the optic sulcus and in the pre-tectum (Fig. 2A). At 9.0-9.5 dpc, strong expression was observed in the optic vesicle and in the pre-tectum, with a striking resemblance to the *Pax6* expression pattern in these structures (Fig. 2B-E). *Necab* expression also spreads at the midline position in the roof of the midbrain, starting at 9.5 dpc (Fig. 2B,F; Fig. 3E), in contrast to *Pax6*. On examining sections of 9.5 dpc embryos, we observed that the *Necab* expression pattern in the optic vesicle mimicked the gradient expression pattern of *Pax6* in this structure. Both genes are expressed in the entire neural ectoderm, but with stronger expression in the laterodistal region of the optic vesicle (the future ciliary margin) (Fig. 2G,H). This is in contrast to *Rx* and *Six3*, both of which are highly expressed in the retinal plate (the future neural retina) (Fig. 2I; Fig. 4C (inset)) and to *Lhx2*, which is mainly expressed in the inner region of the optic vesicle (Fig. 2J). At 10.5 dpc, *Necab* expression is detected in the olfactory placodes, like *Pax6* (Fig. 3C,D). At 12.5-13.5 dpc, *Necab* is expressed in the retinal-pigmented epithelium (rpe) and in the neural retina, with strong expression in the ciliary margin (Fig. 2K; Fig. 3G). In the brain, the Necab gene is active in the pre-tectum, the epithalamus (not shown), where *Pax6* is also expressed, and in the dorsal thalamus, where *Pax6* is not expressed (compare Fig. 3E with 3F). In the neonatal eye, *Necab* expression becomes highly restricted to the iris and to the ciliary margin of the retina, with low levels of expression in the inner portion of the neural retina (Fig. 3I). When compared with *Pax6*, and *Lhx2* (not shown), we observed that all three genes were coexpressed in the ciliary margin of the retina (Fig. 3J,K). In the adult mouse, the ciliary margin of the retina, which is at the junction between the neural retina and the iris, has been shown, to contain a subpopulation of pigmented cells with stem cell properties (Tropepe et al., 2000). It is therefore possible that early retinal determinant genes are also involved in retina stem cells maintenance in the mature eye.

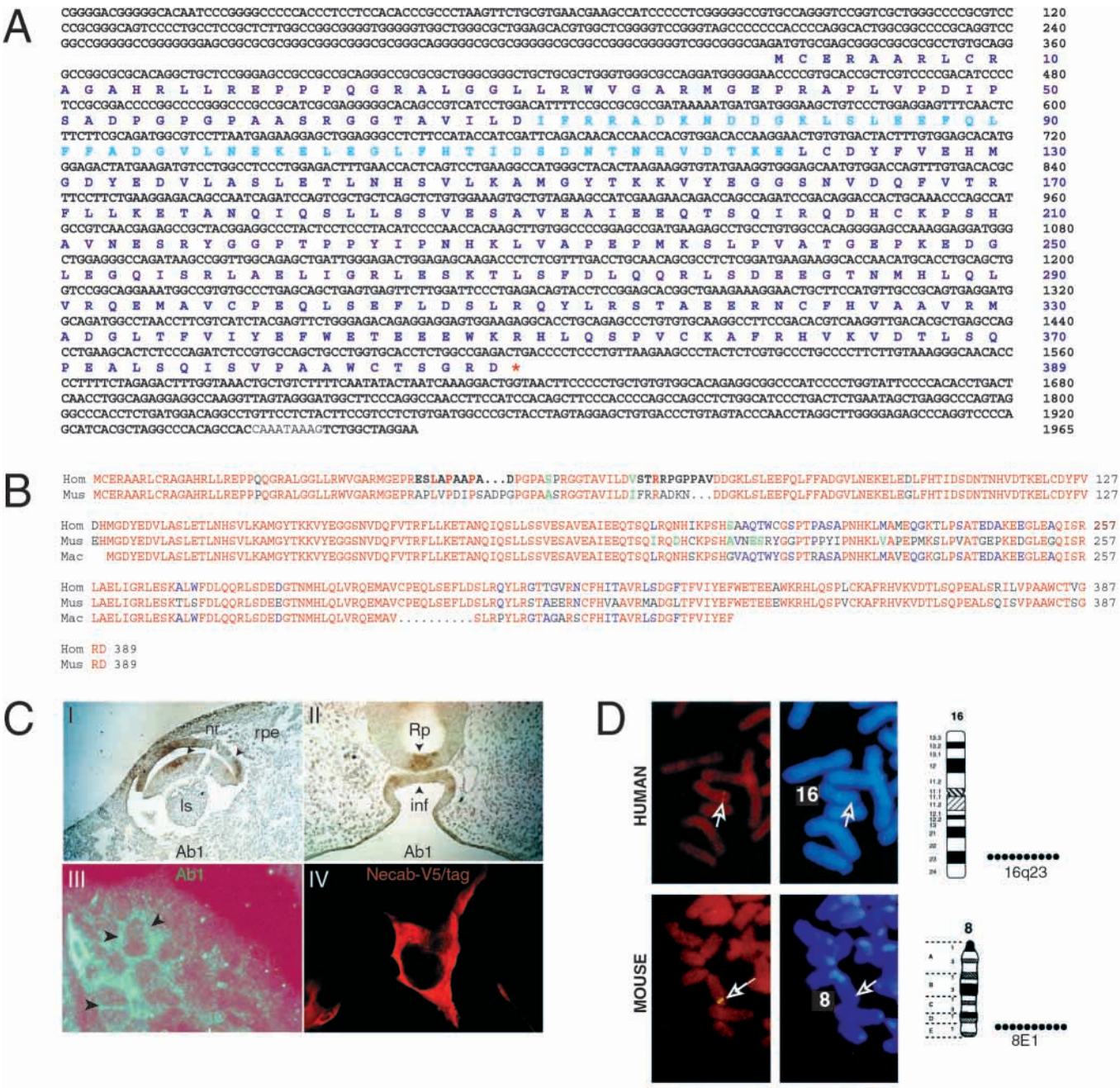

Fig. 1. *Necab* encodes a phylogenetically conserved mammalian protein. (A) Nucleotide sequence and amino acid translation of mouse *Necab* cDNA. *Necab* encodes a 389 amino acid protein (dark blue) with an EF-hand Ca^{2+}-binding domain (in pale blue). A polyadenylation signal is present at position 1944 to 1953 of the nucleotide sequence. (B) Alignment of mouse (*Mus musculus*; Accession Number AF411253), macacus (*Macaca fascicularis*; Accession Number AB046031) and human (*Homo sapiens*) Necab amino acid sequence. Red, amino acid identity in all shown sequences; blue, amino acid identity in two out of three species; green, amino acid similarity; black, divergence. The amino acid stretch in bold of the human sequence represents possible mistakes in the predicted translation product of *NECAB* cDNA. (C, part I) Immunohistochemistry with Ab1 antibody at 10.5 dpc (coronal sections) showing immunoreactivity in the retinal-pigmented epithelium (rpe) and in the neural retina (nr). No signal is detected in the in the lens (ls) or in the surrounding eye mesenchyme. (C, part II) Immunoreactivity of Ab1 in the Rathke's pouch (Rp) and in the infundibulum recess (inf). (C, part III) Immunofluorescence at 17.5 dpc (cross sections) with Ab1 antibody. Predominant immunoreactivity is found in the cytoplasm of the ganglion cells (arrowheads). (C, part IV) A Necab/v5 epitope fusion protein is localized to the cytoplasm in COS cells. (D) FISH on metaphase chromosomes. *NECAB* is localized to human chromosome 16q23 and *Necab* is localized to mouse chromosome 8E1.

Necab is downstream of *Pax6*

In *Pax6* mutant embryos, the optic vesicle initially forms (Fig. 4A) but does not progress to the optic cup stage (Grindley et al., 1995; Hogan et al., 1986). Although the optic vesicle is morphologically abnormal, it contains cells that express *Pax6*, as demonstrated using an anti β-galactosidase antibody in *Pax6*$^{lacz/lacz}$ embryos

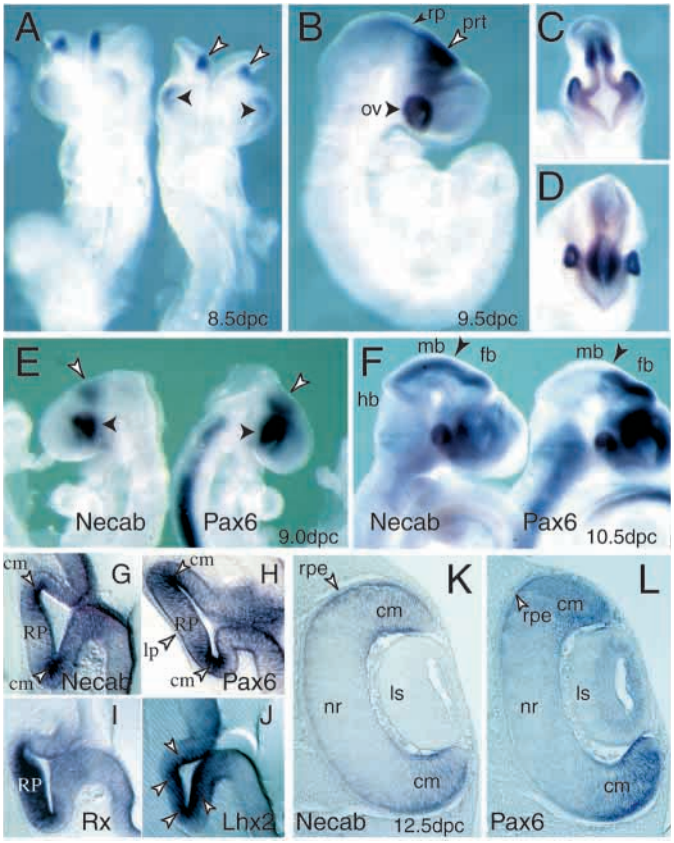

Fig. 2. *Necab* co-expresses with *Pax6* in the developing optic vesicles and in the pre-tectum. (A-D) Whole-mount in situ hybridization showing *Necab* expression in the optic sulcus, optic vesicles (black arrowhead) and pretectum (white arrowhead). (C,D) Frontal and top views of the embryo, respectively, showing the sharp expression domain of *Necab* in the pretectum. (E) *Necab* mimics *Pax6* expression pattern in the embryonic forebrain. (F) *Necab* expression is detected in the midbrain at 10.5 dpc, in contrast to *Pax6*. The arrowhead shows the forebrain/midbrain boundary. (G-J) *Necab* is expressed in a gradient manner in the optic vesicle (cross sections). Strong expression is observed in the presumptive ciliary margin region (G), similar to *Pax6* (H). By contrast, (I) *Rx* expression is predominant in the retinal plate (RP) and (J) *Lhx2* expression is predominant in the inner portion of the optic vesicle (arrowheads). (K) *Necab* expression is detected in the retinal-pigmented epithelium (rpe) and is predominant in the ciliary margin (cm) of the neural retina (nr), like *Pax6*. (L). fb, forebrain; hb, hindbrain; lp, lens placodes; ls, lens; mb, midbrain; ov, optic vesicle; prt, pre-tectum; rp, roof plate of the midbrain.

(Fig. 4A (inset); St-Onge et al., 1997). We tested for expression of *Otx2*, *Rx*, *Six3*, *Lhx2* and *Necab* in the *Pax6* mutant background at 9.5 dpc (Fig. 4B-F) by whole-mount in situ hybridization. Only *Necab* expression was affected in *Pax6* mutants (Fig. 4F). In the optic vesicles and in the pre-tectum, *Necab* expression was absent (*n*=6). However, expression in the midbrain was maintained. Thus, *Necab* expression in *Pax6* mutants is only affected where *Pax6* expression is normally found.

Pax6 can induce ectopic expression of *Necab*

Previous overexpression experiments in medaka and in *Xenopus* embryos have revealed that the midbrain region of vertebrates is competent to form retinal tissue when under the influence of Six3, Six6 (Optx2) or Pax6 transcription factors (Loosli et al., 1999; Chow et al., 1999; Bernier et al., 2000). Using DNA electroporation, we tested whether *Pax6* could induce ectopic expression of *Necab* in vivo in the midbrain region of 8.5 dpc mouse embryos. Embryos were electroporated, cultured for 6 hours, and processed for double in situ hybridization (Fig. 5A). Using a DIG-labelled *Necab* riboprobe (in blue), we detected endogenous expression in the optic vesicles and in the pre-tectum

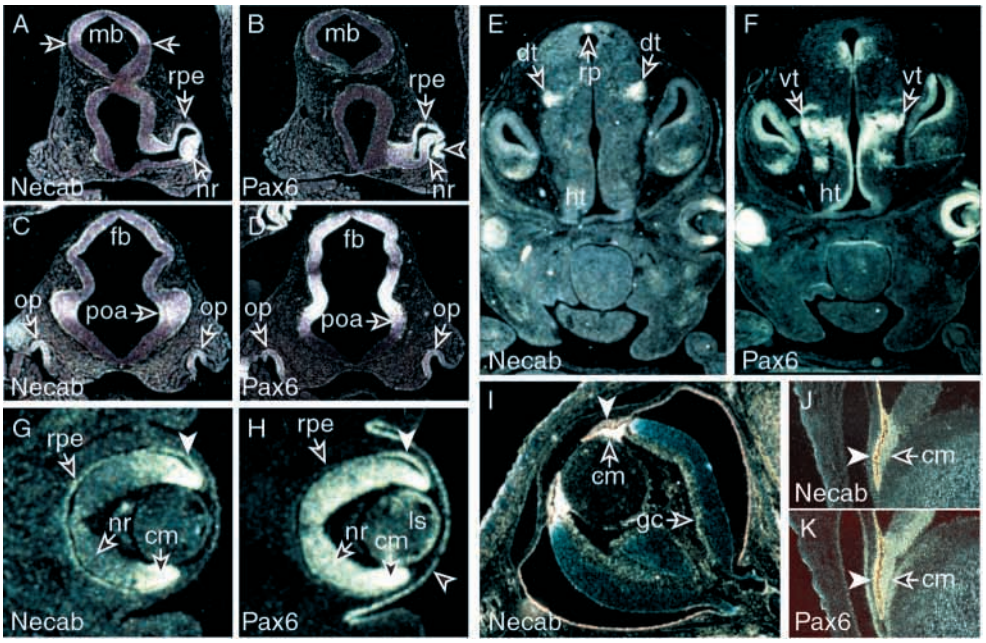

Fig. 3. *Necab* coexpresses with *Pax6* in the olfactory placodes, the iris and the ciliary margin of the retina. (A-D) Radioactive in situ hybridization at 10.5 dpc (coronal sections) showing co-expression of *Necab* and *Pax6* in the retinal-pigmented epithelium (rpe), the neural retina (nr), the olfactory placodes (op), the forebrain (fb) and the pre-optic area (poa). *Necab* is also expressed in the dorsal portion of the midbrain (A, arrows). Arrowhead in B indicates the lens pit. (E,F) *Necab* is expressed in the dorsal thalamus (dt), the roof plate of the midbrain (rp), and at low level in the hypothalamus (ht), (coronal sections, 13.5 dpc). (G,H) *Necab* and *Pax6* expression is detected in the distal portion of the retina (cm) and in the future iris (white arrowhead). The black arrowhead indicates the cornea. (I) *Necab* is expressed in the ciliary margin (cm) and iris (17.5 dpc), and at low level in the ganglion cells layer (gc). (J,K) *Necab* and *Pax6* are co-expressed in the ciliary margin and in the iris (white arrowhead) (17.5 dpc). ls, lens; vt, ventral thalamus.

(Fig. 5B). In addition, a few ectopic *Necab*-expressing cells were observed in the lateral portion of the midbrain. By contrast, the expression domain of the *Pax6* transgene (in red) was much broader, but colocalized with *Necab*-positive cells (Fig. 5C). To see whether this effect was specific to *Pax6* activity, embryos were electroporated with a *Six3* expression vector, cultured for 6 hours and tested for *Necab* expression. In all embryos tested (n=12), ectopic expression of *Necab* was not observed (Fig. 5G-I). To test whether the limited activation of *Necab* by *Pax6* was due to a restriction in cellular competence to express *Necab* or was simply dependent on time, we electroporated the *Pax6* construct in 8.5 dpc mouse midbrain explants and cultivated them for 24 hours. By in situ hybridization, we observed that most of the explants were expressing the transgene (n=22; Fig. 5D). After the second colour reaction, *Pax6*-positive cells revealed to be also positive for *Necab* expression (n=22; Fig. 5E). Control explants (n=8), cultured for 24 hours but not electroporated, were negative for both the transgene and for *Necab* expression (Fig. 5F). We concluded that *Pax6* could induce ectopic expression of *Necab* in vivo but that it requires at least 6 hours to induce this activity.

Necab overexpression can induce ectopic expression of *Chx10*

Although the biochemical function of *Necab* cannot be predicted at the moment solely based on its amino acid sequence except for its Ca^{2+} binding activity (T. C. Sudhof, personal communication), we tested using electroporation whether *Necab* would induce the ectopic expression of the homeobox gene *Chx10* (Liu et al., 1994). *Chx10* is the gene mutated in the ocular retardation (or) mouse mutant (Burmeister et al., 1996). Homozygous mutants are microphthalmic and have a thin retina with no optic nerve. We chose *Chx10* as putative target for *Necab* activity because its expression is first detected at 9.5 dpc in the neural ectoderm of the optic vesicle, thus about 1 day later than *Necab*. We microinjected and electroporated a *Necab* expression vector (see Materials and Methods) in the midbrain region of 8.5 dpc embryos and cultured them for a period of 6 hours. Using in situ hybridization, we observed ectopic expression of *Chx10* in the lateral region of the midbrain, where *Chx10* is not normally expressed and before endogenous *Chx10* expression in the retina (Fig. 5J). By revealing the transgene expression domain, we observed that ectopic *Chx10* expression was present in only a subset of transgenic cells (Fig. 5K). Other experiments using the same conditions revealed that *Necab* misexpression did not induce ectopic expression of *Pax6* (not shown). In addition, we did not observed *Chx10* activation by *Pax6* misexpression after 6 hours of embryos culture (not shown). *Chx10* activation was however observed when *Pax6* was electroporated in midbrain explants that were cultured for 24 hours (not shown). These results suggest that *Necab* might have a function in regulating gene expression during retina development.

DISCUSSION

Pax6 plays a central role in regulating eye development in mammals. However, few genes are known to be dependent on *Pax6* activity for their expression during eye development, impeding the elucidation of the genetic cascade. To this end, we have identified a novel gene expressed during early eye development. *Necab* is expressed in the optic sulcus and in the pretectum at 8.5 dpc, thus about 6 to 12 hours later than *Pax6*. In the neural ectoderm of the optic vesicle, *Necab* co-expressed with Pax6, both genes showing a unique expression pattern not observed with other retinal markers. In *Pax6* mutant embryos, *Necab* expression is lost in the optic vesicles and in the pretectum, indicating that *Necab* is downstream of *Pax6*. Using gain-of-function experiments, we have shown that *Pax6* is able to induce ectopic expression of *Necab*, further supporting the notion that *Necab* is within the *Pax6* genetic pathway. Finally, we have found that *Necab* misexpression can induce ectopic expression of *Chx10*, suggesting that it might be involved in regulating gene expression.

Necab is genetically downstream of *Pax6* in the retina

To our knowledge, *Necab* is the first described gene that coincides with *Pax6* expression in the developing optic vesicle and in the pretectum and is *Pax6* dependent for its expression. The importance of this discovery is highlighted by the observation that *Otx2*, *Rx*, *Lhx2* and *Six3* expression is not affected by the *Pax6* mutation. A previous report has shown that the expression of another homeobox gene expressed in the optic

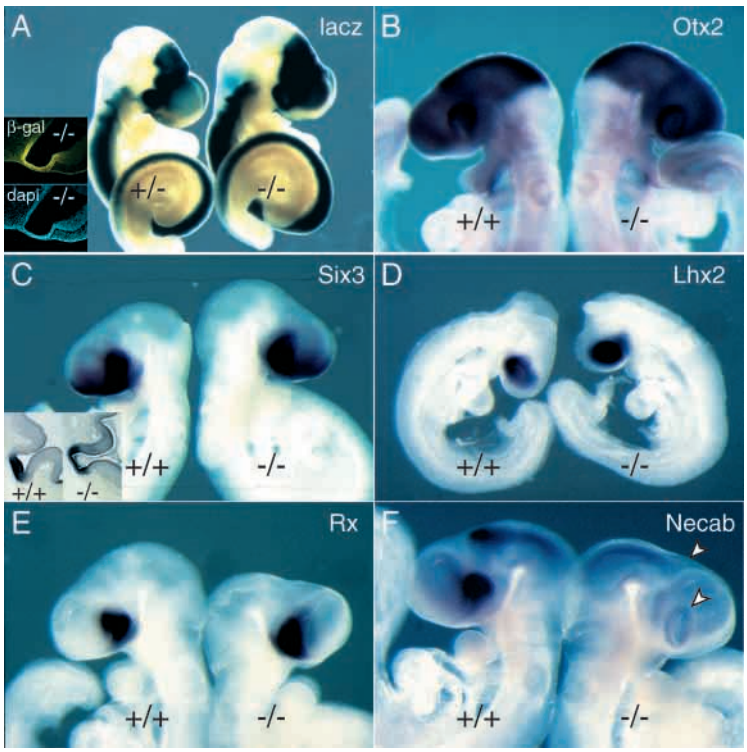

Fig. 4. *Necab* is downstream of *Pax6*. (A) X-gal staining showing *Pax6* expression in the spinal cord, forebrain and optic vesicles. Immunofluorescence showing β-galactosidase-positive cells in the optic vesicle of a *Pax6* mutant embryo (Inset). (B-F) In situ hybridization showing that, in contrast to *Otx2* (B), *Six3* (C), *Lhx2* (D) and *Rx* (E), *Necab* expression is absent in the optic vesicle and in the pretectum of *Pax6* mutant embryos (arrowhead, F). Wild type, +/+; heterozygous *Pax6* mutant, +/–; homozygous *Pax6* mutant, –/–.

primordium, *Six6* (*Optx2*), is also *Pax6* independent (Jean et al., 1999). The hypothesis that *Necab* is strictly dependent on *Pax6* for its expression in the optic vesicle is also strengthened by our result showing that *Six3* misexpression could not induce ectopic expression of *Necab* under the same experimental conditions, showing that *Six3* alone is not sufficient for *Necab* activation. However, Pax6 cannot be the only transcription factor that regulates *Necab* activity as *Necab* expression is also detected in body areas outside the *Pax6* expression domain, such as the midbrain and the trigeminal and spinal ganglia (not shown).

Necab can induce gene expression

We showed that ectopic expression of *Necab* is sufficient to induce the expression of *Chx10*. This result correlates well with the relatively earlier expression of *Necab* in the developing retina and suggests that *Necab* might regulate *Chx10* expression. However, *Necab* null mouse mutants are not microphthalmic (G. B. and P. G., unpublished) suggesting either functional redundancy with the other *Necab* family members or that *Necab* has a more subtle function during eye development. In addition, care should be taken in deciphering direct genetic interaction between genes while using overexpression experiments, as eye-specific genes form complex regulatory network, as demonstrated in the fly, the fish and the frog. Regardless of this, our results demonstrate that *Necab* misexpression can induce the transcription of a retina specific gene in vivo. How this is achieved at the molecular level is presently not known. Immunolocalization experiments revealed that Necab is mainly present in the cytoplasm. Interestingly, *Eya1*, *Eya2* and *Eya3* genes, the mouse orthologues of *eye absent* in *Drosophila*, also encode cytoplasmic proteins (Xu et al., 1997; Ohto et al., 1999). Co-transfection experiments revealed that Eya proteins could be translocated into the nucleus by direct interaction with Six2, Six4 and Six5 proteins (Ohto et al., 1999). Although Eya proteins are mainly cytoplasmic and do not have a DNA-binding domain, overexpression of *eya* alone in *Drosophila* has been shown to induce ectopic eye formation (Bonini et al., 1997). It is therefore possible that a similar mechanism applies for *Necab*, if it could act as a transcriptional co-activator. However, many other options exist that do not require nuclear translocation of Necab in order to induce gene transcription. For example, the cytoplasmic Ca^{2+}-sensitive protein calcineurin can regulate gene transcription by inducing the nuclear translocation of NF-AT transcription complex via its phosphatase activity; see review by Crabtree (Crabtree, 2001). Similarly, RNA injections in *Xenopus* embryos of the Wnt transmembrane receptor frizzled (Xfz3) resulted in ectopic gene expression and in the formation of ectopic eyes, showing that signal transduction pathways can regulate gene expression during eye development (Rasmussen et al., 2001).

Necab is part of a novel gene family

Necab is part of a novel gene family that is conserved in mammals. The gene family is composed of three members, all containing a putative EF-hand Ca^{2+}-binding domain. This Ca^{2+}-binding domain appears to be functional (T. C. Sudhof, personal communication). Although *Necab* expression was restricted to the nervous system and some epithelial derivatives (e.g. the

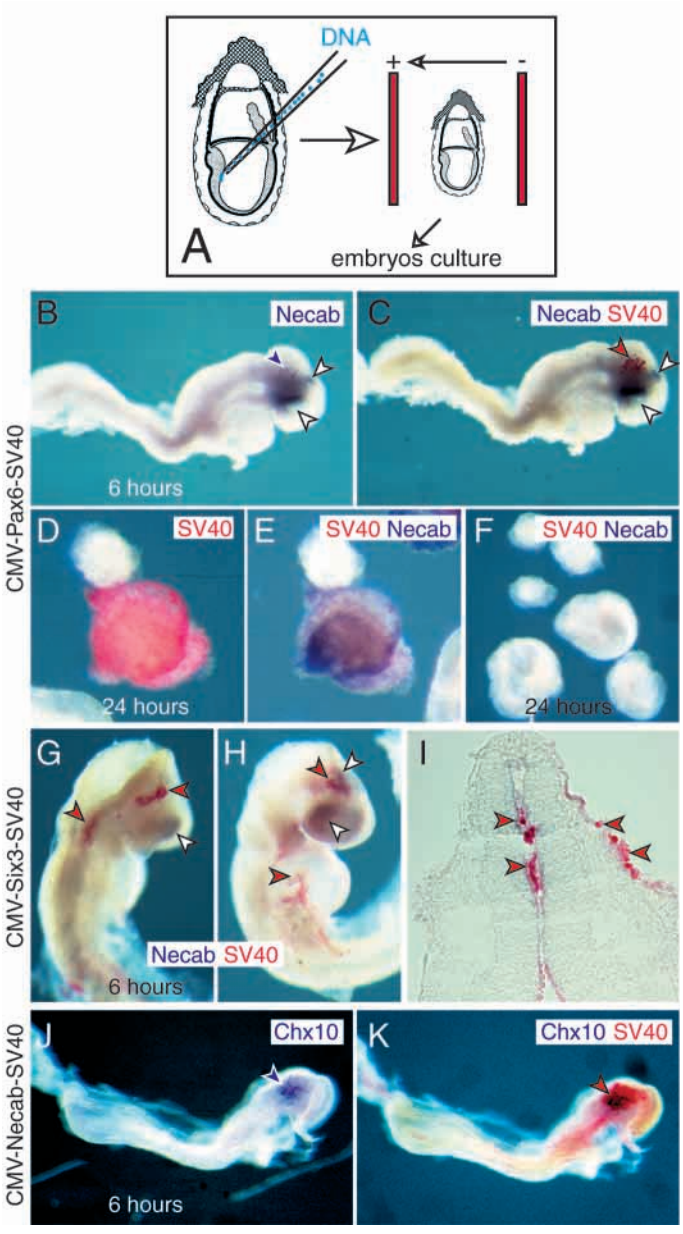

Fig. 5. *Pax6* can induce ectopic expression of *Necab*. (A) Schematized view of the electroporation procedure. Mouse embryos are microinjected with a needle containing the DNA solution (in blue) and electroporated. (B,C) Double whole-mount in situ hybridization (8.5 dpc), 6 hours after electroporation with a *Pax6* expression vector. (B) Ectopic expression of *Necab* is detected in a subset of cells (blue arrowhead). Endogenous expression is detected in the pretectum and optic vesicle (white arrowheads). (C) The expression domain of the *Pax6* transgene (red arrowhead) is broader but overlaps ectopic *Necab*-positive cells. (D-F) Double in situ hybridization (8.5 dpc) on midbrain explants. (D,E) Twenty four hours after electroporation with a *Pax6* expression vector, cells expressing the transgene (in red) are also expressing *Necab* (in blue). (E) Control explants (no electroporation) cultured for 24 hours and hybridized with both probes. **(**G,H**)** Double in situ hybridization (8.5 dpc), 6 hours after electroporation with a *Six3* expression vector. Transgene expression is present (red arrowhead) but ectopic *Necab*-expressing cells are not detected. (I) Cross section at the hindbrain level of the embryo in G, showing that the transgenic cells do not expressed *Necab*. (J,K) Six hours after the electroporation of a *Necab* expression vector, ectopic expression of *Chx10* is detected in the midbrain region (J) where transgenic cells are present (K). Red arrowhead, transgene expression; blue arrowhead, ectopic expression of the target gene; white arrowhead, endogenous expression.

olfactory placodes) by in situ hybridization, we also observed strong expression in the dermomyotome with our polyclonal antibodies, suggesting crossreactivity with the other Necab family members. It is thus likely that one of the *Necab* genes is involved in early myogenesis. Characterization of the expression pattern of the other members should highlight possible functional redundancies of this gene family during development.

In conclusion, we have reported on the isolation of a novel gene expressed in the mouse optic vesicle using a conventional large-scale in situ screen. This gene is a component of the genetic cascade governed by *Pax6* during eye formation. In the near future, the combination of whole genome projects and of DNA chip microarray analysis should lead to the identification of the complete set of genes regulated by *Pax6* during eye morphogenesis. However, functional characterization of each of these genes will be required in order to decipher the molecular mechanisms of *Pax6* activity in the developing eye.

We thank Michael Kessel and Hendrik Knötgen for useful discussions, Roderick McInnes and Thomas Theil for providing cDNA, Petros Petrou and Martin Wagner for technical help. Clones 9066 and 9075 were isolated in project 01KW9603/9 funded by the German Human Genome Project from the German Ministry of Education and Research. This work was supported by the Max-Planck-Society and by EU Contracts B104-CT96-0378 QLG3-2000-00158. W. V. is a Starke-Werner PhD fellowship recipient and G. B. is an EMBO fellowship recipient.

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