Regulation of Prox 1 during Lens Regeneration

Katia Del Rio-Tsonis,[1] *Stanislav I. Tomarev,*[2] *and Panagiotis A. Tsonis*[1]

Purpose. To determine the expression pattern of Prox 1 during the process of lens regeneration in the urodele *Notophthalmus viridescens*.

Methods. Polymerase chain reaction was performed to amplify a partial newt *Prox 1* sequence. In situ hybridization and immunodetection methods were used to detect the *Prox 1* mRNA and the Prox 1 protein, respectively.

Results. *Prox 1* mRNA was present in the retina and in the lens (in the epithelium and bow region) of the intact eye. Prox 1 protein was found to be predominantly present in the lens and dorsal iris of the intact eye, although some trace levels of Prox 1 protein were detected in the ventral iris as well. After lentectomy, expression of the mRNA was also pronounced in the dorsal dedifferentiating iris and the regenerating lens. The ventral iris also expressed Prox 1 but seemingly at lower levels. Although Prox 1 protein showed upregulation in the dorsal iris during the process of lens regeneration, trace levels were also detected in the ventral iris. In the retina, Prox 1 protein was distributed in horizontal cells of the inner nuclear layer, whereas the mRNA was expressed in all layers of the retina.

Conclusions. Prox 1 was unevenly distributed in the intact cells of the newt iris, with significantly higher levels of Prox 1 protein present in the dorsal versus the ventral margin. This protein was differentially regulated during the process of lens regeneration, with obvious upregulation in the dorsal iris. Prox 1 is the first transcriptional factor to be shown to be regulated in the dorsal versus ventral iris during the process of lens regeneration. (*Invest Ophthalmol Vis Sci.* 1999;40: 2039–2045)

One of the most intriguing cellular events is the transdifferentiation of one terminally differentiated cell type to another. Lens regeneration in adult urodele amphibians occurs through such an event. After lentectomy, the pigmented epithelial cells at the dorsal margin of the iris (but not the ventral) undergo a reprogramming that directs them to proliferate, dedifferentiate, and finally, transdifferentiate into lens cells, producing a lens vesicle. This vesicle continues to grow, and the cells of the vesicle begin to differentiate into lens epithelium and lens fibers. Once the lens epithelial cells are established, they continue with their fated process of differentiating to lens fibers and thus give rise to the new lens.[1–3] Although this phenomenon is unique only to some urodeles,[4] the process can be mimicked in vitro by using pigment epithelial cells from dorsal or even ventral iris from a wide variety of species.[5] This implies that the potential to transdifferentiate is intrinsic to the pigment epithelial cells. That in vivo transdifferentiation can only occur from the dorsal pigmented epithelial cells of some urodeles is indicative of some sort of spatial regulation. This regulation must involve key molecules that control the fate of cell lineages, growth, and differentiation. One group of molecules that has been shown to be involved in processes such as the ones mentioned include homeobox-containing genes.

Homeobox-containing genes have been implicated as playing an important role during the process of eye development.[6] In fact, one of these genes, *Pax-6*, which, in addition to containing a homeobox also contains a paired-box domain, has been declared the eye-determining gene.[7] *Pax-6* mutations cause eyeless phenotype in *Drosophila*, small eye in mouse, and aniridia in humans.[8–10] In addition, ectopic expression of the *Drosophila*, mouse, and squid genes in *Drosophila* cells destined to become legs or antennae, can lead to the formation of a complete eye in those structures,[7,11] and ectopic expression of *Pax-6* in *Xenopus* embryos can create ectopic lenses.[12] Another homeobox-containing gene that has been able to create ectopic lenses is *Six-3*, the vertebrate homologue of *Drosophila* sine oculis.[13] There are other homeobox-containing genes that have been shown to be required for the development of the visual system. Mutations in genes such as *Rx* and *Lhx-2* and disruption of *Msx-1* and *Msx-2* interrupt or even halt the normal development of the eye.[14–16]

Consequently, we have initiated studies to examine the role of *Hox* genes during the process of lens regeneration. We have previously reported that *Pax-6* was expressed during the process of lens regeneration.[17] Recently, we have reported the expression patterns of six homeobox-containing genes during the process of lens regeneration in the urodele amphibian *Notophthalmus viridescens.*[18] The common pattern of expression appeared to be a general downregulation of these genes in the retina of the eyes undergoing lens regeneration with no apparent regulation in the dorsal–ventral iris.

From the [1]Laboratory of Molecular Biology, Department of Biology, University of Dayton, Ohio; and the [2]Laboratory of Molecular and Developmental Biology, National Eye Institute, National Institutes of Health, Bethesda, Maryland.

Supported by National Institutes of Health Grant EY10540.

Submitted for publication July 14, 1998; revised March 3, 1999; accepted April 8, 1999.

Proprietary interest category: N.

Reprint requests: Katia Del Rio–Tsonis, Laboratory of Molecular Biology, Department of Biology, University of Dayton, Ohio Dayton, OH 45469-2320.

```
TTTTTTAATGCCATGAATTCCCAAGGTTCTGAGCAGGATGTTGAGTATTCCGTGGTGCAGCATGCAGATGGGGAGAAGTCAAACGTGCTACGCAAGCTGC 100
F  F  N  A  M  N  S  Q  G  S  E  Q  D  V  E  Y  S  V  V  Q  H  A  D  G  E  K  S  N  V  L  R  K  L  L 34

TGAAGAGGGCAAACTCGTATGAAGATGCCATGATGCCTTTTCCAGGGGCAACCATAATTTCCCAGCTGTTGAAAAATAACATGAACAAAAATGGTGGCAA 200
   K  R  A  N  S  Y  E  D  A  M  M  P  F  P  G  A  T  I  I  S  Q  L  L  K  N  N  M  N  K  N  G  G  N  67

TGAGCCCAGTTTCCAGGCCAGTGGTCTCTCCAGTACGGGCTCGGAAATCCACCAGGAGGACATGTGCAGCAACTCTTCGAGAGATAGTCCCCAAGAGTGT 300
 E  P  S  F  Q  A  S  G  L  S  S  T  G  S  E  I  H  Q  E  D  M  C  S  N  S  S  R  D  S  P  Q  E  C   100

CTTTCTCCTTTTGGCAGACCCCCAATGAGCCAGTTTGATATGGAACGGTTGTGTGATGAGCACCTGAGAGCAAAGCGTGCCCGTGTTGAGAACATCATCC 400
L  S  P  F  G  R  P  P  M  S  Q  F  D  M  E  R  L  C  D  E  H  L  R  A  K  R  A  R  V  E  N  I  I  R 134

GAGGGATGAGCCATTCTCCGAATGTGTCAATGAGGGGCAATGAAAATGAGAGAGAGGGAGCCCCGCAGTCCATAAGTCCCCGAGAAAGTTACAGAGAAAA 500
   G  M  S  H  S  P  N  V  S  M  R  G  N  E  N  E  R  E  G  A  P  Q  S  I  S  P  R  E  S  Y  R  E  N  167

CAAACGCAAGCAAAAGCTGCCTCAACAGCAGCAGCAGAGTTTCCAGCAGCTGGTTTCAGCTAGAAAAGAACAGAAGCGCGAGGAGCGCAGACAGCTGAAA 600
 K  R  K  Q  K  L  P  Q  Q  Q  Q  Q  S  F  Q  Q  L  V  S  A  R  K  E  Q  K  R  E  E  R  R  Q  L  K   200

CAGCAGCTGGAGGACATGCAGAAACAGCTGCGCCAGCTGCAGGAGAAGTTTTACCAAATCTATGACAGCACCGATTCTGAAAATGATGGTAACCTGTCCG 700
Q  Q  L  E  D  M  Q  K  Q  L  R  Q  L  Q  E  K  F  Y  Q  I  Y  D  S  T  D  S  E  N  D  G  N  L  S  E 234

AAGACAGCATGCATTCCGAAATCATGGATGGCAGAGCAGCGGACTCGGTTGGGAGGTCAGATCACGAATTGTTTGATTTAGACCCCGGACAGTTCATTGA 800
   D  S  M  H  S  E  I  M  D  G  R  A  A  D  S  V  G  R  S  D  H  E  L  F  D  L  D  P  G  Q  F  I  D  267

CCGGGCACGGGCCCTGATTAGGGAACAGGAAATAGCTGCTGAGAATAAGCCTAAAAGAGAAGGTCACAGAGAAAAAGACCATGGGCACAACTCTTGCCAT 900
 R  A  R  A  L  I  R  E  Q  E  I  A  A  E  N  K  P  K  R  E  G  H  R  E  K  D  H  G  H  N  S  C  H   300

CCAGAAGGCAAGCATTTAGCCGAAACACTAAAACAAGAGCTAAACACTGCCATGTCACAAGTCGTGGACACAGTGGTTAAGGTTTTTTCATCCAAACCCT 1000
P  E  G  K  H  L  A  E  T  L  K  Q  E  L  N  T  A  M  S  Q  V  V  D  T  V  V  K  V  F  S  S  K  P  S 334

CCCGCCATCTTCCTCAGGACTTCCCACCTCTCCAGATCCCCCAAGCCAGATTTGCAGTGAATGG                                    1064
   R  H  L  P  Q  D  F  P  P  L  Q  I  P  Q  A  R  F  A  V  N  G                                     355
```

FIGURE 1. Newt Prox 1 partial sequence (accession number, AF070733).

Investigators in the field of lens regeneration have not identified a dorsal-specific transcriptional factor that could be paramount for grasping the molecular mechanisms of the process of transdifferentiation and lens regeneration. In the present study, we show that Prox 1 protein (homologue of *Drosophila prospero*)[19–21] is detected primarily in the dorsal epithelial cells of the iris, becoming the first transcriptional factor to show a specific regulation in the intact dorsal iris cells and during the process of lens regeneration.

MATERIALS AND METHODS

Animals, Operations, and Tissue Collection

Adult newts (*Notophthalmus viridescens*) were purchased from Mike Tolley, Nashville, Tennessee. Lentectomy was performed in animals under anesthesia, and their eyes were collected at different stages of regeneration. The collection occurred at 10, 15, 20, and 25 days. The eyes were embedded in paraffin, sectioned, and processed for in situ hybridization. Some of the tissues were frozen in optimum cutting temperature compound (Tissue-Tek, Torrance, CA) sectioned, and processed for immunohistochemistry, or the tissues were collected to make protein extracts for further western blot analyses. Experiments were conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Cloning of the Newt Prox 1

Newt genomic DNA (1.25 μg) was used in each of a series of polymerase chain reactions (PCRs) that amplified overlapping regions of newt Prox 1. The primers used included: The 5′-oligo 1: 5′-CAAACCAAGAGAAGAAG(A/G)GTTGA(C/T)AT-3′, which corresponds to positions 13 to 21 in the human Prox 1 amino acid sequence,[22] and the 3′-oligo 1: 5′-GGAATGATGACATCACC(A/G)AA(A/G)CA(T/C)TG-3′, which corresponds to positions 410 to 418 in the human Prox 1 amino acid sequence. PCR was performed with *Taq* polymerase (Perkin Elmer, Norwalk, CT) using a thermal cycler (PTC-200; MJ Research, Watertown, MA) with the following conditions: 94°C for 1 minute 30 seconds; then 94°C for 40 seconds, 55°C for 1 minute 15 seconds, and 72°C for 1 minute 15 seconds (31 times). Final annealing was for 5 minutes at 72°C. Fragments were separated by agarose gel electrophoresis, and a weak band was cut out and purified using a kit (Geneclean II; Bio 101, San Diego, CA). This fragment was reamplified using two combinations of oligos: 1) 5′-oligo 2: 5′-AAACGGGCCCGGGT(T/G)GA(G/A)AA(T/C)AT-3′, which corresponds to positions 165 to 172 in the human aa sequence) and 3′-oligo 1. This combination produced fragment 1. 2) 5′-Oligo 1 was the same as in the first PCR reaction, and 3′-oligo 2 was 5′-TGGCGCAGCTGCTTCTGCAT(G/A)TC(C/T)TC-3′, which corresponds to positions 244 to 252 in the human aa sequence. This combination produced fragment 2. Conditions for PCR were: 94°C for 1 minute 30 seconds; then 94°C for 40 seconds, 55°C for 45 seconds, and 72°C for 1 minutes 30 seconds (25 times). Final annealing was at 72°C for 5 minutes. The fragments were separated by agarose gel electrophoresis, purified as before, and sequenced with fluorescent dideoxynucleotides on an automated sequencer (model 310; Applied Biosystems, Foster City, CA).

Probes

Riboprobes were prepared using the newt cDNA sequence shown in Figure 1. Primers were designed so that in one set the 3′ half of the primer corresponded to regions of the newt Prox 1 (underlined sequence), whereas the 5′ half included the sequence for the T3 phage promoter (bold sequence). The sense oligo pair included 5′-oligo #3: 5′-GGAGCA**ATTAACCCTCACTAAAGGG***GAATGTGTCAATGA*-3′ 3′-oligo #2: 5′-*TGGCGCAGCTGCTTCTGCAT(G/A)TC(C/T)TC*-3′. The antisense oligo pair included 5′-oligo #2: 5′-*AAACGGGCCCGGGT(T/*

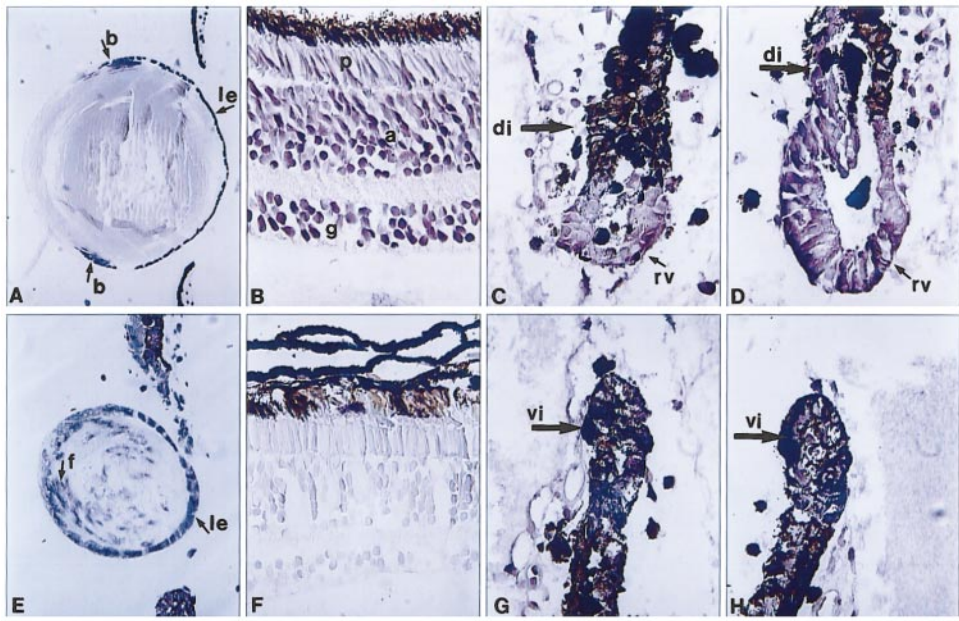

FIGURE 2. In situ hybridization of Prox 1 in newt lens regeneration. Expression in the intact eye (**A**, **B**, **F**). Expression during the process of lens regeneration (**C**, **D**, **E**, **G**, **H**). (**A**) Prox 1 mRNA was present in the lens epithelium (le) of the intact lens and in the cells of the bow region (b). (**B**) Prox 1 was expressed in the retina of the intact newt eye in all cell layers: ganglion (g), amacrine (a), and photoreceptor (p). (**C**) Expression 10 days after lentectomy. Prox 1 mRNA was expressed in the dorsal iris (di) and regenerating vesicle (rv). (**D**) At 15 days after lentectomy, Prox 1 was strongly expressed in the regenerating vesicle (rv) and the dorsal iris (di). (**E**) At 20 days, Prox 1 was expressed in the lens epithelium (le) and the differentiating fibers (f). (**F**) Negative control with the sense probe in a retina from an intact eye. (**G**, **H**) Expression of Prox 1 in the ventral iris (vi) of the newt eye 10 and 15 days after lentectomy, respectively.

G)GA(G/A)AA(T/C)AT-3′ and 3′-oligo #3: 5′-GGAGCA**ATTA-ACCCTCACTAAAGGG***CTAGCTGAAACCAG*-3′. Amplifications were performed for 42 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute 20 seconds, with a final cycle of 72°C for 5 minutes. The amplified fragments were separated through a 1.2% agarose gel and then eluted, using the squeeze-and-freeze method.[23] The purified fragments were then subjected to riboprobe labeling using the dioxigenin (DIG) labeling kit (Boehringer Mannheim, Indianapolis, IN) and T3 RNA polymerase.

In Situ Hybridization

Slides containing paraffin sections were deparaffinized in xylene and subsequently hydrated through an ethanol series. The slides were rinsed in 1× PBS and then fixed in 4% paraformaldehyde for 15 minutes. After a rinse with 1× PBS, the slides were incubated with 250 μg/ml pepsin at 37°C for 15 minutes. Subsequently, the slides were rinsed again with 1× PBS and then treated with 0.1 M triethanolamine/0.25% acetic anhydride. The slides were then washed one last time with 1× PBS and dehydrated using ethanol series. After 1 hour of air drying, the sections were hybridized at 55°C to 60°C for 16 hours with hybridization solution (50% formamide, 1 mM EDTA, 10 mM Tris-HCl [pH 7.5]), 600 mM NaCl, 0.25% sodium dodecyl sulfate [SDS], 10% polyethylene glycol 6000, 1× Denhardt's, 200 μg/ml tRNA, and 1 μg/ml DIG-labeled probes). The next day, the slides were washed with 4× SSC followed by a treatment with 50 μg/ml RNase at 37°C for 1 hour. Subsequently, the slides were incubated in 2× SSC at 55°C to 60°C two times for 30 minutes each time, and then in 0.1× SSC at 55°C to 60°C two times for 30 minutes each. For immunologic detection, the slides were rinsed in buffer 1 (0.1 M Tris-HCl [pH 7.5], 0.15 M NaCl) and then incubated in buffer 2 (buffer 1 with 10% horse serum) for 1 hour at room temperature. The sections were then incubated with anti-DIG antibody–alkaline phosphatase conjugate in buffer 2 (horse serum at 1%) at 1:1500 for 2 hours at room temperature. After three washings with buffer 1 for 15 minutes each, the slides were incubated in buffer 3 (0.1 M Tris-HCl [pH 9.5], 0.1 M NaCl, 50 mM $MgCl_2$) for 10 minutes and later incubated in the same solution plus nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt) for 16 to 24 hours. The reaction was stopped with 10 mM tris pH 8.0, 1 mM EDTA, and the sections were mounted with crystal mount (Biomeda Corp., Foster City, CA). Pictures were produced with a video printer (Sony, Tokyo, Japan).

Immunostaining

Polyclonal rabbit antibodies against the homeodomain and C-terminal domain (aa 546–736) of the human Prox 1 were used.[24] The specificity of the antibody for newt eye tissues was determined by western blot analysis and showed primarily the same 90-kDa band that chick and mouse lens have shown in the past[24] (see the Results section). OCT-embedded tissues were sectioned and incubated in methanol at −20°C for 10 minutes. Then the slides were rinsed with 1× PBS three times. The sections were then blocked with 10% normal goat serum for 20 minutes. The primary antibody was used at a dilution of

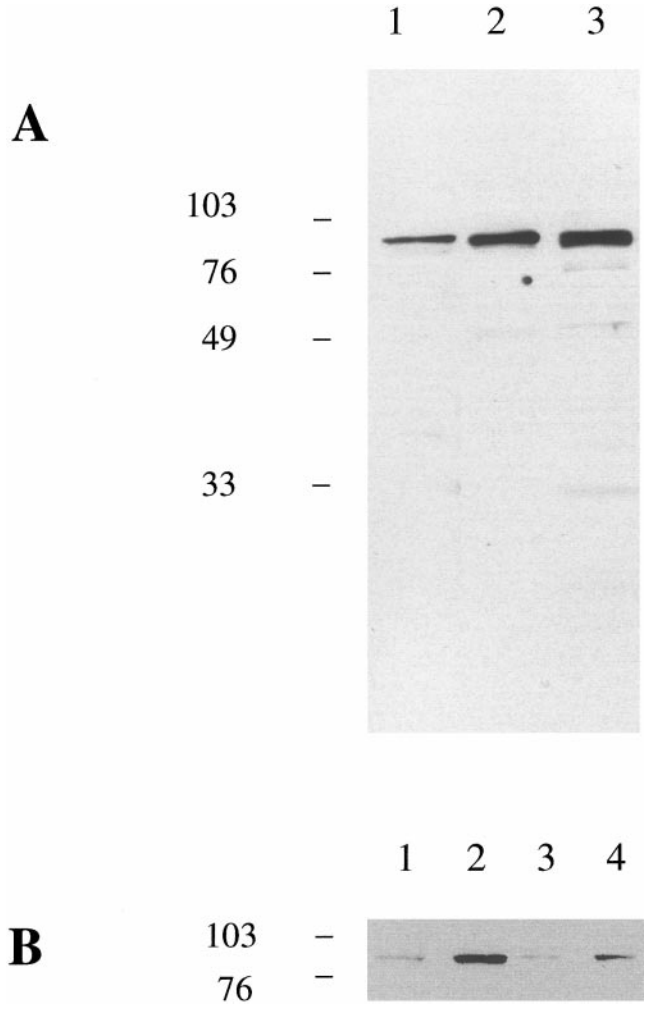

FIGURE 3. Western blot analysis using Prox 1 antibodies. (**A**) *Lane 1*, total mouse eye extract (20 μg); *lane 2*, embryonic chick nuclear extract (8 μg); and *lane 3*, extract from newt lenses (20 μg). The correct 90-kDa band was recognized in all three species. (**B**) *Lane 1*, extracts from isolated ventral iris of the regenerating eye; *lane 2*, extracts from isolated dorsal iris of the regenerating eye; *lane 3*, extracts from the ventral iris of the intact eye; *lane 4*, extracts from the dorsal iris of the intact eye. Thirty micrograms of protein extracts were loaded in each lane.

1:200 overnight at 4°C. After three washings with 1× PBS, the sections were then treated with anti-rabbit IgG conjugated with fluorescein isothiocyanate (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at room temperature. After three washings with 1× PBS, the slides were mounted with glycerol and observed by fluorescence microscopy. Negative controls consisted of samples that were not incubated with the primary antibody but were processed similarly otherwise.

Western Blot Analysis

Dorsal and ventral irises from lentectomized eyes 14 days after operation and from intact eyes were carefully dissected, and protein extracts were prepared by homogenizing the samples in extraction buffer (50 mM Tris [pH 8.0], 1 mM EDTA, 5 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 100 μg/ml phenylmethylsulfonyl fluoride [PMSF], 1% Triton X-100, 0.1% SDS) for 15 minutes at 4°C. The extracts were then centrifuged for 10 minutes and supernatants used to determine protein concentration using a protein assay kit (Bio-Rad, Hercules, CA). The chick nuclear extract was obtained from Dr. Ales Cvekl (Albert Einstein College of Medicine, Bronx, NY) and was prepared according to Shapiro et al.[25] Equivalent amounts of protein extracts were loaded per lane in a 12% acrylamide gel. Samples were blotted onto nitrocellulose and blocked in 5% dry milk in TBST (10 mM Tris [pH 8.0]), 150 mM NaCl, 0.05% Tween-20) overnight, and then incubated in TBST with 1% dry milk and 1:2000 dilution of Prox 1 antibodies for the blot shown in Figure 3A and 1:4000 for the blot in Figure 3B. Horseradish peroxidase anti-rabbit IgG (Amersham, Arlington Heights, IL) was used at a 1:2000 dilution in TBST. The signal was detected using the SuperSignal Kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

RESULTS

Cloning of the Newt Prox 1

A partial DNA sequence was obtained after using overlapping sets of PCRs. The sequence was obtained using degenerate primers that flanked part of the first coding exon of the human Prox 1 (Fig. 1). This sequence is approximately 86% homologous at the nucleotide level with chicken and human Prox 1.

Expression of Prox 1 during Lens Regeneration

After removal of the lens, the dorsal pigmented epithelial cells from the iris begin to proliferate and to dedifferentiate by loosing their pigment. Such events are most pronounced in the pupillary region of the dorsal iris and lead to the enlargement of the cytoplasm and the appearance of extensive intercellular spaces. As a result, the dorsal iris is significantly uncompacted when compared with the one in the intact eye. The ventral iris undergoes some of these changes but not as extensively as the dorsal iris, and it does not contribute to lens regeneration.[1–3] By 10 days the cells of the dorsal iris have completely depigmented and have formed a dedifferentiated lens vesicle. At 14 to 16 days after lentectomy, these cells begin to transdifferentiate into lens epithelial and lens fiber cells. At approximately 25 days after lentectomy, this vesicle has produced a complete lens with a definite lens epithelial layer covering a mass of lens fibers.[1–3] To study the possible role(s) that Prox 1 may play during the process of lens regeneration in the adult newt, we performed in situ hybridization and immunodetection studies.

In situ hybridization studies showed that Prox 1 was expressed in the intact newt eye and throughout the process of lens regeneration. *Prox 1* transcript was found in the intact eye, in the lens epithelial cells, and in the bow region of the lens (Fig. 2A). It was also found in all layers of the retina (Fig. 2B). As was mentioned earlier, the pupillary region of the intact iris is too compacted to allow clear detection of Prox 1 transcript. After lentectomy, however, depigmentation and other cellular alterations allowed detection. *Prox 1* mRNA was predominantly expressed in the dorsal iris epithelial cells that dedifferentiate and transdifferentiate into lens cells. At 10 days after lentectomy, the vesicle of dedifferentiated cells expressed Prox 1 throughout (Fig. 2C), and this expression pattern continued up to 15 days after lentectomy (Fig. 2D). At 20 days, most of the cells expressing Prox 1 were lens epithelial cells and the newly differentiating fibers (Fig. 2E). *Prox 1* also was

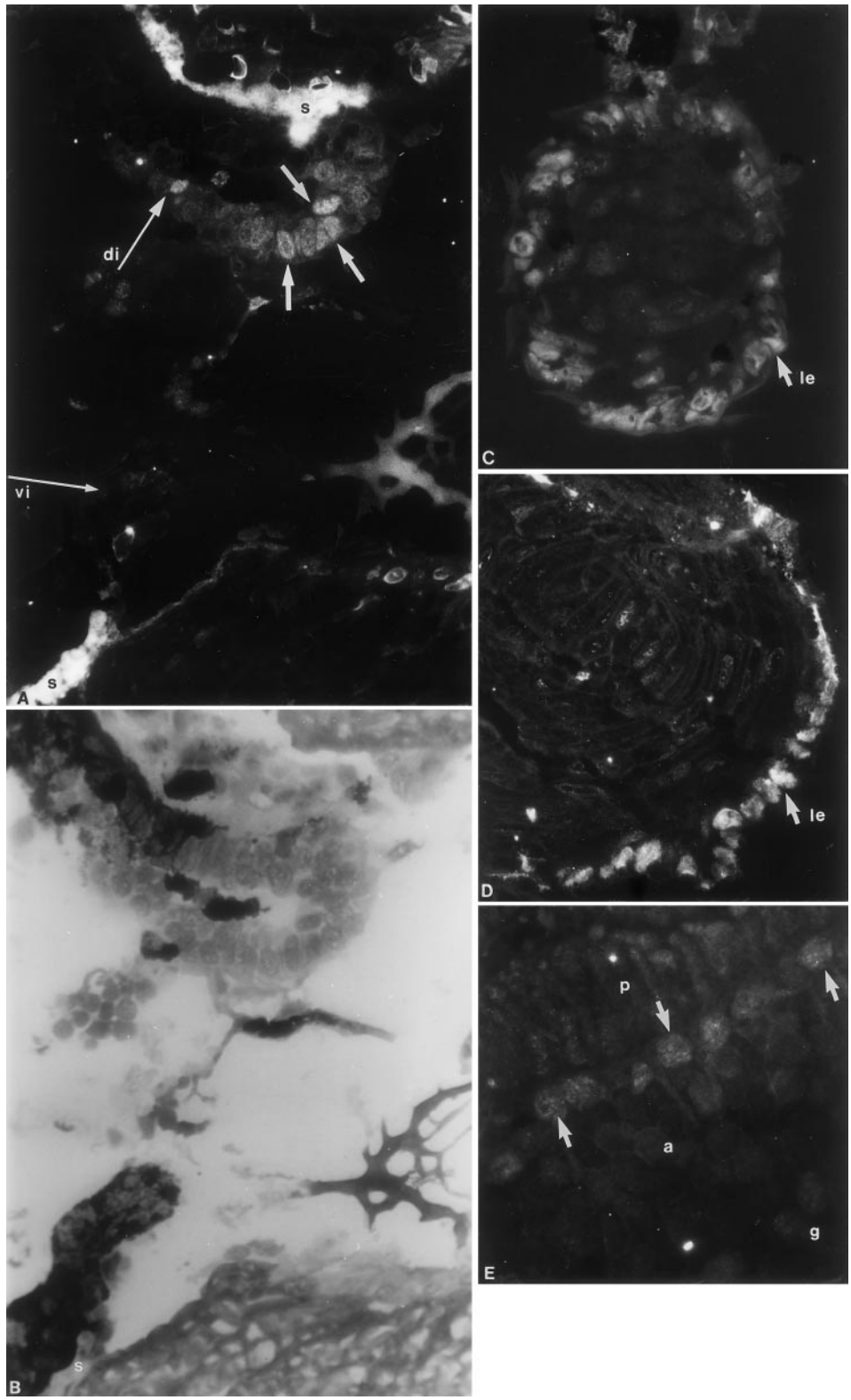

FIGURE 4. Immunohistochemical detection of Prox 1 protein in newt lens regeneration. (**A**) A section through the dorsal (di) and ventral (vi) iris of a newt eye 10 days after lentectomy. Presence of Prox 1 protein is shown in the dedifferentiating dorsal iris. Note the characteristic nuclear staining in cells (*short arrows*); the ventral iris (vi) was completely negative. Stroma (s) always autofluoresces. (**B**) A bright-field image of (**A**) to illustrate the structures of the eye. (**C**, **D**) Expression of Prox 1 protein in a section through a regenerating lens 17 and 20 days after lentectomy, respectively. Note the high expression mainly in the lens epithelium (le). (**E**) Presence of Prox 1 protein was mainly in the horizontal cells (*arrows*) in the retina of the newt eye. p, photoreceptor layer; a, amacrine layer; g, ganglion layer.

expressed in the cells of the ventral iris (Fig. 2G, 2H). Although these experiments were not quantitative and the ventral iris is more pigmented, expression in the ventral iris seemed to be at lower levels than in the dorsal iris. The retina expressed Prox 1 throughout the process of lens regeneration with the same pattern as in the intact eye (not shown).

The possibility that there could be a gradient of *Prox 1* along the dorsal–ventral axis prompted us to investigate the distribution of Prox 1 protein in the newt eye undergoing lens regeneration, by using antibodies against Prox 1 that specifically recognize the newt Prox 1 protein (Fig. 3A). We initially detected Prox 1 protein by use of immunohistochemistry.

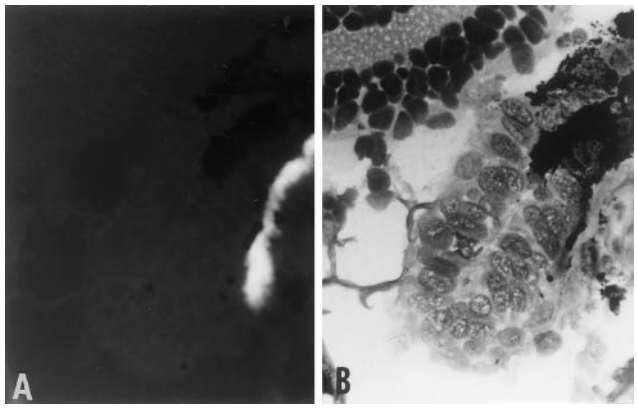

FIGURE 5. Negative control for immunofluorescence. (**A**) A section through a newt eye 10 days after lentectomy. There was no apparent positive signal in the regenerating vesicle. (**B**) Bright field of A.

Again, the pupillary region of the intact dorsal and ventral iris was compacted and, as in the case of mRNA, it hindered any possible detection of the Prox 1 protein. However, we circumvented this problem by using western blot analysis with isolated intact dorsal and ventral irises (see later description). During the process of lens regeneration, Prox 1 protein was readily detected by immunostaining, during dedifferentiation, vesicle formation, and transdifferentiation. In fact, it was exclusively localized in the dorsal iris where the events of lens regeneration take place (Figs. 4A through 4D). At 10 days after lentectomy, the Prox 1 protein was only detected in the dedifferentiated lens vesicle of the dorsal iris but not in the ventral iris (Figs. 4A, 4B). At later stages of regeneration (17 and 20 days after lentectomy), Prox 1 was primarily present in the lens epithelial cells of the regenerating lens vesicle (Figs. 4C, 4D). No protein was detected in the ventral iris at any stage. In the neural retina of the intact and regenerating eye, the horizontal cells of the inner nuclear layer (Fig. 4E) and a few scattered cells in the amacrine layer (not shown) were the ones showing the presence of Prox 1 product.

To determine whether Prox 1 protein was present in the intact iris, which could not be achieved with immunohistochemical studies, we isolated intact dorsal and ventral irises, and their protein extracts were examined by western blot analysis. Prox 1 protein was detected in the dorsal iris of the intact eye and in the dorsal iris undergoing lens regeneration. Prox 1 protein was found predominantly in the dorsal iris, even though trace levels were detected in the ventral iris as well. The levels of Prox 1 protein in the dorsal iris were higher during regeneration, indicating upregulation (Fig. 3B).

DISCUSSION

Our results showed regulation of *Prox 1* mRNA and protein during the process of lens regeneration. Prox 1 protein seems to be confined preferentially in the dorsal iris cells that are capable of transdifferentiation during lens regeneration. Immunohistochemistry experiments showed preferential presence in the dorsal pupillary iris during the process of lens regeneration. Supplemental experiments using western blot analysis with isolated irises confirmed that the Prox 1 protein is highly expressed in the dorsal iris, but these experiments also showed trace amounts of the protein in the ventral iris. It should be remembered, however, that isolation of such a small area from the eye is tedious, and some contamination cannot be ruled out. We believe that in situ methods using sections are more informative when it comes to detection of a protein in tissues composed of few cells, such as the tip of the iris. Of course, the possibility exists that the ventral iris produced some Prox 1 protein at low levels, which could be detected with more sensitive methods, such as western blot analysis. Whatever the case may be, our data show conclusively that Prox 1 protein is present in the newt iris (intact or undergoing lens regeneration) as a gradient with much higher levels in the dorsal than in the ventral iris.

The observation of the unique regulation of Prox 1 protein during the process of lens regeneration represents the first identification of a transcriptional factor that is regulated during the process of lens regeneration along the dorsal–ventral axis. To date, only extracellular or cell surface proteins have been shown to be regulated in the dorsal iris, with fibroblast growth factor receptors being the only known molecules with such regulation.[26–32] Possible scenarios for such unique distribution and regulation of molecules during lens regeneration may include differential splicing, differential regulation of transcription or translation, or differential interactions with other proteins.

Interestingly, differential protein distribution of Prox 1 has been observed in other systems. In mouse *rd3* mutants the Prox 1 protein shows differential distribution in the lens and retina when compared with the wild-type mouse. In the retina, whereas the wild-type mouse expresses Prox 1 product in the horizontal cells of the inner nuclear layer, the *rd3* mutant failed to show any presence of the protein in the neural retina. Similarly, the distribution in the lens differs between mutant and wild-type mice. In the mutant, Prox 1 protein is present in the more mature fibers.[33]

The fact that Prox 1 was regulated in the dorsal–ventral iris (both intact and regenerating) suggests that this molecule plays an important role in the process of lens regeneration. It has been shown that Prox 1 is present in chick developing lens cells undergoing differentiation, and it has been suggested that Prox 1 can regulate crystallin expression.[19,34] Therefore, Prox 1 in the dorsal dedifferentiated iris and subsequent regenerating lens could control events that lead to lens-specific regulation and differentiation.

Finally, a note on the expression of Prox 1 in the retina: Preferential localization of Prox 1 protein in the newt retina horizontal cells is similar to its distribution in the developing chicken retina.[24] However, in the chicken, *Prox 1* mRNA is also preferentially located on the horizontal cells,[19,24] whereas in the newt, *Prox 1* mRNA was found in all retina layers. This suggests some sort of posttranscriptional control of Prox 1 expression in the newt retina. In addition, this is the first homeobox-containing gene that has not shown a regulation of the mRNA in the retina during the process of lens regeneration.[18]

The information obtained in this study reiterates the importance of regulatory genes in the process of lens regeneration and pinpoints Prox 1 as one of the key transcriptional factors involved in the process of transdifferentiation and subsequent differentiation of lens cells.

Acknowledgment

The authors thank Mike Trombley for technical assistance.

References

1. Eguchi G. Electron microscopic studies on lens regeneration, I: mechanism of depigmentation of the iris. *Embryologia.* 1963;8: 45–62.
2. Reyer RW. The amphibian eye: development and regeneration. In: Crescitelli F, ed. *Handbook of Sensory Physiology*. Vol. 7. Berlin: Springer–Verlag; 1977;309–390.
3. Yamada T. Control mechanisms in cell-type conversion in newt lens regeneration. In: Wolsky A, ed. *Monogr Dev Biol*. Basel: 1977;13:1–119.
4. Stone LS. An investigation recording all salamanders which can and cannot regenerate a lens from the dorsal iris. *J Exp Zool* 1967;164: 87–104.
5. Eguchi G, Kodama R. Transdifferentiation. *Curr Opin Cell Biol.* 1993;5:1023–1028.
6. Beebe DC. Homeobox genes and vertebrate eye development. *Invest Ophthalmol Vis Sci.* 1994;35:2897–2900.
7. Halder G, Callaerts P, Gehring WJ. Induction of ectopic eyes by targeted expression of the eyeless gene in Drosophila. *Science.* 1995;267:1788–1792.
8. Quiring R, Walldorf U, Kloter U, Gehring WJ. Homology of the eyeless gene of Drosophila to the small eye in mice and Aniridia in Humans. *Science.* 1994;265:785–789.
9. Ton CCT, Hirvonen H, Miwa H, et al. Positional cloning and characterization of a paired box- and homeobox-containing gene from the aniridia region. *Cell.* 1991;67:1059–1074.
10. Hill RE, Favor J, Hogan BLM, et al. Mouse small eye results from a paired-like homeobox-containing gene. *Nature.* 1991;354:522–525.
11. Tomarev SI, Callaerts P, Kos L, et al. Squid Pax-6 and eye development. *Proc Natl Acad Sci USA.* 1997;94:2421–2426.
12. Altmann CR, Chow RL, Lang RA, Hemmati–Brivanlou A. Lens induction by Pax-6 in *Xenopus laevis. Dev Biol* 1997;185**:**119–123.
13. Oliver G, Loosli F, Koster R, Wittbrodt J, Gruss P. Ectopic lens induction in fish in response to the murine homeobox gene Six-3. *Mech Develop.* 1996;60:233–239.
14. Porter FD, Drago J, Xu Y, et al. Lhx2, a LIM homeobox gene, is required for eye, forebrain, and definitive erythrocyte development. *Development.* 1997;124:2935–2944.
15. Mathers PH, Grinberg A, Mahon KA, Jamrich M. The Rx homeobox gene is essential for vertebrate eye development. *Nature.* 1997; 387:603–607.
16. Foerst–Potts L, Sadler TW. Disruption of Msx-1 and Msx-2 reveals roles for these genes in craniofacial, eye, and axial development. *Dev Dyn.* 1997;209:70–84.
17. Del Rio–Tsonis K, Washabaugh CH, Tsonis PA. Expression of pax-6 during urodele eye development and lens regeneration. *Proc Natl Acad Sci USA.* 1995;92:5092–5096.
18. Jung JC, Del Rio–Tsonis K, Tsonis PA. Regulation of homeobox-containing genes during lens regeneration. *Exp Eye Res.* 1998;66: 361–370.
19. Tomarev SI, Sundin O, Banerjee–Basu S, Duncan MK, Yang JM, Piatigorsky J. Chicken homeobox gene Prox 1 related to Drosophila prospero is expressed in the developing lens and retina. *Dev Dyn.* 1996;206:354–377.
20. Oliver G, Sosa–Pineda B, Geisendorf S, Spana EP, Doe CQ, Gruss P. Prox, a prospero-related homeobox gene expressed during mouse development. *Mech Develop.* 1993;44:3–16.
21. Doe CQ, Chu–LaGraff Q, Wright DM, Scott MP. The *prospero* gene specified cell fates in the *Drosophila* central nervous system. *Cell.* 1991;65:451–464.
22. Zinovieva RD, Duncan MK, Johnson TR, Torres R, Polymeropoulos MH, Tomarev SI. Structure and chromosomal localization of the human homeobox gene Prox 1. *Genomics.* 1996;35: 517–522.
23. Sambrook J, Fritsch EF, Maniatis T. In: Ford N, Nolan C, Ferguson M, eds. *Molecular Cloning: A Laboratory Manual* Cold Spring Harbor, NY; Cold Spring Laboratory Press; 1989.
24. Belecky–Adams T, Tomarev SI, Li HS, et al. Prox 1, Pax-6 and Chx10 homeobox gene expression correlate with phenotypic fate of retinal precursor cells. *Invest Ophthalmol Vis Sci.* 1997;38: 1293–1303.
25. Shapiro DJ, Sharp PA, Wahli WW, Keller MJ. A high-efficiency HeLa cell nuclear transcription extract. *DNA.* 1988;7:47–55.
26. Zalik SE, Scott V. Sequential disappearance of cell surface components during dedifferentiation in lens regeneration. *Nature.* 1973; 244:212–214.
27. Tsonis PA, Del Rio–Tsonis K. Protein separation techniques in the study of tissue regeneration. *J Chromatogr A.* 1995;698: 361–367.
28. Ortiz JR, Vigny M, Courtois Y, Jeanny JC. Immunohistochemical study of extracellular components during lens and neural retina regeneration in adult newt. *Exp Eye Res.* 1992;54:861–870.
29. Tsonis PA, Doane K, Del Rio–Tsonis K. Expression of integrins during axolotl limb regeneration. *Dev Growth Differ.* 1997;39:9–14.
30. Eguchi G. Cellular and molecular background of Wolffian lens regeneration. In: Eguchi G, Saxen, L, Okada L, eds. *Regulatory Mechanisms in Developmental Processes.* Dublin: Elsevier; 1988: 147–158.
31. Del Rio–Tsonis K, Trombley MT, McMahon G, Tsonis PA. Regulation of lens regeneration by fibroblast growth factor receptor 1. *Dev Dyn.* 1998;213:140–146.
32. McDevitt DS, Brahma SK, Courtois Y, Jeanny JC. *Dev Dyn.* 1997; 208:220–226.
33. Tomarev SI, Zinovieva RD, Chang B, Hawes NL. Characterization of the mouse Prox1 gene. *Biochem Biophys Res Comnum.* 1998; 248:684–689.
34. Duncan MK, Tomarev SI, Cvekl A, Piatigorsky J. The developmental regulation of Beta B-crystallin (abstract). *Exp Eye Res.* 1996; 63(Suppl. 1):S57.