Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 5092–5096, May 1995
Developmental Biology

Expression of *pax-6* during urodele eye development and lens regeneration

KATIA DEL RIO-TSONIS*, CHARLES H. WASHABAUGH*, AND PANAGIOTIS A. TSONIS

Laboratory of Molecular Biology, Department of Biology, University of Dayton, Dayton, OH 45469-2320

Communicated by Susumu Ohno, Beckman Research Institute of the City of Hope, Duarte, CA, February 21, 1995

ABSTRACT **Regeneration of eye tissues, such as lens, seen in some urodeles involves dedifferentiation of the dorsal pigmented epithelium and subsequent differentiation to lens cells. Such spatial regulation implies possible action of genes known to be specific for particular cell lineages and/or axis. *Hox* genes have been the best examples of genes for such actions. We have, therefore, investigated the possibility that such genes are expressed during lens regeneration in the newt. The *pax-6* gene (a gene that contains a homeobox and a paired box) has been implicated in the development of the eye and lens determination in various species ranging from *Drosophila* to human and, because of these properties, could be instrumental in the regeneration of the urodele eye tissues as well. We present data showing that *pax-6* transcripts are present in the developing and the regenerating eye tissues. Furthermore, expression in eye tissues, such as in retina, declines when a urodele not capable of lens regeneration (axolotl) surpasses the embryonic stages. Such a decline is not seen in adult newts capable of lens regeneration. This might indicate a vital role of *pax-6* in newt lens regeneration.**

Some urodele amphibians are the gifted animals capable of regenerating the lens throughout their life following lentectomy (1). Except for two reports of positive lens regeneration in freshwater fish and in avian embryos, no other species has been catalogued as capable of regeneration (see ref. 1). The regenerative ability in those two species, however, is restricted to a very specific and limited time during development. On the other hand, regeneration of lens has been reported in adult rabbit after removal of the lens and only after implantation of cytolysing fetal tissue (2). Among urodeles the ability is not universal. The axolotl, for example, a salamander with good regenerative abilities of the limb and tail, is not able to regenerate the lens. Such restrictions pose interesting questions as to why this selection exists.

Once the lens is removed the process of regeneration is initiated by dedifferentiation of the dorsal iris pigment epithelium (3–5). About 6 days later, transdifferentiation of the iris into lens cells begins. The regenerating lens starts as a budding process of the dorsal iris cells. Formation of the lens vesicle by the depigmented progenies of the iris cells is evident between 9 and 15 days. Between 12 and 15 days after lentectomy the internal layer of the lens vesicle thickens and synthesis of β- and γ-crystallins starts. Following that period lens fibers are produced in the internal layer of the vesicle and β-crystallin appears in the external layer. By day 20 the fiber complex grows further and α-crystallin accumulates in the lens fibers and in the external layer. By day 25 we have definite lens tissue and dividing cells are observed only in the lens epithelium. The ventral iris does not contribute to this phenomenon (5). Work with the developing lens in frogs and in the newt *Notophthalmus viridescens* has shown that synthesis of crystallins parallels the steps seen during lens regeneration. In this sense similar events might take place during development and regeneration of the lens, even though inductive interactions such as the ones seen during lens development (surface ectoderm with optic vesicle) are not necessary for lens regeneration (6, 7).

The dedifferentiation of the iris cells and their subsequent transdifferentiation to lens cells is a switch from one cell type to another, likely to be controlled by genes responsible for differentiation of cell lineages, such as homeobox-containing genes. The homeobox was first identified in segmentation genes and subsequently in other developmental genes in *Drosophila* (8, 9). These genes define borders and axes in the developing body where certain regions of particular cell lineages will develop. The homeobox is a DNA binding domain, similar in sequence and in structure to the helix–turn–helix-containing bacterial repressors (10). During the past 10 years the presence and roles of homeobox-containing genes (*Hox* genes) in vertebrate development have been studied. Their presence is always correlated with active processes of differentiation and pattern formation (11). One of the *Drosophila* segmentation genes, the paired, contains a homeobox and also contains another domain called the paired domain (12). Genes with paired boxes have also been isolated from vertebrates and grouped in the Pax family by virtue of sequence similarity to the paired box domain of *Drosophila* (13). The paired domain has also DNA binding activity and has been implicated in developmental processes (14).

Hox and *Pax* genes have already been implicated in vertebrate eye development. In mouse, *Hox-7.1* (*Msx-1*) is expressed after formation of the optic cup marking the presumptive ciliary body; *Hox-8.1* (*Msx-2*) is expressed in regions corresponding to the future corneal epithelium and neural retina (15). Other studies implicating *Hox* genes in eye development have shown that the δ-crystallin enhancer binding protein contains a homoeodomain (16). Homeobox genes have been also detected in the retina of adult goldfish. Among these homeoboxes, one homologous to the paired homeobox was also found (17). The *pax-2* gene is expressed during development of the mouse eye and its expression is restricted to the ventral optic cup and stalk (18). The *pax-6* gene is expressed in the developing mouse eye, including the lens (19). Furthermore, the mutation aniridia in humans and the equivalent small eye of the mouse are induced by a deletion in a gene containing a paired domain and a homeodomain (*pax-6*) (20, 21). Most interesting, however, is the fact that the same gene (*pax-6*) is involved in the development of the eye in *Drosophila* in spite of the different morphology and mode of development (22). The mouse mutation microphthalmia is associated with mutations in a different type of nuclear transcriptional factor containing a helix–loop–helix–zipper motif (23). These studies provide convincing evidence of the regulatory role of such genes during eye development.

The hypothesis is that a unique regulatory event, after lentectomy in amphibia, results in regulation of molecules necessary for dedifferentiation and the initiation of lens differentiation. DNA binding proteins could be involved in

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

*K.D.R.-T. and C.H.W. contributed equally to this work.

such a case. And since *Hox* and *Pax* genes are determinants of cell lineages, their role in this event becomes very possible. In fact, *pax-6* has been directly implicated in lens determination in chicken, where it is first expressed in a region of the future head ectoderm close to the anterior margin of the early neural plate, an area where lens induction happens (24). As a first step we have examined the expression of *pax-6* during urodele eye development and lens regeneration. The role of *pax-6* in eye determination renders it the best candidate as a starting point. In this paper we report expression of a *Pax* gene in the developing and regenerating urodele eye. Such results open a new avenue in the investigation of *Hox* and *Pax* genes during such phenomena as transdifferentiation and regeneration.

MATERIALS AND METHODS

Animals. Two urodeles were used in the present study. Adult newts (*N. viridescens*) were purchased from Amphibia of North America (Nashville, TN). Axolotls (*Ambystoma mexicanum*) were provided as embryos or larvae by the axolotl colony, Indiana University, Bloomington. Newt embryos are not readily available and this is a deterrent when developmental studies are concerned. The use of axolotls solves this problem and also provides an additional benefit of comparative studies, because the axolotl is not able to regenerate the lens.

Design of Pax Oligonucleotide Primers. The use of degenerate oligonucleotides to amplify *pax* sequences has not yet been applied or found in the literature. Therefore, we proceeded in designing our own primers. Taking into consideration the sequences from the *Drosophila* paired, the human aniridia, and the mouse *pax-1*, *-2*, *-7*, and *-8* genes, we identified two very conserved regions in all of the genes (21). Region 1 encodes GCVSKIL, and region 2 encodes WEIRDR. The degenerate primers for these two regions are as follows: no. 1, GGNTGYGTN(A,T)(G,C)(T,C,A)AARAT(T,C,A)CT (sense), and no. 2, C(T,G)RTCNC(T,G)DATYTCCCA (antisense). R is A or G, Y is C or T, and D is A, G, or T. The use of nos. 1 and 2 amplifies a fragment of about 160 nucleotides (170 when primers have sites). These Pax primers can successfully amplify the expected size fragment when mouse *pax-6* sequence was used as template (not shown).

Reverse Transcription–Polymerase Chain Reaction. RNA was isolated from whole eye tissues. The eyes were homogenized in 4 M guanidine thiocyanate and layered over CsCl. After centrifugation at 36,000 rpm the pellet was extracted, precipitated with ethanol, and then washed extensively with 70% ethanol. The total RNA (at least 2 μg is needed) was reverse transcribed in a reaction containing oligo(dT) primer, dNTPs (0.5 mM), and 100 units of reverse transcriptase. The Red Module kit from Invitrogen was used. The reaction was carried out at 42°C for 60 min. For subsequent amplification, usually one-half of the reverse transcription reaction (10 μl) was used. For PCR, a fraction of the DNA was used along with *Taq* polymerase (2.5 units/100-μl reaction), 200 μM dNTPs, and 4 μM primers. Temperature cycling was as follows. For the initial denaturation, 95°C for 5 min, followed by the following cycles: 95°C for 1 min, 37–42°C for 1–2 min, 72°C for 1–2 min for 35 cycles, and a final extension for 5 min at 72°C. After the amplification was over, the samples were run on an agarose gel and analyzed for the particular band of the expected size.

Cloning. The amplified DNA fragments of the expected size were cloned into the TA cloning vector pCRII (Invitrogen). Ligation was performed with 3–5 Weiss units of T4 ligase in the presence of $MgCl_2$ and ATP at 12–16°C for 16 hr. The plasmid with the ligated insert was transfected into competent (50 mM $CaCl_2$) *Escherichia coli* (JM109 or DH5). Bacterial cells were heat-shocked (42°C for 30–45 sec) and allowed to recover for 30–60 min at 37°C in LB broth before spreading on LB plates with ampicillin (100 μg/ml), isopropyl β-D-thiogalactoside (100 mM), and 5-bromo-4-chloro-3-indolyl β-D-galactoside (2%). Plasmids were isolated and restricted to identify the correct size of the insert.

Sequence Analysis of the Inserts. We used the commercially available pCRII vector from Invitrogen (TA cloning) and the Sequenase kit from United States Biochemical employing the method of Sanger *et al.* (25) and following the manufacturer's directions. The plasmid DNA was denatured (0.2 M NaOH/0.2 mM EDTA), neutralized by 0.5 M ammonium acetate (pH 5.4), and ethanol precipitated. One microgram of denatured DNA was annealed by incubation in 100 mM Tris/10 mM $MgCl_2$/10 mM dithiothreitol (DTT)/2 mM EDTA, pH 7.5, in the presence of 25–50 ng of primer. The universal primers provided for the TA cloning vector were employed. Twenty-five picomoles of [α-[^{35}S]thio]dATP and 5 units of Klenow polymerase were added to the annealing reaction above and the mixture was equally divided among four tubes. Each tube containing the appropriate deoxy/dideoxy mix (G, A, T, and C) was incubated for 20 min at 37°C followed by addition of dNTP chase mix and further incubation for 15 min at room temperature.

***In Situ* Hybridization.** For these experiments, the tissues were isolated, frozen in OCT medium, and processed for *in situ* hybridization as described (26, 27). Briefly, sections were mounted on baked Depc-washed slides treated with silane and kept at −70°C until ready to use. The slides were brought to room temperature and fixed in fresh 4% paraformaldehyde for 20 min. The slides were then rinsed in 1× phosphate-buffered saline and immersed in triethanolamine (TEA) buffer for 5 min and in TEA buffer plus 0.25% acetic anhydride for another 10 min. After rinsing the slides in 2× standard saline citrate (SSC) for 10 min, the slides were dehydrated to 100% ethanol through a graded series and then air dried for 1 hr. Riboprobes (antisense and sense) were made using either T7 or SP6 RNA polymerase and [α-[^{35}S]thio]UTP. After purification of the probe through Stratagene's Nuctrap columns, 1×10^7 cpm/ml was added to hybridization buffer that contained 1.2 M NaCl, 20 mM Tris·HCl (pH 7.5), 4 mM EDTA, 2× Denhardt's solution, 20% dextran sulfate, 40% formamide, 1 mg of yeast tRNA per ml, and 50 mM DTT. About 200 μl of the hybridization solution was added on each slide, which were then placed in a humidified environment at 50–55°C overnight. The slides were then washed in 2× SSC for 15 min and then placed at the corresponding hybridization temperature in a solution (solution 1) containing 50% formamide, 1× SSC, and 0.1% β-mercaptoethanol for 30 min. The slides were then transferred to a solution containing 0.5 M NaCl, 10 mM Tris·HCl (pH 8.0), and 20 μg of RNase A per ml for 30 min at 37°C. Subsequently, the slides were washed again at the corresponding hybridization temperature in solution 1 for another 30 min and then the slides were incubated in another solution (solution 2) containing 1× SSC and 0.1% β-mercaptoethanol for 30 min at 37°C. Finally, the slides were dehydrated through a graded series of ethanol and then let air dried for about 1 hr. Autoradiography was performed using Kodak NTB-2 emulsion and developed following instructions of the manufacturer. The slides were

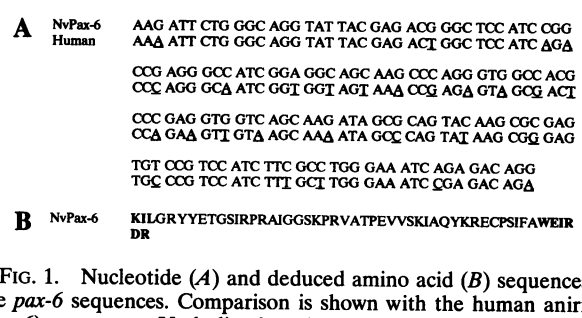

FIG. 1. Nucleotide (*A*) and deduced amino acid (*B*) sequences of the *pax-6* sequences. Comparison is shown with the human aniridia (*pax-6*) sequences. Underlined nucleotides are different among the two species. Degenerate primers were made from amino acids presented in bold-face type.

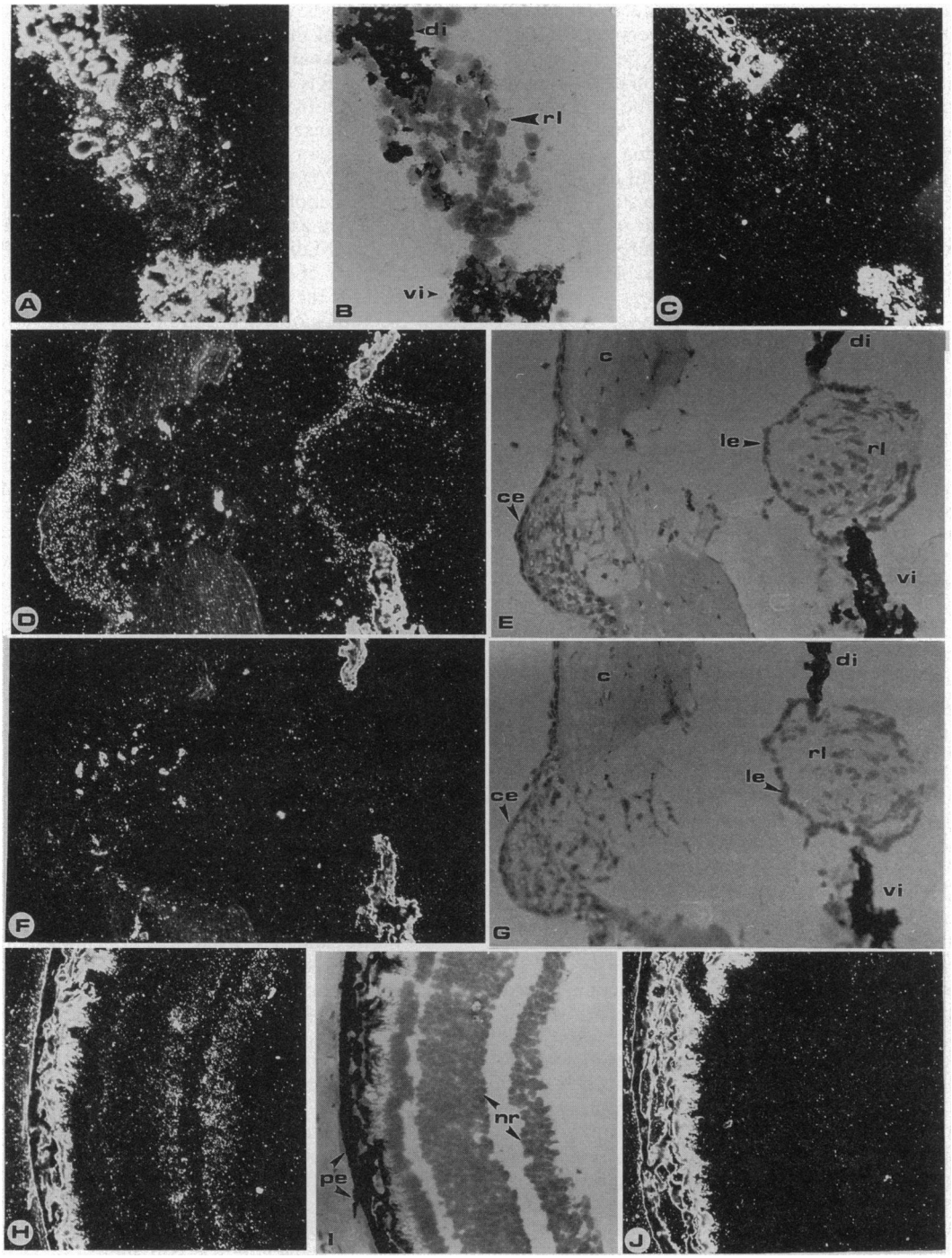

FIG. 2. *In situ* hybridization using the newt *pax-6* riboprobe, labeled with ^{35}S. (*A–C*) Sections through the dorsal–ventral iris 15 days after lentectomy. (*D–G*) Sections through the dorsal–ventral iris 20 days after lentectomy. (*H–J*) Sections through the intact retina of an adult newt eye. (*A*, *D*, and *H*) Hybridization with antisense probe. Positive reaction can be seen in the regenerating lens (rl), the cornea epithelium (ce), and the neuroretina (nr). At day 15 the expression seems uniform in all lens cells (*A*), but at day 20 most of the expression is confined at the external layer of the lens, the lens epithelium (le) (*D*). Strong expression is also seen in the cornea epithelium (ce). Some distortion of the regenerating tissue is unavoidable with frozen sections through the whole eye. (*B*, *E*, *G*, and *I*) Bright-field picture of the sections seen in *A*, *D*, *F*, and *H*, respectively. (*C*, *F*, and *J*) Negative control, hybridization with the sense probe. Note the absence of silver grains above the background and the artifactual grains in the irises and pigment epithelium (pe). di, Dorsal iris; vi, ventral iris; c, cornea. (×60.)

then stained with hematoxylin/eosin, mounted in Permount, and observed.

RESULTS

Cloning of Pax Sequences. The amplification of newt eye RNA with the designed Pax primers resulted in a prominent band of the expected size (170 bases). This fragment was cloned into the TA vector and, after verification, was subjected to sequencing reactions as described in *Materials and Methods*. The newt *pax-6* nucleotide sequences included in the cloned fragment are 81% and 82% homologous to the human and mouse counterpart, respectively, but the translational products are identical (Fig. 1). With the same region from the eyeless gene of *Drosophila*, there is 96% identity at the amino acid level and 73% homology at the nucleotide level.

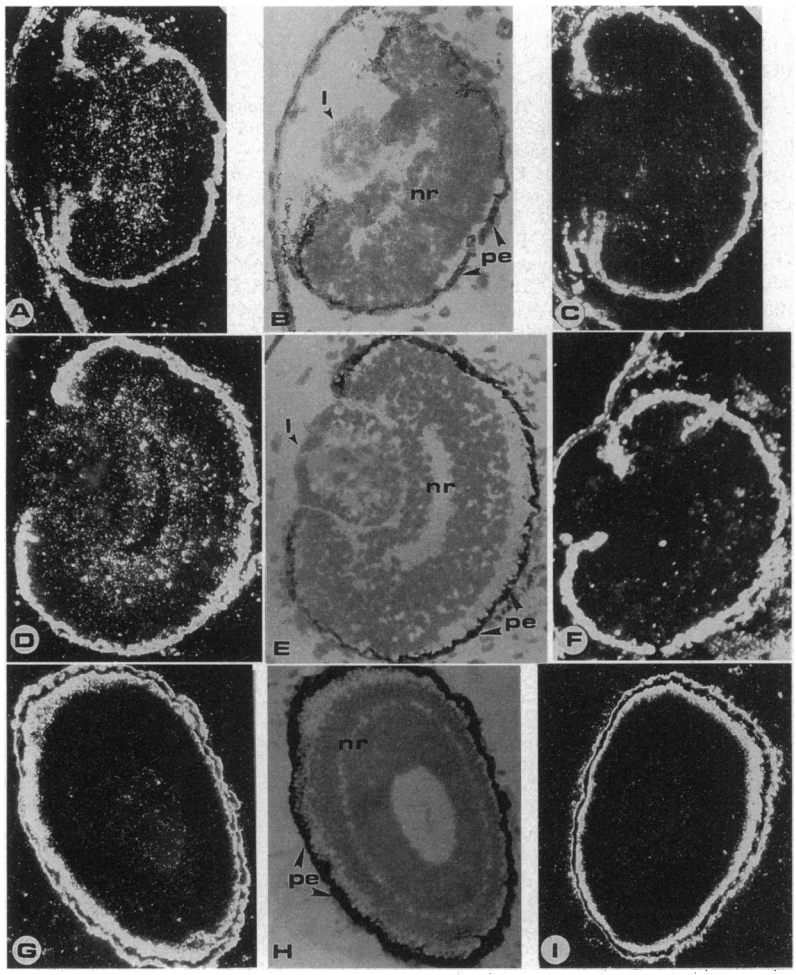

FIG. 3. *In situ* hybridization using the newt *pax-6* riboprobe, labeled with ^{35}S. (*A–C*) Sections through the eye of a stage 40–41 developing axolotl. (*D–F*) Sections through the eye of a hatched axolotl. (*G–I*) Sections through the retina of a larvae axolotl. (*A*, *D*, and *G*) Dark-field picture after hybridization to antisense probe (experimental). The neuroretina (nr) and the lens (l) are positive. The positive reaction in the outer layer (pigmented layer) (pe) is artifactual due to the pigment (pigment granules are refractile under dark-field imaging). (*B*, *E*, and *H*) Bright-field pictures of the tissue seen in *A*, *D*, and *G*, respectively. (*C*, *F*, and *I*) Negative control, hybridization with the sense probe. Note the absence of silver grains in the neuroretina and lens and the artifactual grains in the pigmented retina. (×60.)

Expression in the Intact Newt Eye. *pax-6* transcripts were localized in the neural retina, the lens, and the cornea epithelium. In fact, this is a pattern similar to aniridia gene expression in the developing human eye (21).

Expression in the Regenerating Newt Eye. Sections were taken from different stages of lens regeneration (11, 13, 15, 16, 17, 18, 19, 20, and 30 days after lentectomy). These stages represent the formation of the lens vesicle by the depigmented progenies of the iris cells, the growing of the fiber complex, and the completion of regeneration (see Introduction). *pax-6* transcripts were detected in all stages of lens regeneration. Examples of *pax-6* expression are shown in Fig. 2. At day 15 of regeneration (stage VI, see ref. 5) we can see accumulation of grains in the growing lens vesicle and the differentiating lens fibers (Fig. 2*A*). Some distortion of the regenerating lens is unavoidable with frozen sections through the whole eye but the accumulation of grains is obvious. At day 20 (stage X), the external layer of the lens showed most of the expression (Fig. 2*D*). The retina (Fig. 2*H*) of the eye undergoing lens regeneration and the cornea epithelium were positive as well.

Expression in the Developing Axolotl Eye. We used stage 40–41, hatched, and larvae eye. Expression was seen in the retina and the lens of stage 40–41 (Fig. 3*A*) and hatched (stage 45) eye (Fig. 3*D*). This is a very similar pattern to the newt eye (intact or lentectomized). In fact, similar to the regenerating eye, the external layer of the lens showed somewhat stronger expression. Most striking, however, was the observation that, as opposed to the newt, expression of *pax-6* in the retina declined as the animal became older (Fig. 3*G*).

DISCUSSION

The confinement of the ability for lens regeneration to the dorsal iris only implies strong spatial regulation. Knowledge gained from other developing systems has pinpointed several genes that are specific to certain axes during morphogenesis, specifying, thus, pattern formation. Based on this, we believe that similar genes might govern the spatial regulation seen during the differentiation events preceding lens regeneration after lentectomy. We, therefore, decided to clone and investigate the role of *pax-6*, which belongs to the group of genes responsible for morphogenesis and, in our case, especially of the eye. Studies utilizing *Hox* or *Pax* genes are completely lacking in this field and are likely to contribute to our understanding of gene regulation during lens regeneration.

The cloned newt fragment of the paired box of *pax-6* shows an astonishing homology at the amino acid level with the human and mouse counterpart. Such homologies usually indicate conservation of function as well. The newt *pax-6* transcripts were present in the adult newt eye and the regenerating lens. Expression in the regenerating lens was obvious throughout all stages examined and confined to the outer lens layer as regeneration reached completion. Similarly, we observed expression in the developing axolotl eye in the same tissues—namely, retina and lens. The only apparent difference in the axolotl was the decreased expression in the larval eye as the animal grew older. Given the inability of lens regeneration in axolotl, this observation might be indicative of a role of *pax-6* in lens regeneration. Such a hypothesis might explain the apparent expression of Hox and Pax sequences in adult newt tissues capable of regeneration. Expression of *Hox* genes has been readily observed in adult newt limbs (28–30) and eye (unpublished data and the present study), and this is in contrast to the view that *Hox* genes are expressed only during development. Such a phenomenon might indicate that *Hox* genes are necessary for regeneration and, therefore, they are present in

the adult tissues that are capable of regeneration. If this is true the regenerative tissues of urodeles might display very unique and interesting regulatory properties. Viewing our results from such a perspective, it is tempting to speculate that *pax-6* plays an important role in lens regeneration.

Regulation along the dorsal–ventral axis of the eye should be subject to specific expression of transcriptional factors that are responsible for the dedifferentiation process. The iris cells are usually in a G_0 or arrested state of the cell cycle. After lens removal they enter G_1 and at about day 5 they enter the S phase. These events are accompanied by structural changes in the DNA marked by nicks, gaps, and single-stranded material (31, 32). In this respect, it is important to note that amplification of rRNA sequences has been reported in the dorsal iris undergoing dedifferentiation. In fact, these cells contain 60% more rRNA cistrons (33). Usually these molecular alterations suggest active transcription (34). The *myc* oncogene, for example, has also been found to be actively transcribed during the dedifferentiation of the pigmented epithelial cells (35).

One idea is that dedifferentiation involves regulation in the synthesis of extracellular matrix molecules from the dorsal iris only. Several cell surface molecules (such as proteoglycans, laminin, and melanosomal matrix protein) seem to sequentially disappear or are down-regulated from the dorsal iris after lentectomy (36–39). Such alterations could implicate molecules such as fibroblast growth factor (FGF), which binds proteoglycans. Indeed, it has been reported that basic FGF is one of the essential factors that enhances and regulates lens transdifferentiation of the pigmented epithelial cells (40). Eguchi *et al.* (41, 42) have recently described a monoclonal antibody that is directed against a cell surface antigen involved in cell adhesion and expressed in the iris pigmented epithelial cells among other tissues. This antigen disappears during the process of dedifferentiation after lentectomy. More striking, however, is the fact that ventral iris treated with the antibody *in vitro* and implanted into a newly lentectomized eye is able to produce a lens (35). The immediate conclusion from such results is that disappearance of cell adhesion molecule(s) is necessary and sufficient for the dedifferentiation and the subsequent events of regeneration to occur. Interestingly, the same antigen disappears from the regenerating limb blastema as well (41, 42). The identity of this molecule is not known.

Differential regulation in the dorsal–ventral axis exists during eye development as well, indicating that the establishment of the dorsal and the ventral retina is governed by distinct mechanisms. Several molecules have been found to be specifically expressed in the dorsal retina. These include the modified GD3 ganglioside (43), the ribosome binding protein p40, which shows extensive homology with the laminin receptor, and aldehyde dehydrogenase, which is imperative for the synthesis of retinoic acid (44–46). On the other hand, a different dehydrogenase is confined to the ventral retina during development (46). Regulatory factors involved in transcription and confined to the ventral retina include the retinoid X receptor α (RXRα) and pax-2. Knock-out experiments involving RXR and retinoic acid receptor have shown that the ventral retina develops short (47).

The search for transcriptional factors specific for lens regeneration would undoubtedly provide the means to study the molecular events during lens regeneration. In this respect factors as the one reported in the present paper should prove valuable in the study of lens transdifferentiation from the dorsal iris.

1. Stone, L. S. (1967) *J. Exp. Zool.* **164,** 87–104.
2. Steward, D. S. & Espinasse, P. G. (1959) *Nature (London)* **183,** 1815.
3. Eguchi, G. (1963) *Embryologia* **8,** 45–62.
4. Dumont, J. N. & Yamada, T. (1977) *Dev. Biol.* **29,** 385–401.
5. Yamada, T. (1977) *Monogr. Dev. Biol.* **13.**
6. McDevitt, D. S., Meza, I. & Yamada, T. (1969) *Dev. Biol.* **19,** 581–607.
7. McDevitt, D. S. & Brahma, S. K. (1981) *Dev. Biol.* **84,** 449–454.
8. Scott, M. P. & Weiner, A. J. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 4115–4119.
9. Akam, M. (1987) *Development (Cambridge, U.K.)* **101,** 1–22.
10. Kissinger, C. R., Liu, B., Martin-Bianco, E., Kornherg, T. B. & Pabo, C. O. (1990) *Cell* **63,** 579–590.
11. Wright, C. V. E. (1991) *Curr. Opin. Cell Biol.* **3,** 976–982.
12. Frigerio, G., Borri, M., Bopp, D., Baomgartner, S. & Noli, M. (1996) *Cell* **47,** 735–746.
13. Hill, R. E. & Hanson, I. M. (1992) *Curr. Opin. Cell Biol.* **4,** 967–972.
14. Chapelakis, G., Fritsch, R., Fickenscher, H., Deutsch, U., Goulding, M. & Gruss, P. (1991) *Cell* **66,** 873–881.
15. Monaghan, A. P., Davinson, D. R., Sime, C., Graham, E., Baldock, R., Bhattacharya, S. S. & Hill, R. E. (1991) *Development (Cambridge, U.K.)* **112,** 1053–1061.
16. Funahashi, J., Sekido, R., Murai, K., Kamachi, Y. & Kondoh, H. (1993) *Development (Cambridge, U.K.)* **119,** 433–446.
17. Levine, E. M. & Schechter, N. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 2729–2733.
18. Nornes, H. O., Dressler, G. R., Knapik, E. W., Deutsch, U. & Gruss, P. (1990) *Development (Cambridge, U.K.)* **109,** 797–809.
19. Walther, C. & Gruss, P. (1991) *Development (Cambridge, U.K.)* **113,** 1435–1449.
20. Hill, R. E., Favor, J., Hogan, B. L. M., Ton, C. C. T., Saunders, G. F., Hanson, I. M., Prosser, J., Jordan, T., Hastie, N. D. & van Heyningen, V. (1991) *Nature (London)* **354,** 522–525.
21. Ton, C. C. T., Hirvonen, H., Miwa, H., Weil, M. M., Monaghan, P., Jordan, T., Van Heyningen, V., Hastie, N. D., Meijers-Heijboer, H., Drechsler, M., Royeker-Pokora, B., Collins, F., Swaroop, A., Strong, L.C. & Saunders, G.F. (1991) *Cell* **67,** 1059–1074.
22. Quiring, R., Walldorf, U., Kloter, U. & Gehring, W. J. (1994) *Science* **265,** 785–789.
23. Hodginson, C. A., Moore, K. J., Nakayama, A., Steingrimsson, E., Copeland, N. G., Jenkins, N. A. & Arnheiter, H. (1993) *Cell* **74,** 395–404.
24. Li, H.-S., Yang, J.-M., Jacobson, R. D., Pasko, D. & Sundin, O. (1994) *Dev. Biol.* **162,** 181–194.
25. Sanger, F., Nicklen, S. & Coulson, A. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
26. Onda, H., Poulin, M. L., Tassava, R. A. & Chiu, I.-M. (1991) *Dev. Biol.* **148,** 219–232.
27. Poulin, M. L., Patrie, K. M., Betelho, M. J., Tassava, R. A. & Chiu, I.-M. (1993) *Development (Cambridge, U.K.)* **119,** 353–361.
28. Beauchemin, M. & Savard, P. (1992) *Dev. Biol.* **154,** 55–65.
29. Belleville, S., Beauchemin, M., Tremblay, M., Noiseux, N. & Savard, P. (1992) *Gene* **114,** 179–186.
30. Beauchemin, M., Noiseux, N., Tremblay, M. & Savard, P. (1994) *Int. J. Dev. Biol.*, **38,** 641–649.
31. Collins, J. M. (1974) *J. Biol. Chem.* **249,** 1839–1847.
32. Collins, J. M. (1974) *Biochem. Biophys. Res. Commun.* **57,** 359–364.
33. Collins, J. M. (1972) *Biochemistry* **11,** 1259–1263.
34. Roseman, B., Lough, J., Houkom, E. & Herman, T. (1986) *Biochem. Biophys. Res. Commun.* **137,** 474–476.
35. Eguchi, G. (1988) in *Regulatory Mechanisms in Developmental Processes*, eds. Eguchi, G., Okada, T. S. & Saxen, L. (Elsevier, Limerick, Ireland), pp. 147–158.
36. Zalik, S. E. & Scott, V. (1973) *Nature (London) New Biol.* **244,** 212–214.
37. Tsonis, P. A. & Del Rio-Tsonis, K. (1995) *J. Chromatogr.*, in press.
38. Ortiz, J. R., Vigny, M., Courtois, Y. & Jeanny, J.-C. (1992) *Exp. Eye Res.* **54,** 861–870.
39. Mochii, M., Agata, K., Kobayashi H., Yamamoto, T. S. & Eguchi, G. (1988) *Cell Differ.* **24,** 67–74.
40. Hyuga, M, Kodama, R. & Eguchi, G. (1993) *Int. J. Dev. Biol.* **37,** 319–326.
41. Imokawa, Y., Ono, S.-I., Takeichi, T. & Eguchi, G. (1992) *Int. J. Dev. Biol.* **36,** 399–405.
42. Imokawa, Y. & Eguchi, G. (1992) *Int. J. Dev. Biol.* **36,** 407–412.
43. Constantine-Paton, M., Blum, A. S., Mendez-Otero, R. & Barnstable, C. J. (1986) *Nature (London)* **324,** 459–462.
44. Rabacchi, S. A., Neve, R. L. & Drager, U. C. (1990) *Development (Cambridge, U.K.)* **109,** 521–531.
45. McCaffery, P., Tempst, P., Lara, G. & Drager, U. C. (1991) *Development (Cambridge, U.K.)* **112,** 693–703.
46. McCaffery, P., Posch, K. C., Napoli, J. L., Gudas, L. & Drager, U. C. (1993) *Dev. Biol.* **158,** 390–399.
47. Kastner, P., Grondona, J. M., Mark, M., Gansmuller, A., LeMeur, M., Decimo, D., Vonesch, J.-L., Dolle, P. & Chambon, P. (1994) *Cell* **78,** 987–1003.