Retinal regeneration in amphibians

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ABSTRACT This review is a comparative analysis of retina regeneration in different amphibians. Special attention is given to the newt, which, unlike other vertebrates, retains the capacity for the regeneration of eye structures for all life. The review focuses on the sources of the cells which contribute to retina regeneration, proliferative activity of cells participating in regeneration, the factors which control the process, and the genes expressed during the course of regeneration.

KEY WORDS: *retina regeneration, cell sources, signal molecules, gene expression, amphibians*

Introduction

Regeneration potencies in vertebrates are expressed in different degree and are most pronounced in adult, in which the lens and retina may be fully restored (Fig. 1). The first studies on eye regeneration in amphibians (newts) were carried out by the famous naturalist Bonnet in 1781 (Dinsmore, 1992). The experimental studies of retinal regeneration in amphibians were initiated at the end of last century (Philipeau, 1880; Griffini and Marcchio, 1889) and have been developed most intensively in our century (Fujita, 1913; Wachs, 1920; Stone, 1950a,b; Hasegawa, 1958, 1965; Hendrickson, 1964; Williams, 1964; Mitashov, 1968; Reyer, 1971, 1977; Keefe, 1973 a-d; Levine, 1975, 1977; McDevitt, 1989; Klein *et al.*, 1990).

Studies on developing and regenerating amphibian eye in Russia were started by Dragomirow (1932, 1936) and Lopashov (1949, 1955). Based on the accumulated experimental materials, we continued these investigations and attempted to look for the approaches to study the mechanisms of retinal regeneration in lower vertebrates. Our approach is based on the use of various nuclear and cytoplasmic markers, mono- and polyclonal antibodies, and molecular-biological methods for comparative studies of cell sources, proliferation, cell differentiation and gene expression during retinal regeneration in amphibians.

The question about cell sources of retinal regeneration is the key one because our theoretical concepts about the mechanisms of retinal regeneration largely depend on whether reserve undifferentiated cells or the participation of differentiated cells are involved in the regeneration. The essence of the question is considerably deeper than simply the correct explanation of a specific regeneration event: the question concerns the genetic information which may be expressed in the cells participating in regeneration. Transition to this level of research became possible only through the use of molecular-biological methods for experimental studies of the retinal regeneration.

Cell sources of retinal regeneration in amphibians

The eye has a similar structure in all vertebrates (Fig. 2). The newt eye is characterized by a high level of myopia determined by the structure of a large spherical lens attached to the falciform process. The retina of adult newt comprises the following layers from the basal retina surface bordering with the internal limiting membrane to the apical surface bordering with the external limiting membrane: ganglion cell layer, inner plexiform layer, internal nuclear layer, outer plexiform layer, external nuclear layer, and layer of rods and cones (Fig. 3). At the level of outer segments of the photoreceptors, the retina is in close contact with the uniserial layer of intensely pigmented epithelial cells called retinal pigment epithelium (RPE). The RPE cells fulfil certain physiological functions of the retina: they participate in visual processes, supply of the retina with nutrients, and phagocytosis of shed disks of the outer photoreceptor segments.

The conclusions about the cell sources of retinal regeneration were for a long time drawn only from the analysis of proliferative activity of the cells located in different areas of the eye. For understanding the mechanisms of retina regeneration it is essential that the two areas are present in the eye, where proliferation of the cells can be activated (1) undifferentiated cells in the periphery of the eye, and (2) the differentiated RPE cells (Fig. 4). The peripheral area of the eye was called the growth zone because the cells of this area participate in growth of the retina during development (see next section).

In a number of studies, especially in the earliest ones carried out without safe cell markers, it was stated with a varying degree of

Abbreviations used in this paper: RPE, retinal pigment epithelium; DOPA, dihydroxyphenylalanine; N-CAM, neural cell adhesion molecules; aFGF, bFGF, acidic (basic) fibroblast growth factor; GFAP, glial fibrillar acidic protein; NF, neurofilaments; EGF, epithelial growth factor.

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substantiation that the cells of the growth zone (Philipeau, 1880; Griffini and Marcchio, 1889; Fujita, 1913; Stone and Zaur, 1940; Gaze and Watson, 1968) or only differentiated pigment epithelium cells (Stone, 1950a) are cell sources of retinal regeneration in adult newts.

The role of cells of the growth zone in retinal growth and regeneration

The dispute about the cell sources of retinal regeneration in amphibians seemingly ended by the mid1970s when the now widespread viewpoint was formulated that both concepts are correct and the retina in adult newt is restored both at the expense of proliferation of a ring of the cells located in the growth zone and pigment epithelium cells (Fig. 4. Hasegawa, 1958, 1965; Mitashov, 1968; Reyer, 1971; Keefe, 1973a,d). However new data recently obtained from studies on retina growth and regeneration in amphibians, fish, birds, and mammals suggest that eye growth and retina regeneration, if it takes place, are provided by different cell sources. A better understanding of the cell sources of regeneration is especially important with regard to the mechanisms regulating eye growth and retina regeneration.

Little differentiated precursor cells were found in the peripheral retina of all studied vertebrates (amphibians, fish, birds and mammals) using ^{3}H-thymidine, retroviruses, and fluorescent dyes as nuclear and cytoplasmic markers (Hollyfield, 1968, 1971; Straznicky and Gaze, 1971; Johns, 1977; Johns and Easter, 1977; Meyer, 1978; Beach and Jacobson, 1979; Morris and Cowan, 1984; Hunt *et al.*, 1987; Reh, 1987, 1992; Turner and Cepko, 1987; Holt *et al.*, 1988; Wetts and Fraser, 1988, 1991; Wetts *et al.*, 1989; Fekete *et al.*, 1990, 1994; Turner *et al.*, 1990; Cepko *et al.*, 1996; Alexiades and Cepko, 1997). The cell population of this zone is heterogenous: it comprises uni- and multipotent cells producing photoreceptors, interneurons, and glia. The precursor cells of this zone are a source of the differentiated retina growth during development. The growth zone of the eye reflects the growth potential of the vertebrate eye, which can markedly vary in animals of different ages according to the number of produced cells.

In amphibians in which the retina regenerates, proliferating little differentiated cells in the growth zone of the eye were observed (Gaze and Watson, 1968; Mitashov, 1968; Reyer,1971; Keefe, 1973a,d; Levine, 1981; Mitashov and Maliovanova, 1982; Svistunov and Mitashov, 1985). The peculiarities of eye growth in amphibians, such as newt, axolotl, *Ambystoma* and *Xenopus*, were studied in the experiments with repeated injections of ^{3}H-thymidine in adult animals and subsequent fixation of the eyes one, two, three, four, five or six months later. The most intensive growth of the retina was observed in the actively growing axolotl and *Xenopus* and the least intensive one, in the newt and *Ambystoma*. After removal or destruction of the retina under ischemic conditions, the most complete regeneration occurs in adult newts and partial regeneration only in the periphery of the eye, in axolotl and *Xenopus*. In the periphery of the eye, only a small volume of the retina forms from the cells of growth zone (Mitashov, 1968; Mitashov and Maliovanova, 1982; Svistunov and Mitashov, 1983a, b). The similar type of retinal regeneration as a result of proliferation of the cells located in the growth zone was observed in larvae of some anuran amphibians (Dabagyan and Sheresheva, 1966; Dabagyan and Tret'yakova, 1968). Until recently these data were considered as a manifestation of the role of different cell sources during retinal regeneration in different amphibians. However this conclusion is wrong. The growth zone of the eye in all studied amphibians contains the cells only for the retinal growth, rather than for regeneration. This conclusion is very important for understanding the mechanisms of retinal regeneration.

No full restitution of the retina from the precursor cells localized in the growth zone was ever observed. Only the cell volume of the retina, which corresponds to a possible eye growth for the amphibians of a given age, is replenished. This volume will always correspond to the age of animal: it is highest in the larvae and growing animals and lowest in adults. The growth potential, rather than the regeneration potential, is realized in all studied amphibians during both the eye growth and retina regeneration.

The results of experiments on *Xenopus laevis* (Mitashov and Maliovanova, 1982), axolotl (Svistunov and Mitashov, 1983b, 1984) and larvae of some anuran amphibians (Dabagyan and Sheresheva, 1966; Dabagyan and Tret'yakova, 1968) provide additional convincing evidence on the role of the cells of growth zone in retina growth. In these amphibians, the pigment epithelium cells are not involved in retina regeneration, while proliferation of the precursor cells localized in the growth zone leads to the formation of a small retina. As a result, the retina is not fully restored. The restored retina volume fully corresponds to that provided by the cell population of the growth zone, thus suggesting that the growth zone contains only enough precursor cells for retina growth during ontogenesis, rather than for its full restitution. In adult newts, retinal regeneration in the central area of *fundulus oculi* is realized in the uniserial layer of pigmented epithelium (Fig. 4). If the growth zone is removed in adult newts, the retina is fully restored only from the pigment epithelium cells. A paradox of retinal regeneration consists in that there are little differentiated cells in the retina but full restitution of the removed or destroyed retina does not occur. The reason is clear: the volume of cells in the growth zone is limited and surfaces only for growth of the retina rather than for its regeneration.

The role of pigment epithelium cells in retinal regeneration

The retina and pigmented epithelium have a common origin in embryogenesis. In vertebrate animals, the eye is formed as a result of evagination of the intermediate lobe wall of the forebrain. At the eye vesicle stage, the eye rudiment consists of a row of undifferentiated neuroepithelium cells. The retina is formed from the distal part of the eye vesicle as a result of cell invagination. After intense cell proliferation and subsequent differentiation, a multilayer retina is formed, while the external layer cells form RPE.

At the early embryonic stages, the pigmented epithelium displays pronounced plasticity. The pigment epithelial cells can be induced for transdifferentiation into the retina cells at the early stages of their transdifferentiation in all studied vertebrates: fish (Dabagyan, 1959, 1960; Sologub, 1975), newts and axolots (Dragomirow, 1932, 1936; Detwiler and Van Dyke, 1953, 1954), frogs (Ikeda, 1937; Lopashov, 1949, 1955; Lopashov and Sologub, 1972; Reh and Nagy, 1987; Reh *et al.*, 1987), chicken (Orts-Llorca and Genis-Galvez, 1960; Coulombre and Coulombre, 1965, 1970; Tsunematsu and Coulombre, 1981; Park and Hollenberg, 1989, 1991; Pittack *et al.*, 1991, 1997; Reh *et al.*, 1991; Guillemot and

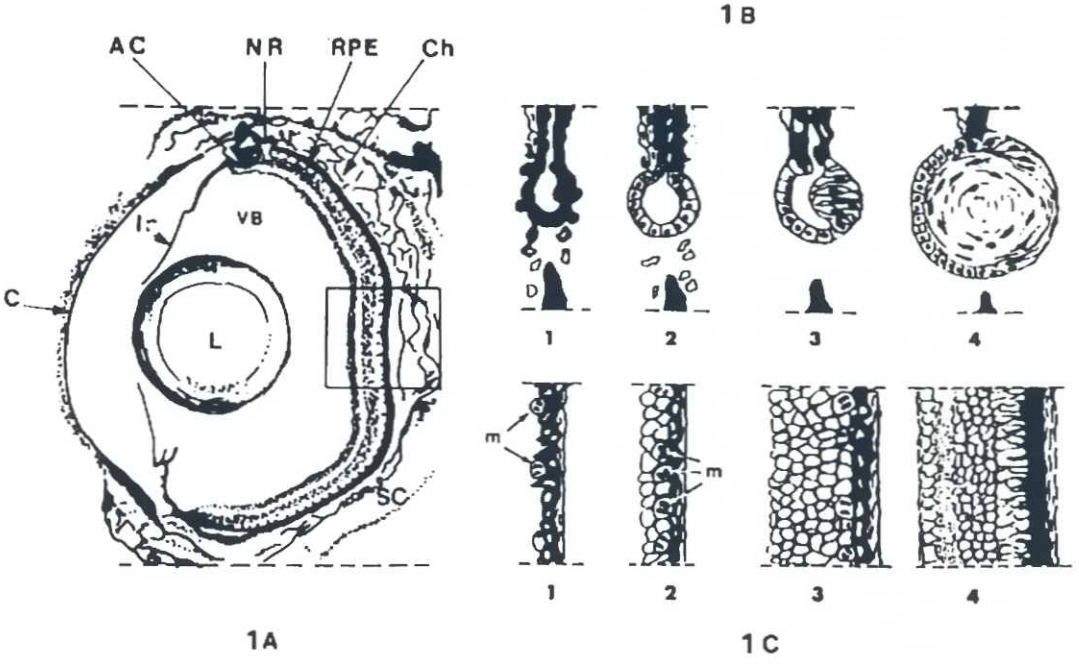

Fig. 1. Schematic representations of normal eye and the regeneration stages of lens and retina in the adult newt. (**A**) *Vertical, meridional section through the adult normal eye. NR, neural retina; RPE, retinal pigmented epithelium; CH, choroid; SC, sclera; VB, vitreous body; L, lens; Ir, iris; AC, anterior complex with ora serrata and ciliary epithelium; C, cornea.* (**B**) *Lens regeneration stages (1-4). Each step is represented in a section through the dorsoventral axis of the iris. (1-2) 10-15 days after lentectomy. Formation of a vesicle of depigmented epithelial cells from the inner and outer laminae of dorsal iris. (3) 20 days after lentectomy. (4) 30-35 days after lentectomy. Regenerating lens containing fibers.* (**C**) *Retinal regeneration stages (1-4) from the RPE. Only parts of retinal regenerates corresponding to the normal adult retinal central area enclosed in (A) are presented (1) 5-10 days after retinectomy. Dedifferentiation and proliferation of the RPE cells begin. m, mitotic cells. (2) 14 days after retinectomy. Double-layered retinal rudiment lying next to a monolayer of repigmented epithelial cells. (3) 20 days after retinectomy. Retinal regenerate appears multilayered but no differentiated layers can be seen. (4) 30-35 days after retinectomy. Retinal regenerate displays differentiated cell and fiber layers. (Schemes drawn from Reyer, 1977; Eguchi, 1979 and our observations; from Mitashov et al., 1995).*

Cepko, 1992; Opas and Dziak, 1994), and rats (Stroeva, 1960). During development, this capacity is lost in frogs, axolotls, fish, birds, and mammals, but is preserved in some Urodelan amphibians during all their life. In representatives of Urodelan amphibians (newts), the pigment epithelium cells are the main source of regeneration of the removed or destroyed retina in adult animals.

During regeneration, the differentiated pigment epithelium cells form cells of the neuroepithelium rudiment as a result of transdifferentiation. To understand the mechanisms underlying retina regeneration in adult, it is necessary above all to elucidate the mechanisms of formation of the neuroepithelium rudiment, since this is the key event of regeneration.

In experimental-morphological studies of the retina regeneration, the natural cytoplasmic component of the cells, melanin, served as marker for visualization of gradual transformations of the pigment epithelium cells during formation of the neuroepithelium. The pigment epithelium cells are gradually depigmented, proliferate, and form the neuroepithelium rudiment. The initial pigment epithelium cells are so intensely pigmented that their depigmentation takes a rather long time. The cells of a nascent two- to three-serial neuroepithelium rudiment contain a sufficient amount of pigment, thus allowing us to follow their gradual migration from the pigment epithelium layer in the vitreous body cavity at subsequent transitional stages of regeneration. At the stage of formation of the two- to three-serial pigmented neural epithelium, a boundary between the pigmented neuroepithelium in the central part of *fundus oculi* and neuroepithelium forming from the growth zone, whose cells are not pigmented, is quite distinct in sections (Fig. 5).

Later, the role of pigment epithelium in the retina regeneration was finally proved in the experiments with the use of ^{3}H-thymidine as a nuclear marker (Mitashov, 1968; Reyer, 1971, 1977; Keefe, 1973a, d; Stroeva and Mitashov, 1983) and staining by monoclonal antibodies specifically binding to the pigment epithelium cells as a cytoplasmic marker (Klein *et al.*, 1990). Proliferating cells of the pigment epithelium were labeled with ^{3}H-thymidine and then, upon fixation of the experimental materials within several days at the moment of the neuroepithelium rudiment formation, the labeled cells were found in the neuroepithelium. The intensity of labeling was weaker as a result of preceding divisions. Analysis of the intensity of labeling of the initial pigment epithelium cells and nascent neuroepithelium cells convincingly suggest the origin of neuroepithelium from the pigment epithelium cells (Stroeva and Mitashov, 1970, 1983; Reyer, 1971; Keefe, 1973a,d).

When using monoclonal antibodies REP-1 against specific antigens in the cytoplasm of the pigment epithelium cells and their descendants, the origin of neuroepithelium from the pigment epithelium cells during retina regeneration was also convincingly demonstrated (Klein *et al.*, 1990).

The labeled specific precursor of melanin synthesis ^{3}H-dihydroxyphenylalanine (^{3}H-DOPA) proved to be an exceedingly successful cytoplasmic marker, which made it possible to estimate the number of the pigment epithelium cells forming the neuroepithelium rudiment (Mitashov, 1976, 1980). When studying synthesis of the specific product of the pigment epithelium cells melanin during retina regeneration, an important phenomenon was found (Fig. 4). During formation of the neuroepithelium rudiment, ^{3}H-DOPA is not incorporated in those cells of the pigment epithelium in the central zone of *fundus oculi*, which are a source of its formation, i.e., in a certain subpopulation of cells, production of the component specific for the pigment epithelium is inhibited. At the same time, in the eye periph-

ery, where a small part of the neuroepithelium rudiment, as was already mentioned, is formed at the expense of proliferation of little differentiated cells of the eye growth zone, the pigment epithelium cells are not involved in retina regeneration. Cell interactions appear to determine the ratio of regeneration to growth potencies of the eye tissues in adult newts. In the eye periphery, the pigment epithelium cells are partially depigmented and immediately, in response to depigmentation, melanin is resynthesized in these cells, according to incorporation of ^{3}H-DOPA in the cell cytoplasm.

The data on ^{3}H-DOPA incorporation in the pigment epithelium cells suggest that approximately 60-70% of the pigment epithelium cells are involved in the neuroepithelium rudiment formation after the retina removal and preservation of a population of the precursor cells providing for the retina growth in the periphery (Fig. 4). Correspondingly, in different experimental models (retina removal or transection of the optic nerve and blood vessels), the number of the pigment epithelium cells involved in the neuroepithelium formation is different, which is determined not only by the type of operation, but also by the number of precursor cells preserved in the growth area after the operation.

In the experiments with transplantation of the pigment epithelium cells from the central area of *fundus oculi* without any admixture of the retinal cells in the eye cavity of newts, the involvement of all transplanted pigment epithelium cells in retina regeneration was also convincingly demonstrated (Stone and Steinitz, 1957; Mitashov and Grigoryan, 1984).

How is the retina (neuroepithelium) rudiment formed from the pigment epithelium cells? Two main pathways of formation of the neuroepithelium cells from the pigment epithelium cells were described in various experimental models. In the experiments with removal of the retina, depigmented pigment epithelium cells proliferate and the daughter cells are displaced to the vitreous body cavity, where they acquire properties of the neuroepithelium rudiment cells. Mitotic figures perpendicular to the choroid were found in the proliferating pigment epithelium cells. As a result of cell division with such orientation, the daughter cells are displaced in the vitreous body cavity and become cells of the neuroepithelium rudiment, while the initial cells remain in the pigment epithelium layer and are repigmented. The same mechanism of the formation of a multilayered neuroepithelium was supposed in chicken (Opas and Dziak, 1994).

In all studies, asynchronous formation of the neuroepithelium cells was noted. In crested newts, two zones of predominant cell proliferation were found: around the optic nerve and ring blood vessel. In the experiments with eye devascularization, the initial retina is first degenerated and during the retina degeneration, an intense immigration of the pigmented cells from the pigment epithelium layer was found. These cells migrate over significant distances in the vitreous body cavity. The cells emigrated from the pigment epithelium layer are depigmented, proliferate and form depigmented neuroepithelium, which is a source of restitution of the destroyed retina. The cells remaining in the pigment epithelium layer are involved in elimination of the degenerating retina debris and thus fulfil, together with the macrophages migrating in the eye cavity, the macrophagal function. These pigment epithelium cells are also partially depigmented and then repigmented to destitute the initial pigment epithelium layer (Keefe, 1973d).

Since the adult newts have such pronounced regeneration potencies of the eye tissues, a possible existence of little differentiated cells as a source of retina regeneration was discussed for a long time. Small oval cells referred to as little differentiated special cells were found among the differentiated retinal cells of adult crested newts (Keefe, 1973d). Keefe failed to elucidate their role in retina regeneration and their nature is so far unknown. Structurally, these cells resemble those actively migrating from the blood flow, rather than the neural type cells. No population of little differentiated cells was found among the pigment epithelium cells, which could be a special source of regeneration.

The pigmented epithelium is characterized by a distinct macrophagal function, which is especially expressed, as was shown for the experimental model with retina devascularization. It appears to be a subject to stochastic determination which cells fulfill the macrophagal function and eliminates the destroyed retina and are involved in the neuroepithelium formation.

Peculiarities of RPE cells proliferative activity during retina regeneration

One of the earliest events in transdifferentiation of the pigmented epithelium cells into the neuroepithelium is proliferation of the pigmented epithelium. Formation of a retinal rudiment requires several divisions of RPE cells.

Initiation of DNA synthesis in the RPE cells was detected on the second to fourth day following removal of the neural retina or dissection of the optic nerve and blood vessels (Mitashov, 1969, 1970; Reyer, 1971, 1977; Parshina and Mitashov, 1978). Incorporation of ^{3}H-thymidine in the cell nuclei of control eyes was also found in an insignificant percentage of cases (Mitashov, 1970). The labeling index of RPE cells markedly increases shortly after the DNA synthesis initiation. A maximum proliferative activity is reached on the fourth to eighth day after surgery and remains high until the 14th to 17th day, fluctuating from 30% to 54% in different newt species (Fig. 6). This corresponds to a level of proliferative activity of the RPE cells after a single injection of ^{3}H-thymidine (Parshina and Mitashov, 1978). Repeated injections of 3H-thymidine allow to labeling of 70-91% of the pigmented epithelium cells (Mitashov, 1969). By resorting to the technique of reusing the labeled precursors of DNA synthesis, up to 98.5% of the cells can be labeled (Parshina and Mitashov, 1978). This is the period when the first cells of retinal rudiment are formed. The ^{3}H-thymidine labeling index of the retinal rudiment cells reaches 65-71% after a single injection of ^{3}H-thymidine. Soon after formation of the retinal rudiment, the RPE cells gradually cease to proliferate, although their proliferative activity continues at a lower level up to the 30th-40th day after surgery (Fig. 6).

Another specific feature of the proliferative activity of RPE cells and retinal rudiment consists in that the duration of the total cell cycle in the retinal rudiment becomes 1,5-2.0 times shorter than in the layer of pigmented epithelium (T= 43; 21.0-23.5 respectively).

Proliferative activity of RPE cells in amphibians, in which the retina does not regenerate from RPE cells

We have also studied proliferative activity of the RPE cells in amphibians (axolotl and *Xenopus*) in which the RPE cells do not participate in successful retinal regeneration (Mitashov and Maliovanova, 1982; Svistunov and Mitashov, 1983a, b; 1984, 1985). We observed a small number of ^{3}H-thymidine labeled cells

in the control non operated axolotl and *Xenopus* eyes. Moreover, the embryonic slit was observed in the growing axolotls in which the RPE cells actively proliferate (labeling index 20-24%). A part of the retina is restored by the cells of the growth zone, rather than by the RPE cells. In this case, the proliferative activity of RPE cells leads to overgrowth of the pigmented epithelium layer. After removal of the retina in *Xenopus laevis*, the RPE cells are also activated for proliferation. However the level of proliferative activity is significantly lower (labeling index 9-14%), than in the pigmented epithelium layer in axolotls. The RPE cells were involved in formation of an atypical small retinal regenerate only in albino *Xenopus laevis* individuals. A retina of small size is restored by the cells of growth zone in *Xenopus laevis*.

Changes of RPE cell characteristics forming retinal rudiment in newts

The key stage during retinal regeneration is the formation of retinal neuroepithelium cells from the RPE cells. In the process of retinal rudiment formation, the RPE cells are gradually depigmented. Depigmentation of the RPE cells involves changes in differentiation of the initial cells and is essential for understanding cell transdifferentiation mechanisms. Melanin synthesis is a specific characteristic of the pigmented cells of newt eyes. The precursor of melanin synthesis DOPA forms melanin in the pigmented cells of newts under the effect of tyrosinase, just as in those of mammals. The latter is deposited on special protein structures, premelanosomes. Simultaneous use of ^{3}H-DOPA and ^{3}H-thymidine revealed a subpopulation of cells in the RPE incorporating both precursors during different stages of retinal regeneration (Mitashov, 1976, 1978, 1980; Grigoryan and Mitashov, 1979). Figure 4 gives the labeling index values of the cells containing ^{3}H-thymidine and ^{3}H-DOPA for different zones of pigmented epithelium as restitution of the retina proceeds.

In the following paragraph, the basic data for the pigmented epithelium and retinal neuroepithelium cells are compared. We have already considered the detailed spatial distribution of cells in the pigmented epithelium once melanin synthesis has been initiated. This enabled determination of the number of RPE cells involved in restitution of the neural retina. We shall merely note here that against the background of melanin biosynthesis, the peripheral pigmented epithelium zones contain only 4-6% of cells with ^{3}H-thymidine. The RPE cells in the *fundus oculi* area contain no ^{3}H-DOPA throughout the entire period of transdifferentiation into the retinal rudiment whereas the index of ^{3}H-thymidine-labeled cells reaches nearly 50% after a single injection of the DNA precursor (Fig. 6). Thus, in this area with a high RPE cell proliferation level, the synthesis of specific melanin granules is arrested.

At the early stages of retina restitution, the retinal neuroepithelium cells contain melanin granules since they were formed from RPE cells, but they did not incorporate ^{3}H-DOPA and synthesize melanin. Thus, transformation of the RPE cells into the cells of another type of differentiation takes place upon termination of the synthesis of specific products characteristic of the initial cell type.

At which stages of retina regeneration will the retinal neuroepithelium cells begin producing specific proteins of the neural type of differentiation? To answer this question, we carried out immunocytochemical studies of the retina regeneration with antibodies against N-CAM, GFAP and NF (Mitashov *et al.*, 1993, 1995). We found that the neuroepithelium cells produce antigens specific for

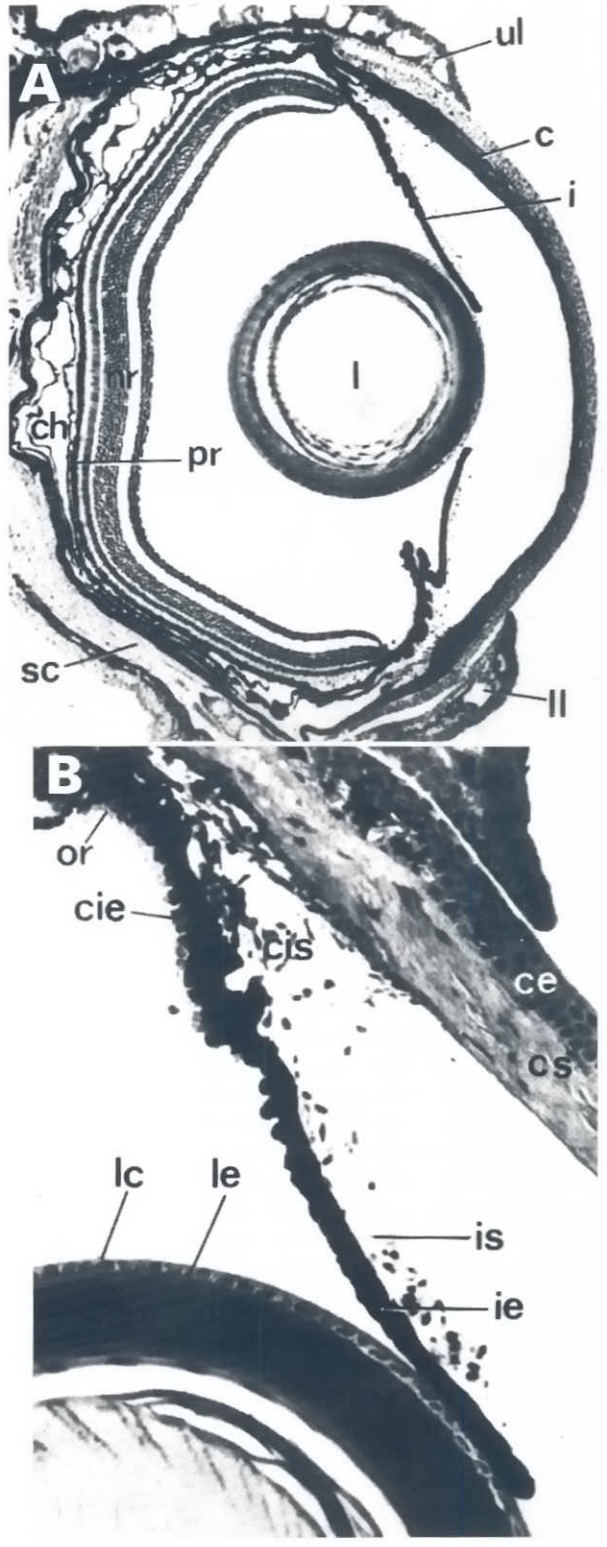

Fig. 2. Histological preparations of the eye of the adult newts, *Notophthalmus viridescens*. (**A**) *Vertical meridional section through the eye. c, cornea; l, lens; i, iris; nr, neural retina; pr, pigment epithelium; ch, choroid; ll, lower lid; sc, scleral; ul, upper lid.* (**B**) *Detailed representation of the dorsal iris region. cie, ciliary epithelium; le, lens epithelium; le, lens ; ie, iris epithelium; cis, stroma of ciliary body; is, iris stroma; or, ora serrata; ce, corneal epithelium; cs, corneal stroma (substantia propria) (from Reyer, 1977).*

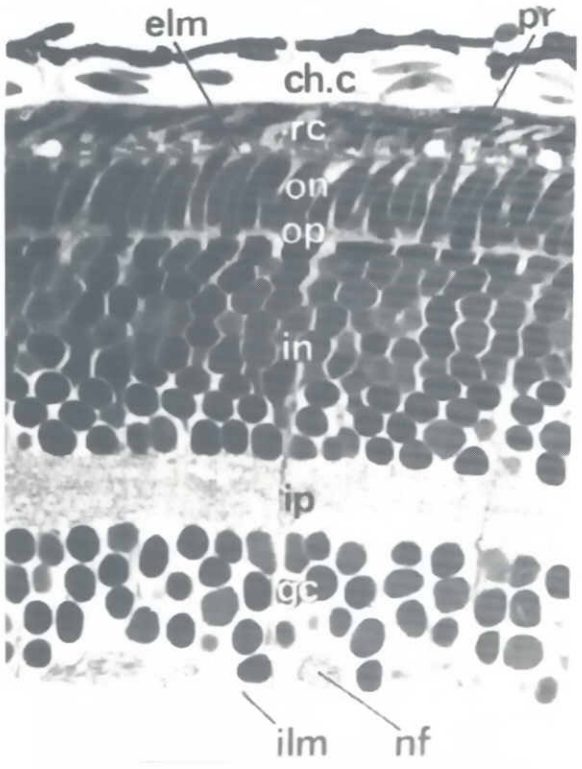

Fig. 3. Histological preparation of neural and pigmented retina. *ilm, internal limiting membrane; nf, nerve fiber layer; gc, ganglion cell layer; ip, inner plexiform layer; in, inner nuclear layer; op, outer plexiform layer; on, outer nuclear layer; elm, external limiting membrane; rc, layer of rods and cones; pr, pigmented retinal epithelium; ch.c, choriocapillaris (from Reyer, 1977).*

cells of the neural type of differentiation already at the early stages of regeneration. We tried also to determine whether the initial pigment epithelium cells produce any antigens that are produced normally by the differentiated retina cells. We found no expression of retina-specific antigens in the pigment epithelium cells under the normal conditions and at the early stages of RPE transdifferentiation. Neurospecific proteins were expressed only in the cells of the neuroepithelium rudiment after formation of one- to two-layered depigmented retinal rudiment. We observed also expression of bFGF and tenascin (Markitantova *et al.*, 1995).

Signal molecules controlling retina regeneration in lower vertebrates

Comparative analysis of the retina regeneration in vertebrates suggests that the complete retina restitution is possible only in the presence of the cells providing not only for growth of the retina but also for its regeneration. These are the pigment epithelium cells (Figs 1 and 4). In this model of the retina regeneration, the initial cells pass through gradual transformation, during which their fate changes and they acquire characteristics of the presumptive neuroepithelium (Figs. 4 and 5).

Let us consider the main regulatory events in the RPE. It is evident that the initial pigment epithelium cells are differentiated cells. But the degree of their differentiation in the embryonic stages and adult animals (newts) differs with respect to the genome expression, factors stabilizing the differentiated state, chromatin packaging, and intensity of cell-to-cell and cell-to-substrate contacts. A 80-200 kDa glycoprotein produced by the pigment epithelium cells of the retina and iris and maintaining a stable differentiated state was isolated (Imokawa *et al.*, 1992; Eguchi, 1993). This glycoprotein was also found in the intercellular space and on the cell surfaces of limb tissues in adult newts (Imokawa *et al.*, 1992). The glycoprotein disappears from the dorsal iris at the early stages of lens regeneration. It was proposed that removal of the factor relieves the differentiated state of the cells and they acquire the capacity for formation of the lens rudiment. Similar events related to removal of a factor that determines a stable differentiated state were also found at the early embryonic stages and during limb regeneration in adult newts (Eguchi, 1993). A factor stabilizing the differentiated state is produced in the pigment epithelium, when stratification of the differentiated retina layers is completed (Eguchi, 1993). This allows a suggestion that the factor stabilizing the differentiated state is absent in the pigment epithelium at the early stages of embryogenesis in amphibians, fish, birds, and mammals, when the regeneration potencies of the pigment epithelium cells are quite pronounced. This may explain regeneration potencies of the pigment epithelium at the early stages of eye development.

In adult newts, the glycoprotein stabilizing the differentiated state appears to be lost during migration of the cells to the eye cavity, where the neuroepithelium rudiment is formed. The neuroepithelium cells produce antigens specific for cells of the neural type of differentiation already at the early stages. Using antigens against the glial fibrillar acidic protein (GFAP), neurofilaments (NF), and adhesive proteins of neural type (N-CAM), we tried to determine whether the initial pigment epithelium cells produce any antigens that are produced normally by the differentiated retina cells (Mitashov *et al.*, 1993). We found no expression of retina-specific antigens in the pigment epithelium cells under the normal conditions and at the early stages of RPE transdifferentiation (Mitashov *et al.*, 1995). The neurospecific proteins were expressed only in cells of the neuroepithelium rudiment after formation of one- to two- layered depigmented retinal rudiment. Expression of the antigens specific for the differentiated retinal cells, e. g., opsin, was found in the first differentiating photoreceptors after reproduction of the neuroepithelium cells and formation of a multilayer retinal regenerate (Bugra *et al.*, 1992).

Similar data were also obtained on the model of retina regeneration in fish and amphibian larvae. In fish, cell precursors of the rods do not produce opsin. In the frog larvae also, the antigens of the neural type of differentiation are expressed in depigmenting cells of the nascent neural epithelium, rather than in the initial pigment epithelium cells (Reh and Nagy, 1987).

Thus, definitive cells of the early depigmented retinal rudiment have neuroepithelial features. This is preceded by a long period of cell depigmentation and emigration from the pigment epithelium layer to the vitreous body cavity. All these events are accompanied by cell-to-cell and cell-to-substrate interactions, with the substrates different in the choroid adjoining the pigment epithelium cells and in the vitreous body cavity (Ortiz *et al.*, 1992; Mitashov *et al.*, 1993). The intensity of cell proliferation in the pigment epithelium layer and in the neuroepithelium formed in the vitreous body cavity undergoes changes (Stroeva and Mitashov, 1983). The genes related to

production of the antigens characteristic for the pigment epithelium cells are switched off, while those genes that determine production of the antigens specific for the neuroepithelium cells and differentiating retina are switched on (Mitashov *et al.*, 1995).

Which factors determine changes in the fate of cells from different types of neurons, photoreceptors, and glia during retina regeneration? The molecular mechanisms underlying the choice of cell fate in the newt pigmented epithelium cells are not yet clear. Let us consider, first, the transdifferentiation model of chick embryos. I*n vivo* regeneration of the retina both in adult newts and chick embryos is realized through formation of the neuroepithelium. In the chick embryos, this takes place under the influence of growth factors (aFGF and bFGF) and as a result of active proliferation of the initial pigment epithelium cells. However, differentiated ganglionic cells were found *in vitro* both among dividing pigment epithelium cells and nondividing epithelium cells after their partial depigmentation, although their amount (4-5%) is small (Guillemot and Cepko, 1992). These data allowed a suggestion that the growth factors (FGFs) initiate differentiation of the ganglionic cells, which are the first to differentiate during both normal retina development and its regeneration, directly from the pigment epithelium cells or their descendants after the completion of proliferation. Moreover, transcripts for FGF receptors were found in the pigment epithelium of chick embryos and mammals (Noji *et al.*, 1990; Bost *et al.*, 1992; Tcheng *et al.*, 1994; Torriglia *et al.*, 1994). bFGF was detected in the nucleus and cytoplasm of the RPE cells (Schweigerer *et al.*, 1987; Ishigooka *et al.*, 1992) and during lens regeneration in the newt (Hyuga *et al.*, 1993).

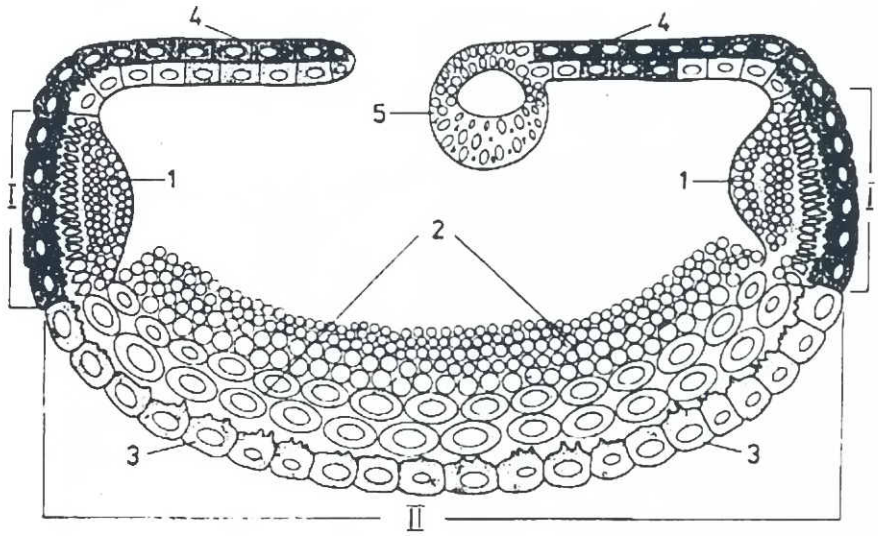

Fig. 4. Schematic drawing of regenerating adult newt eye. *I, peripheral; II, central part of the eyeground; (1) neuroepitelial rudiment formed from ora serrata; (2) neuroepitelial rudiment formed from RPE cells; (3) RPE cells; (4) iris; (5) regenerating lens. At early stages of retina regeneration, all RPE cells at the eye periphery incorporate [^{3}H] DOPA, [^{3}H]thymidine labeling index being 4-6%. During the formation of neuroepithelial rudiment, [^{3}H]DOPA is not incorporated in cells of the central part of the eyeground; [^{3}H]thymidine labeling index in RPE is 40 50%, whereas in the neuroepithelial rudiment it is 70 to 80%. Cells of the two-layered neuroepithelial rudiment contain melanin granules, because the rudiment is formed from RPE, but these granules are absent from multilayered rudiment. The total duration of the cell cycle in the neuroepithelium is 1.5-2.2 times shorter than that in RPE. Expression of NF, N-CAM, and GFAP proteins is observed in the neuroepithelial rudiment but not in RPE (from Stroeva and Mitashov, 1983).*

Hence, the following hypothetical scheme of transdifferentiation of the pigment epithelium cells during retina regeneration is proposed: either preexisting growth factors are activated in the pigment epithelium cells or growth factors come to the pigment epithelium cells from outside as a result of diffusion (Guillemot and Cepko, 1992). Unfortunately, there are no experimental data about the mechanisms underlying the action of growth factors, which realize the choice of differentiation pathway as morphogens. Only specific binding of FGFs to the internal limiting membrane of the retina and Bruch's membrane adjoining RPE was shown (Jeanny *et al.*, 1987; Cirillo *et al.*, 1990; Jacquemin *et al.*, 1993).

What is known about the pigment epithelium of newts, in which retina restitution is most complete? In adult newts at the early stages of retina regeneration before formation of the neuroepithelium rudiment in the pigment epithelium, an intense bFGF expression suggesting the presence of bFGF in the pigment epithelium and in the cells that migrated in the eye cavity was found in the ciliary zone of the iris, macrophages of hemopoietic origin that migrated in the eye cavity, intracellular matrix, and choroid (Mitashov *et al.*, 1993; Markitantova *et al.*, 1995). The macrophages of hemopoietic origin were also intensely stained for tenascin, which contains numerous EGF repeats essential for regulation of cell proliferation. Intense expression of bFGF molecules in the pigment epithelium cells and later in the retinal rudiment and macrophages that migrated in the eye cavity suggest that, just as in the chick embryos, the growth factors act as morphogens to determine changes in the fate of the pigment epithelium cells. Since expression of the growth factors was also found in the macrophages, active contacts of the macrophages with the pigment epithelium and retinal rudiment cells can lead to additional secretion of the growth factors and, possibly, other biologically active factors that transform the pigment epithelium cells in the neuroepithelium rudiment.

Cells of the ciliary area having secretory activity can be another source of biologically active factors. It is also known that the pigment epithelium cells produce factors initiating differentiation of the neural cells (Tombran-Tink *et al.*, 1991, 1992; Steele *et al.*, 1992). These data suggest the presence of intracellular factors in the pigment epithelium that initiate the neural pathway of development. These factors are active in the neuroepithelium rudiment.

On the basis of the available experimental data, I propose the following scheme of sequence of the main events during RPE transdifferentiation in the neuroepithelial rudiment and subsequent formation of the differentiated retina and putative effects of the regeneration-controlling signals.

The retina restitution is realized through formation of an intermediate key structure, regenerate of neuroepithelium. Growth factors (FGFs) and unidentified intra- and/or intercellular biologically active factors are morphogenetic signals that determine changes in

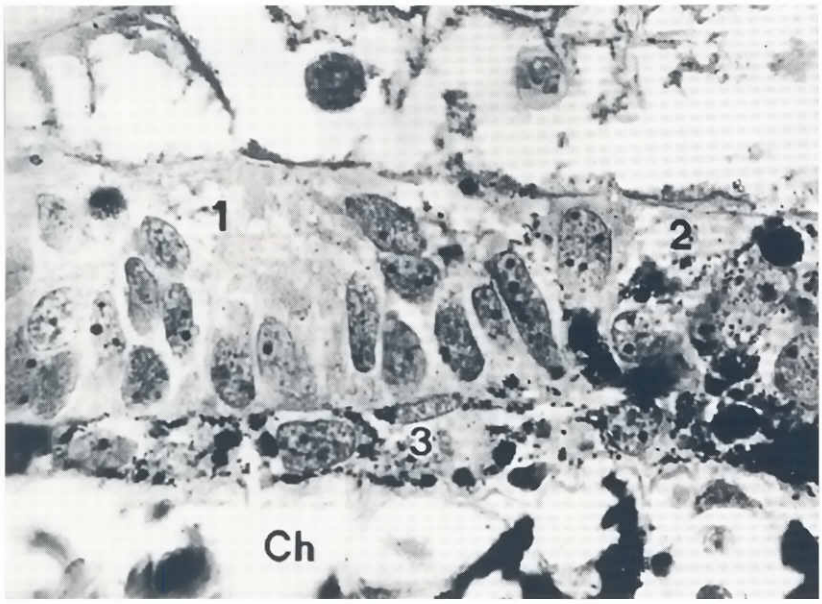

Fig. 5. Two retinal rudiments formed from different cellular sources. *1. Cells of retinal rudiment formed from the anterior complex of the eye; 2. Cells of retinal rudiment formed from RPE cells; 3. RPE cells; Ch, choroid. x280. From Mitashov (1968).*

differentiation of the pigment epithelium cells. The rudiment of neuroepithelium cells form a multilayer neuroepithelium as a result of intense proliferation. The growth factors (FGFs) appear to provide for active proliferation of the neuroepithelium rudiment cells. Differentiation of cells of the ganglionic, internal and external nuclear layers, and glia proceeds under the influence of factors produced as a result of cell-to-cell interactions. The available experimental data suggest that this sequence of events during retina regeneration as a result of RPE transdifferentiation is common for adult newts and early embryos of fish, amphibians, birds and mammals.

During retina regeneration in adult fish, the effects of putative inductive signals depend on character of the retina trauma (Braisted *et al.,* 1994). During restoration of the damaged rods, the local microenvironment is a source of signals of the type of mitogens and differentiation factors. In case of destruction of the photoreceptors (rods and cones), the factors secreted by the pigment epithelium and those of the interphotoreceptor space and external limiting membrane are sources of putative inductive signals. In case of destruction of the photoreceptors and neurons of the internal nuclear layer, inductive signals are produced on both the apical and basal surface of the retina. The signals are yet not identified but there are experimental data suggesting that the growth factors may be such signals (Negishi and Shinagawa, 1993). Although cell sources of regeneration in the both analyzed models of retina regeneration are different, cell transformations connected with the choice of differentiation of the RPE cells and rod precursors have much in common.

And the last question: role of the growth zone cells in retina regeneration. It was repeatedly stressed that involvement of cells of the retina growth zone in retina regeneration in adult newts and fish reflects the growth potencies of precursor cells localized in this area of the retina. Proliferation of the precursor cells in the growth zone is regulated by diffusing growth factors coming from the intercellular matrix and pigment epithelium (Lillien and Cepko, 1992).

Do the different types of cell cycles take place during retinal regeneration?

The main goal in our studies of retina regeneration is to approach the mechanisms of its restoration. I have already mentioned that since the differentiated RPE cells are the cell source of retina regeneration, formation of the neuroepithelium cells is due to transdifferentiation of the RPE cells. Analysis of specific features of proliferative activity of RPE cells in Urodelan and anuran amphibians suggests two types of proliferative activity of the RPE cells connected with cell multiplication, increase in the number of cells. The proliferative activity of this type does not lead to the changes of specific characteristics and their ability to produce the neuroepithelium cells. This type of proliferation of the RPE cells is characteristic of urodelian and anuran amphibians and higher vertebrates and mammals. The RPE cells are currently used for investigation of their behavior and proliferation *in vitro*. The other type of proliferation is most pronounced in adult urodelians and it leads to formation of the neuroepithelium cells, i.e. cells of another type of differentiation. A subpopulation of axolotl RPE cells localized in the embryonic slit area and individual RPE cells of albino *Xenopus* possess the same ability.

Thus, the study of proliferative activity of the RPE cells in urodelian and anuran amphibians during retina regeneration revealed two types of cell cycles: proliferative cell cycles, connected with multiplication of cells in a certain area of the eye and differentiated cell cycles, connected with changes of cell differentiation. Any matrix-bound factors which may influence the cell growth, do not appear to affect the RPE cell transdifferentiation. Which events of retina regeneration are connected with initiation of differentiated cell cycles? Unfortunately we have not yet the answer on this key question. However let us put forward a working hypothesis implying the existence of such event during regeneration. At the early stages of lens regeneration, the changes of transdifferentiating cells connected with removal of glycoprotein from the iris surface were described (Imokawa *et al.,* 1992; Eguchi, 1993). It was proposed that the removal of this factor relieves the differentiated state of cells involved in regeneration and only after this event the cells acquire the capacity for the lens rudiment formation. It is proposed the next hypothetical scheme -passing the RPE cells through differentiated cell cycle occurs after removal from the cells a factor, stabilizing the differentiated state. It is well known that at the early stages of embryogenesis, pigmented epithelium demonstrates a remarkable plasticity. The RPE cells can be induced for transdifferentiation into the retina cells in all studied vertebrates: fish, newts and axolotls, frogs, chicken, and rats. The factor stabilizing the differentiated state was also found on the cell surface of RPE cells, when stratification of the differentiated retina layers is completed (Eguchi, 1993). This suggests that the factor stabilizing the differentiated state is absent in the pigment epithelium at the early stages of embryo-

genesis in amphibians, fish, birds, and mammals, when the regeneration potencies of the RPE cells are quite pronounced. This may explain regeneration potencies of the pigment epithelium at the early stages of eye development.

Gene expression during retina development and regeneration

Search for morphogenetic factors initiating regeneration may also be related to the analysis of gene expression during regeneration. In this area we are at the very beginning. First of all, it is necessary to search for the genes expressed during retina regeneration. Interesting results have been obtained in the studies of gene expression on the *Drosophila melanogaster* embryos. Several of gene families in the *Drosophila* genome responsible for various stages of embryogenesis were established. The success of these studies was determined to a great extent by many extensively characterized mutant strains, including mutations with maternal effects and those specifically affecting early embryogenesis (Lewis, 1978; Kaufman 1983; Gehring, 1987).

Extensive studies are now under way as concerns the analysis of gene expression during normal development of the eye in invertebrate and vertebrate animals. Just as in the studies of normal development, advances in studies of gene expression during development of the eye were determined by the presence of mutations that allowed identification of homeobox-containing genes with the known regulatory function (Matsuo, 1993; Beebe, 1994; Dorn *et al.*, 1994; Quiring *et al.*, 1994; Tremblay and Gruss, 1994; Zuker, 1994; Heberlein *et al.*, 1995; Macdonald *et al.*, 1995; Tomarev *et al.*, 1996).

The most interesting results were obtained by Gehring and his co-workers on ectopic induction of eyes in the wing, limb, and antenna of *Drosophila*, suggesting the presence of genes controlling expression of a cascade of genes involved in regulation of eye morphogenesis (Halder *et al.*, 1995). This function is ascribed to the *Drosophila* gene *ey* (eyeless), whose homologs in mice and humans are genes *Sey* (Small eye or Pax-6) and *Aniridia* (Barinaga, 1995), respectively. Thus, if the genes responsible for morphogenesis of a complex organ, such as the eye, have been identified, are there genes responsible for regeneration of the eye structures? Mutations affecting regeneration are not known and, evidently, studies of genetic mechanisms underlying regeneration require development of alternative methods not based on mutation analysis.

At present, two main approaches are used for identification of the genes expressed during regeneration. One of them is based on production of a bank of the expressed sequences in the regenerating structures and its subsequent screening with the known *Drosophila* probes or vertebrate probes. This approach made it possible to find in the regenerating amphibian limb homeobox-containing genes (Savard *et al.*, 1988; Brockes, 1989, 1992; Tabin, 1989; Beauchamin and Savard, 1992; Simon and Tabin, 1993; Beauchemin *et al.*, 1994; Gardiner *et al.*, 1995), keratin genes (Ferretti *et al.*, 1991), retinoic acid receptors (Ragsdale *et al.*, 1989), tenascin (Onda *et al.*, 1991), FGF receptors (Poulin *et al.*, 1993), and T-box genes (Simon *et al.*, 1997). Expression of the homeobox-containing genes was shown in normal development of the eye in vertebrates and during retina regeneration in the goldfish (Monaghan *et al.*, 1991; Levine and Schechter, 1993; Hitchcock *et al.*, 1995). These genes regulate the boundaries of certain eye structures during development: iris, ciliary body, and retina. Apparently, the genes of this family may fulfil similar functions during retina regeneration.

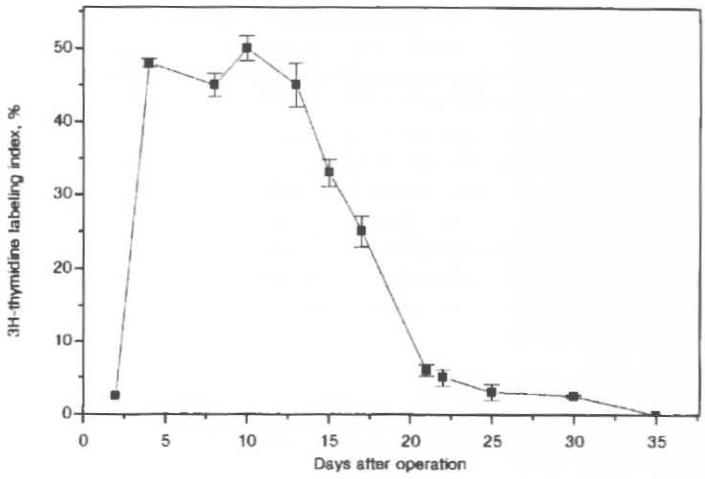

Fig. 6. Change of ^{3}H-thymidine labeling index (%) of RPE cells in adult newt, *T. cristatus* during retina regeneration.

Application of the method of screening of the genome library of the regenerating organs with the known probes allowed identification of genes from the already known families. Analysis of their expression at different stages of regeneration did not yet permit to identify the genes that initiate regeneration of a certain organ or tissue and prove that there are definite, specific «regeneration» genes. The key events of regeneration of a specific organ can be regulated by a specific set of genes that do not have close homologs in the other taxonomic groups. In order to identify these genes, it is necessary to use approaches based on differential expression of genes (Simon and Oppenheimer, 1996; Simon *et al.*, 1997).

Further progress in identification of differentially expressed genes is related to application of gene subtraction (Mitashov *et al.*, 1994; Markitantova *et al.*, 1997). Using this approach we identified expressed new genes during lens regeneration in newts. Expression of these genes was also shown in the early retinal rudiment (Kazanskaya *et al.*, 1995).

It is essential that the identified genes are activated at the early stages of retina regeneration in the neuroepithelium rudiment cells. What is the function of these genes? Is activation of their expression related to synthesis of neurospecific and glial proteins (GFAP, NF, and N-CAM) found by us in the neuroepithelium? Unfortunately, there are no answers to these questions. We recorded expression of the identified genes in the early development of newts and plan to obtain complete copies of cDNA of the identified genes and study their functions.

The experimental data summarized suggest that an extensive information has been accumulated about the cell sources of retina regeneration in vertebrates and about patterns of cell transformations during formation of the neuroepithelium rudiment and subsequent differentiation of the regenerating retina. The main task of the studies of retina regeneration is to understand the mechanisms of restitution. We will be able to better understand the mechanisms of

regeneration, if we identify factors stimulating the RPE transdifferentiation and changing differentiation of the rod precursors, identify the cells producing regulatory morphogenetic factors and their receptors, and identify the genes controlling successive stages of both regeneration and morphogenesis of the eye. All these tasks can be solved in studies with the use of molecular-biological methods. And time for these studies has already come! The model of regenerating eye of the newt is a very convenient models for application of subtracting hybridization. Comparison of the expressed genes in the pigment epithelium, retina, and retinal regenerates will allow identification of the genes specifically expressed in various structures of the eye.

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