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Developmental patterns of crystallin expression during lens fiber differentiation in amphibians

ALEXANDER T. MIKHAILOV[2]*, VLADIMIR N. SIMIRSKII[1], KARINA S. ALEINIKOVA[1], and NIKOLAI A. GORGOLYUK[1]

[1]*Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, Moscow, Russia and* [2]*Institute of Health Sciences, University of La Coruña, La Coruña, Spain*

ABSTRACT Data on activation of crystallin synthesis during lens fiber (LF) formation in amphibians are summarized to point out the questions particularly interesting in the context of lens cell lineage-specific expression programming under different developmental conditions. LFs are known to differentiate throughout life along the same pathway that includes at least five compartments. Using the amphibian eye lens as a model, we have studied how crystallins are expressed in the course of: (1) embryonic LF formation, (2) LF differentiation in adults, and (3) LF transdifferentiation from other (non-lens) eye tissues. Our experiments showed that synthesis of crystallins during morphologically similar LF differentiation in embryonic and adult amphibian lens has different spatial-temporal patterns (i.e., is apparently activated according to different programs). Certain results obtained in our studies suggest the absence of any direct relationship between the capacity of adult newt iris cells to transdifferentiate into LFs and crystallin synthesis («molecular predisposition» to such transdifferentiation) in them. Crystallins appear at the advanced stages of iris transdifferentiation into the lens and dynamics of their synthesis in the «regenerating» lens resembles that in the embryonic lens, although a new lens rudiment develops from the adult iris epithelium. Data on alternative patterns of the crystallin gene activation are summarized and compared with recent observations on spatial-temporal expression of *Pax* genes, which play an essential role in lens cell commitment and crystallin synthesis. On this basis, it is suggested that ontogenetic and tissue- or cell-specific changes in *Pax* gene expression may result in altered programs for activation of crystallin genes in embryonic, adult, and regenerating lens.

KEY WORDS: *lens crystallins, lens fiber differentiation, lens transdifferentiation, amphibians*

Introduction

Amphibian eye lens have been traditionally used in developmental biology studies as a model for analyzing the fundamental biological processes, such as embryonic induction (Mikhailov, 1978, 1988; Servetnick and Grainger, 1991; Li *et al.*, 1994; Grainger, 1996; Servetnick *et al.*, 1996; Altmann *et al.*, 1997), cell differentiation (Piatigorsky, 1981; McDevitt and Brahma, 1982; Mikhailov *et al.*, 1987; Simirskii *et al.*, 1993; Cvekl and Piatigorsky, 1996), transdifferentiation (Okada, 1991; Eguchi, 1993), and molecular evolution (Piatigorsky and Wistow, 1989, 1991; Wistow, 1993; De Jong *et al.*, 1994).

The main bulk of the lens consists of so-called lens fibers (LFs), terminally differentiated translucent cells with specific morphology. They contain high concentrations of lens-specific water-soluble proteins named crystallins. During ontogeny, LFs develop from cells recruited from the lens epithelium (LE), and this process continues throughout life.

Analysis of the published data obtained largely in experiments with mammalian lens suggests that LE probably contains cells manifesting certain properties of stem cells. First, LE cells have the lifelong ability to proliferate (Treton and Courtois, 1981; Chamberlain and McAvoy, 1989; McAvoy and Chamberlain, 1989). Second, some LE cells can form colonies *in vitro* and *in vivo* (McAvoy, 1988). Third, LE cells cloned (cultivated) for long periods of time retain epithelial morphology and the diploid chromosome set (Arita *et al.*, 1988). Some of these cells remain capable of terminal differentiation, as manifested in restricted proliferative potential, formation of

Abbreviations used in this paper: LE, lens epithelium; LF, lens fibers; MW, molecular weight; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; IEF, isoelectric focusing.

***Address for reprints:** Institute of Health Sciences, University of La Coruña, Campus de Zapateira s/n, E-15071 La Coruña, Spain. FAX: (34-81) 16 70 65. e-mail: margot@udc.es

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fiber-like structures, and synthesis of lens-specific markers (crystallins).

Thus, the lens contain cells capable of self-maintenance, proliferation, clone formation, and differentiation. The cells with such parameters were identified in LE of both embryos and adults (see Piatigorsky, 1981; Mikhailov *et al.*, 1987). Lens stem cells are unipotent and, irrespective of extracellular environment, can differentiate only in fiber cells (Simirskii *et al.*, 1991). Some polypeptide factors, acid and basic fibroblast growth factors in particular, stimulate proliferation and differentiation of LE cells (McAvoy and Chamberlaine, 1989; Peek *et al.*, 1992; Robinson *et al.*, 1995).

Differentiation of all LFs, except lens vesicle primary fibers, is morphologically similar and includes a sequence of stages. We distinguish at least five cell compartments in LF lineage (Mikhailov *et al.*, 1996): (1) stem-clonogenic cell compartment, (2) fiber progenitor compartment, (3) differentiating fiber compartment, (4) maturing fiber compartment, and (5) aging fiber compartment (Figs. 2 and 4).

In both embryos and adults, LF differentiation results in activation of crystallin synthesis characterized by specific spatial-temporal pattern. In mammals, differentiation of LE cells into fibers during embryonic development and in the adult state is accompanied by apparently similar patterns of crystallin synthesis (for review, see Simirskii *et al.*, 1993). By contrast, our data on amphibians show that synthesis of crystallins during morphologically similar LF differentiation in embryonic and adult lens of these animals becomes activated according to different programs.

In this paper, we summarize our data on the dynamics of appearance of crystallins during amphibian embryonic development in comparison with that during LF formation in the adult lens. A more general purpose is to show that the approach used in our studies can help to reveal new ontogenetic and morphogenetic peculiarities of crystallin expression patterning in vertebrate eye lens.

In amphibian embryos and adults, LFs can develop under certain conditions not only from LE (normal lens cell lineage) but also from other eye tissues: in particular, from the iris and corneal epithelium (transdifferentiation lens cell lineage). Hence, we present here certain data suggesting that lens transdifferentiation in amphibians is accompanied by activation of crystallin synthesis according to embryo-specific program.

Thus, we have studied how amphibian crystallins are expressed in the course of: (1) embryonic LF formation, (2) LF differentiation in adults, and (3) LF transdifferentiation from the precursor cells of other (non-lens) adult eye tissues.

Our experiments were performed largely on the lens of the common or grass frog, *Rana temporaria L.*, and we considered it appropriate to begin this review from briefly characterizing crystallins of this species.

Rana temporaria eye lens crystallins

Crystallins are generally subdivided into two categories. The first includes α-, β-, and γ-crystallins, which are present in the lens of virtually all vertebrates. The second category comprises taxon-specific crystallins, which occur in several species belonging to different taxonomic groups and are related to metabolic enzymes (Tomarev and Piatigorsky, 1996).

Using biochemical, immunochemical, and molecular-biological methods, we identified in the *R. temporaria* lens not only «canonical» α-, β-, and γ-crystallins, similar in their properties to corresponding proteins of higher vertebrates, but also a taxon-specific protein designated ρ-crystallin. Furthermore, LE and LF of the outer lens cortex proved to contain a major soluble crystallin-like 23-kDa protein immunochemically different from other *R. temporaria* crystallins (Mikhailov *et al.*, 1987; Simirskii *et al.*, 1991, 1993). In this paper, these two proteins receive particular attention.

According to the results of one- and two-dimensional SDS-PAGE, immunoblotting, and IEF, *R. temporaria* α-crystallin is represented by two polypeptide chains: αA (21.6 ± 0.3 kDa, p~ (6.0) and αB (23 ± 0.3 kDa). Note that αA-crystallin is expressed in the lens only, being absent from other organs and tissues. β-Crystallins form five fractions: 24.3 ± 0.4, 25.8 ± 0.3, 27.6 ± 0.2, 28.6 ± 0.2, and 30.8 ± 0.2 kDa (p/6.7-7.3); and γ-crystallin polypeptides form at least three fractions: 18.0, 19.0, and 20.0 ± 0.4 kDa (p/ 7.2 -8.3).

In joint experiments with the Laboratory of molecular genetics, headed by Prof. G.G. Gause (Kol'tsov Institute of Developmental Biology of the Russian Academy of Sciences), we obtained a cDNA library of *R. temporaria* lens (Gause *et al.*, 1980) and used it to identify a clone encoding a novel lens polypeptide (Tomarev *et al.*, 1981, 1984). The latter, designated ρ-crystallin (see: Gause *et al.*, 1986), is a monomeric heparin-binding protein with MW of 36.1 ± 0.4 to 37.0 kDa as determined by SDS-PAGE and gel filtration, respectively. In IEF, purified ρ-crystallin formed three major fractions with p/ 7.4, 7.8, and 8.0 (Simirskii *et al.*, 1990).

This protein belongs to the family of aldehyde/aldose reductases (Carper *et al.*, 1987; Watanabe *et al.*, 1988; Fujii *et al.*, 1990; Dolgilevich *et al.*, 1994) and was detected in the lens of *R. temporaria, R. catesbeiana,* and *R. ridibunda*. In other amphibians studied (*Xenopus laevis, Bufo bufo, Triturus vulgaris,* and *Pleurodeles waltl)* and in mammals, birds, turtles, and fish, the lens proved to be free of ρ-crystallin (Mikhailov *et al.*, 1987; Takenov *et al.*, 1992). Note also that a monomeric 38-kDa protein, designated ρB-crystallin, has recently been identified in the lens of the diurnal-nocturnal gecko, *Lepidodactylus lugubris.* Amino acid sequences of peptides derived from this protein demonstrated 72% identity with the mammalian aldose reductase and 56% identity with the *R. temporaria* ρ-crystallin. As in the case of the *R. temporaria* ρ–crystallin, ρB-crystallin has no enzymatic activity in the gecko lens (Röll *et al.*, 1995).

Unlike α-, β-, and γ-crystallins, ρ-crystallin of *R. temporaria* is not confined to the lens. We revealed its presence in the retina, liver, spleen, testis, kidney, stomach, intestine, lung, and skeletal and heart muscle. Its concentrations, however, were significant only in the lens and retina: up to 10 and 1% of total soluble protein, respectively (Mikhailov *et al.*, 1987; Simirskii *et al.*, 1990, 1991a). In other organs and tissues, ρ-crystallin was detected at housekeeping levels, e.g., no more than 0.05% in the testis and liver, and 0.01% or less elsewhere. These immunochemical results were then confirmed by dot-hybridization with a ρ-crystallin-specific cDNA probe (Dolgilevich *et al.*, 1994).

In the adult frogs, ρ-crystallin was detected in all cells of any region of the lens, including LE, outer and inner cortex, and lens nucleus. In the neural retina, by contrast, this protein was distributed non-uniformly and concentrated only in the inner nuclear layer and ganglionic cell layer.

We have also studied the time-course of appearance and accumulation of ρ-crystallin in embryonic and larval lens and retina of *R. temporaria* (Simirskii *et al.*, 1990). According to immunofluorescence and immunoblotting data, this polypeptide first appears

in the lens, soon after mouth opening in tadpoles (stages 33-35 after Dabagyan and Sleptsova, 1975), and later, in the retina (stages 36-37, by the time of anal orifice opening). The adult level of ρ-crystallin expression is established in the lens and retina by the onset and the end of metamorphosis, respectively. Note that at any developmental stage, its concentration in the lens was approximately five times higher that in the retina. Hence, we proposed that the proportion of ρ-crystallin, specific for the adult lens and neural retina, is already established during larval development.

In general, ρ-crystallin belongs to so-called enzyme-crystallins, i.e., enzymes that have been recruited to perform novel specific functions in the eye tissues (Wistow and Piatigorsky, 1987; Wistow, 1993; Tomarev and Piatigorsky, 1996). Studies on its synthesis in the *R. temporaria* lens and retina could be interesting for analyzing tissue-specific mechanisms of selective overexpression (see Wistow *et al.*, 1994), but this aspect is beyond the scope of our paper.

The crystallin-like 23-kDa polypeptide was discovered in our laboratory accidentally, because of its relatively high concentration in the *R. temporaria* LE (Aleinikova, 1992). We isolated this weakly acidic monomeric polypeptide from LE and lens cortex by multistage ion-exchange chromatography and purified by SDS-PAGE. Immunoblot analysis of its tissue distribution revealed its presence in the lens and retina but not in the liver, kidney, brain, cornea, iris, intestine, and heart of *R. temporaria*. In the lens, 23-kDa polypeptide was detected in the cytoplasm of LE cells and submembrane cytoplasmic regions of LFs. This polypeptide proved to be immunochemically different from αB-crystallin (note that the latter was not detected in the *R. temporaria* neural retina). Among other animals studied, the 23-kDa polypeptide was detected only in the turtle lens, being absent from the mammalian, bird, and fish lens (Takenov *et al.*, 1992).

In the course of this work, we have obtained polyclonal antibodies against different purified crystallins of the *R. temporaria* lens and used them to analyze the patterns of crystallin expression during LF differentiation under different developmental conditions.

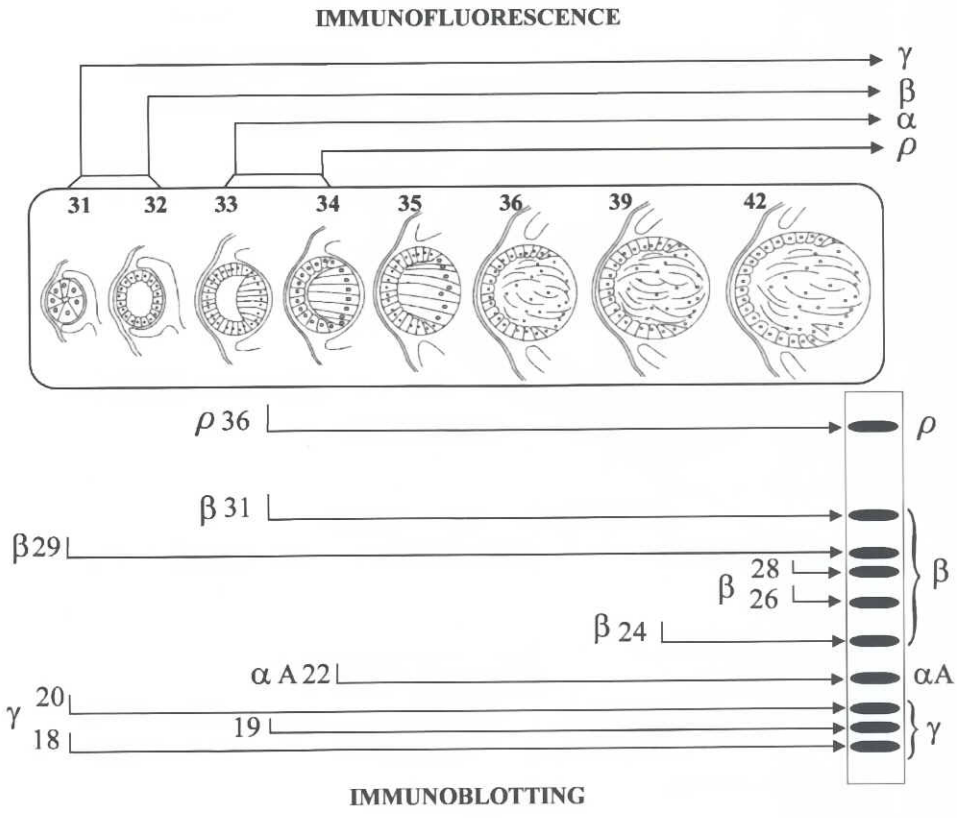

Fig. 1. Appearance of crystallins during embryonic and larval development of *R. temporaria* as determined by immunofluorescence and immunoblotting *(after Mikhailov et al., 1987, modified). Immunofluorescent experiments were performed with paraffin sections of tissues fixed with acetone or Carnoy mixture. Numerals above the lens drawings (31-42) designate the stages of development (Dabagyan and Sleptsova, 1975). In immunoblotting experiments, extracts of tadpole eyes (stages 31-34) or isolated lenses (stages 35-53) were subjected to SDS- PAGE and blotted onto nitrocellulose membranes, which were treated with affinity-purified antibodies against different crystallin polypeptides. Numerals after designations of crystallin polypeptides (18-36) show their apparent molecular weight (kDa).*

Patterns of crystallin expression during embryonic development

We performed experiments with *R. temporaria* embryos (from the stage of lens vesicle formation until the end of metamorphosis, see Fig. 1) using immunofluorescent methods, SDS-PAGE, immunoblotting, and immunoprecipitation.

According to the immunofluorescence data (Mikhailov *et al.*, 1987), individual classes of crystallins appear at different stages of early lens development. Thus, γ- and β-crystallins are first detected in the lens cell cluster facing the eye cup, while αA-, αB- (the latter, see Brahma *et al.*, 1987), and ρ-crystallins appear at later stages, in primary fiber cells filling the lens cavity. Similar dynamics of crystallin expression has been demonstrated for *Xenopus laevis* (McDevitt and Brahma, 1979; Mikhailov and Kornev, 1980) and *Notophthalmus viridescens* (McDevitt and Brahma, 1981a). Note that at the early stages of lens formation, we detected crystallins in LFs only, whereas crystallin-specific immunofluorescent reaction in LE cells was negative.

A similar picture was observed at advanced stages of lens development: expression of different crystallins was restricted to LFs, and fluorescence of LE cells was at the background level. In our previous study, we observed weak and transient fluorescence of LE in lens sections of the *R. temporaria* tadpoles at certain advanced stages of development (Mikhailov and Takenov, 1983). This reaction appeared to be due to the presence of antibodies against non-crystallin proteins in anti-lens antisera used in these experiments.

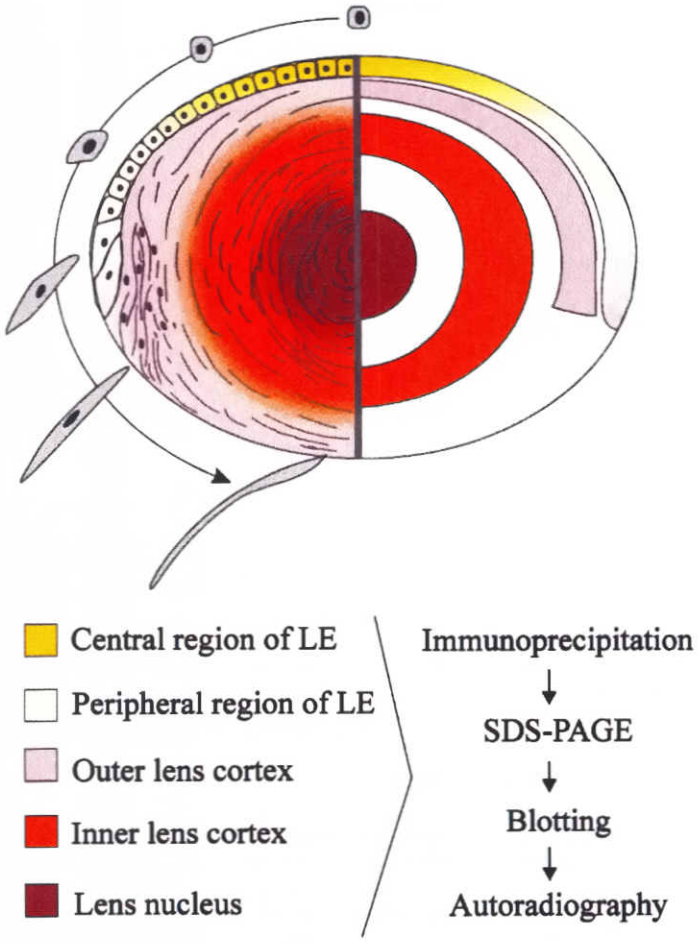

Fig. 2. Diagram showing the structure of amphibian lens (left) and isolated lens regions used in our immunoautoradiographic study (right). *Left: Clonogenic cells are located in the central region of LE. They proliferate, shift toward the lens equator, and begin to differentiate, which is morphologically manifested in cell elongation. Such elongating cells are found at the periphery of LE. Young and mature LFs compose outer layers of the lens cortex, whereas deeper cortical layers consist of terminally differentiated cells. LFs formed during larval and early postlarval development are clustered in the central region of the lens and constitute so-called lens nucleus. Right: Intact lenses or totally explanted LE were incubated in 70% Hanks solution with [^{35}S]-methionine. LE was then divided into the central and peripheral parts, and the lenses were dissected to isolate the outer and inner cortical layers and lens nucleus. The extracts of these lens regions were immunoprecipitated, separated by SDS-PAGE, and blotted onto nitrocellulose membranes. The blots were treated with anti-crystallin antibodies and radioautographed.*

Even in this study, however, we detected no crystallins in the LE of tadpoles completing metamorphosis and in postmetamorphic one-month frogs. Brahma *et al.*, (1987) also showed that α-crystallins are absent from LE of frogs aged two to three weeks.

These results suggest that crystallins, lens-specific polypeptide markers, are not expressed in LE cells during the entire period of embryonic and larval development.

Immunofluorescent methods provide data at the beginning of expression of each crystallin class as a whole. On the other hand, experiments with immunoblotting and immunoprecipitation allowed us to reveal the pattern according to which synthesis of individual polypeptides of a certain crystallin class is activated (Mikhailov *et al.*, 1987; Simirskii *et al.*, 1990). They showed that individual β-crystallin polypeptides appear in the following sequence: 28.6 kDa (stages 31-32), 30.8 kDa (stages 33-34), 24.3 kDa (stage 39), and, finally, 25.8 and 27.6 kDa (stage 42). Thus, activation of synthesis of the 28.6- and 30.8-kDa β-crystallins coincides with the early stages of primary LF formation, whereas other β-crystallin polypeptides appear at more advanced stages of lens morphogenesis, in the secondary LFs. By contrast, ρ- and αA-crystallins first appear in the primary LFs, during a relatively short period of development. All three γ-crystallin polypeptides were detected during the initial phase of primary LF formation.

These results show that, in each class of crystallins, the definitive pattern of expression is established gradually, during consecutive stages of lens development. Hence, fiber cell differentiation in the embryonic lens is accompanied by cascade-like activation of new tissue-specific protein syntheses.

Zinov´eva *et al.*, (1987) analyzed the lens of *R. temporaria* tadpoles to determine the relative contents of different crystallin mRNAs compared to those in the adult frog lens. They found that, at the advanced stages of lens formation (stages 45-46), the proportions of γ4- and ρ-crystallin mRNAs were higher than in poly(A)$^+$RNA isolated from the adult lens, those of γ3-, αA-, and β-crystallin mRNAs were approximately at the adult level, whereas the proportions of γ1- and γ2-crystallin mRNAs were several times lower.

A remarkable feature of the eye lens is that LFs develop *de novo* throughout ontogeny, including adult state. We have addressed the question concerning the pattern of activation of crystallin syntheses during LF formation in the adult amphibian lens: Is it similar to or differs from that in the embryonic lens?

Patterns of crystallin expression in the adult lens

In these experiments, we used metabolic labeling of the lens and isolated LE *in vitro* followed by analysis of their extracts by immunoprecipitation, SDS-PAGE, immunoblotting, and autoradiography (Fig. 2). Synthesis of different crystallins was studied in morphologically distinct parts of the *R. temporaria* lens, including: (1) the central region of LE (contains stem/clonogenic cells), (2) the equatorial region of LE (proliferating and differentiating cells), (3) LFs of the surface (outer) cortex layers (postmitotic differentiated cells), (4) LFs of deep cortical layers (terminally differentiated cells devoid of nuclei), and (5) LFs of the lens nucleus (cells formed during larval and early post-larval development).

At the morphological level, these regions consist of cells at different stages of differentiation, maturation, and aging (Simirskii *et al.*, 1991a,b). Our data on the expression of intermediate filament proteins (vimentin, cytokeratin) and focal adhesion proteins (fodrin and vinculin) in the same regions of the *R. temporaria* lens confirm this conclusion (Table 1; Simirskii et al., 1989). An important fact is that LFs never undergo apoptosis, although the latter occasionally occurs in the LE cells (Morgenbesser *et al.*, 1994).

We used immunofluorescent methods to study spatial distribution of crystallins in paraffin sections of the whole lens and LE cultivated *in vitro* (Simirskii *et al.*, 1991b).

Synthesis of α-crystallins (αA- and αB-chains) was detected in LE and cortex, rather than in the lens nucleus. Immunohistochemi-

cal experiments revealed the absence of these proteins from the central part of LE, a weak positive reaction in the zone of LE cell divisions, and intense labeling in elongating epithelial cells at the lens equator, as well as in the lens cortex fibers (Fig. 3). Thus, activation of α-crystallin synthesis coincides with the earliest stages of LF differentiation.

Synthesis of β-crystallins was observed in the lens nucleus (in traces) as well as in LE and lens cortex. Note that we detected in LE only three out of five β-crystallins (27.6, 28.6, and 30.8 kDa), whereas the inner and outer cortex expressed all five polypeptides. Apparently, the process of terminal LF differentiation is accompanied by activation of synthesis of the 24.3- and 25.8-kDa polypeptides. The appearance of these two β-polypeptides during larval development also coincided with the advanced stages of secondary LF formation (Fig. 1). Immunohistochemical experiments showed that β-crystallins are absent from the central part of LE but appear in elongating epithelial cells (Fig. 3). LFs located at the lens equator and in the outer cortex showed intense immunofluorescence. Thus, β-crystallin synthesis is associated with the early stages of epithelial cell differentiation into LFs, as does α-crystallin synthesis.

Synthesis of γ-crystallins was limited to the lens cortex and nucleus (in traces), being absent from LE. Immunofluorescent reaction for these proteins was negative in all LE regions (Fig. 3) but positive in LFs located in the lens cortex and nucleus. Therefore, γ-crystallins can serve as markers of advanced LF differentiation in the adult lens.

Synthesis of taxon-specific ρ-crystallin was detected in all parts of the adult lens (except lens nucleus) and in all types of lens cells, including those located in the central part of LE. The same applies to the 23 kDa-polypeptide, which proved to be closely associated with the LF plasma membrane. Thus, synthesis of ρ-crystallin and 23-kDa crystallin-like protein in LE is a constitutive process, and activation of their expression is unrelated to differentiation of epithelial cells into LFs.

TABLE 1

EXPRESSION OF INTERMEDIATE FILAMENT AND FOCAL ADHESION POLYPEPTIDES IN *R. temporaria* LENS EPITHELIUM, CORTEX, AND NUCLEUS AS DETERMINED BY IMMUNOBLOTTING

Polypeptide	MW, kDa	Epithelium	Cortex	Nucleus
Vimentin	57	+	+	-
	54	+	-	-
	51*	+/-	-	-
	43*	+/-	-	-
Cytokeratin 8	57	+/-	-	-
α-Fodrin	230	+	+	-
	150*	+/-	+	+
Vinculin	115	+	+/-	-

* Proteolytic fragments (fractions) of vimentin and fodrin.
(+) intensive reaction, (+/-) trace reaction; (-) no reaction in immunoblotting.

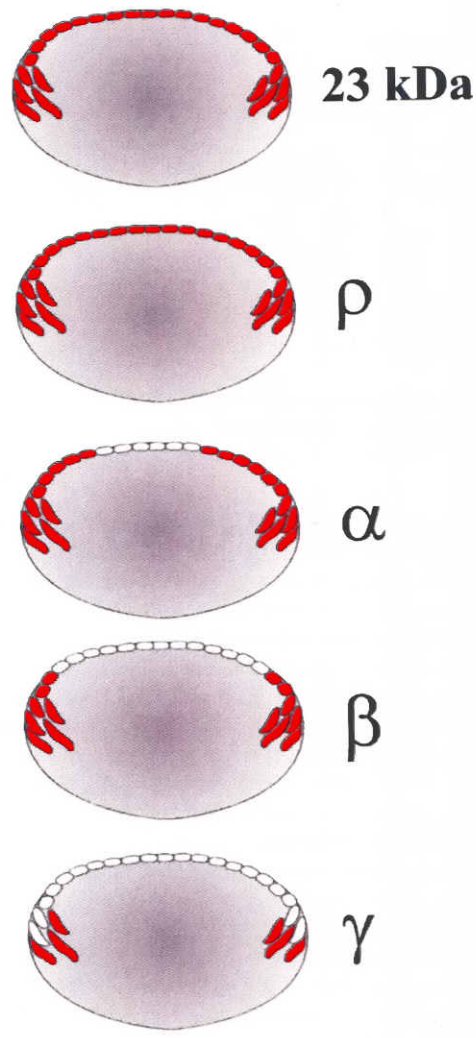

Fig. 3. Scheme showing the distribution of different crystallins in lens epithelium of adult frogs *(data obtained in immunofluorescent experiments with paraffin sections of Bouin-fixed lenses or isolated LE precultivated in vitro). The central region of LE is free of α-, β-, and γ-crystallins and contains only ρ-crystallin and 23-kDa crystallin-like polypeptide; α- and β-Crystallins appear in the peripheral LE regions, and g-crystallins are first detected in differentiating LFs located near the lens equator.*

These results show that the stem/clonogenic cells located in the central part of LE do not contain «canonical» α-, β-, or γ-crystallins (Figs. 3 and 4). While these cells proliferate, move toward the lens equator, and elongate, α- and then β-crystallin syntheses become activated. Synthesis of γ-crystallins begins later, in differentiating LFs located near the lens equator. We observed the same pattern of crystallins appearance in the isolated adult LE cultivated in a medium containing the retina extract. During embryonic development, by contrast, γ- and β-crystallins appear at the earlier stages of LF formation than α- and ρ-crystallins.

Taken together (see Fig. 4), these data suggest that patterning of crystallin synthesis during LF differentiation from embryonic precursor cells, on one hand, and from clonogenic cells of the adult LE, on the other, may depend on different mechanisms.

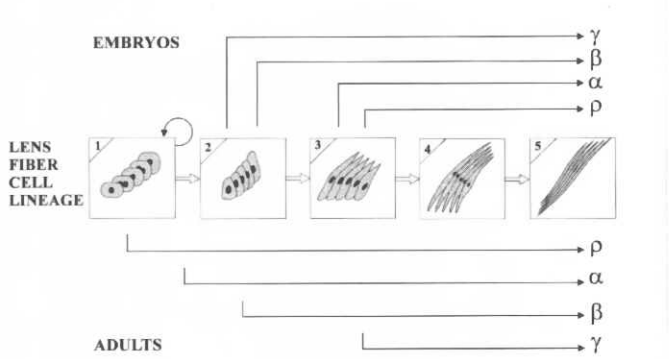

Fig. 4. Appearance of crystallins during LF differentiation from embryonic precursor cells and clonogenic cells of the adult LE *(after Mikhailov et al., 1996, modified). 1-5 - compartments of LF cell lineage (see text).*

At present, we suggest two main ideas at respect, namely: "replacement" and "switching" models. According to a "replacement" model, different crystallin classes are expected to be differently expressed in different precursor (larval LE) or stem-like (adult LE) cells, which start to differentiate into LFs under ontogenetically different conditions. Strikingly, a similar "terminal" cell morphology, as well as a cell lineage can arise from distinct ontogenetic pathways. In contrast, in a "switching" model, the switching in the pattern of crystallin activation may occur in the same stem-like cell population of LE. Note that during the cultivation of chicken or amphibian LE cells, no singes of their "separation" in distinctive subpopulations could be observed (see Piatigorsky, 1981; Simirskii *et al.*, 1991a). These observations favor the idea that, at least in amphibians, the "crystallin pattern switching" may occur in the same stem cell population of LE. The latter may provide a new possibility for investigating the temporal (ontogenetic) regulation of crystallin gene expression during the process of LF differentiation.

It is interesting to compare our results with the data on *Pax* genes, which play an essential role in the eye/lens development and may be required for regulation of crystallin expression patterning (Walther and Gruss, 1991; Cvekl *et al.*, 1994; Li *et al.*, 1994; Richardson *et al.*, 1995; Cvekl and Piatigorsky, 1996; Tomarev, 1997, this issue). In the adult amphibian (newt or axolotl) lens, *Pax-6* expression is generally confined to LE, whereas in the developing or regenerating lens it appears to be uniform in all cells (Del Rio-Tsonis *et al.*, 1995). Hence, ontogenetic changes in *Pax* gene expression may result in altered programs for activation of crystallin genes in the embryonic and adult lens.

Pax-6 can activate or inhibit a number of crystallin genes in both LE and LFs (Cvekl and Piatigorsky, 1996). In chickens, for example, *Pax-6* is expressed at the highest level in LE and may prevent the expression of βA3/A1- and βB1-crystallins in the LE by limiting the access of transcriptional activators for their promoters (Duncan *et al.*, 1997).

In *Xenopus*, ectodermal explants injected with RNA encoding *Pax-6* revealed a positive reaction with antibodies against βB1-crystallin, but did not label with antibodies against the neural-specific markers (Altmann *et al.*, 1997). These results indicated that *Pax-6* provides a lens inducing signal for amphibian gastrula ectoderm, and the establishment of *Pax-6* expression in presumptive lens ectoderm is likely to be an important step in the process of lens commitment and crystallin gene activation.

Ectopic expression of individual crystallins, observed in the cells of several non-lens tissues and organs (Clayton *et al.*, 1986; Kondoh *et al.*, 1987; Sawada *et al.*, 1993; Smolich *et al.*, 1994), suggests that transcription of the genes encoding each individual class of crystallins may be regulated independently. Nevertheless, we believe that normal LF commitment/differentiation (leaving aside the lens transdifferentiation phenomena) is a coordinated unidirectional process involving cascade-like lens-specific activation of crystallin syntheses at the advanced stages.

Moreover, non-crystallin genes identified in chicken LFs (or, to be more precise, their cDNA clones) are also expressed in the lens-specific manner; some of these clones correspond to the genes encoding regulatory proteins (Sawada *et al.*, 1996).

In vertebrates, lens protein composition is characterized by certain species-specific features suggesting spatial-temporal differences in the crystallin gene activation during LF development (Simirskii *et al;*, 1993). In this context, it is appropriate to refer to our results on distribution of crystallins in the adult turtle *(Testudo horsfield)* lens (Takenov *et al.*, 1992). Using SDS-PAGE and immunoblotting, we detected the following crystallins in this species: αA- (20.6 kDa), β- (25.4, 26.3, and 27.5 kDa), τ- (47.3 kDa), δ- (50.1 kDa), and the 23-kDa crystallin-like polypeptide; ρ- and γ-crystallins were absent.

Immunohistochemical experiments revealed the presence of α- and β- crystallins in both LE and LFs. Specific reactions for these proteins were weak in the central region of LE, increased in its equatorial zone, and attained a maximum in the outer cortical layers. Hence, the pattern of α- and β-crystallin expression during LE cell differentiation into LFs in the turtle is more similar to that in birds rather than in amphibians and mammals (see Simirskii *et al.*, 1993).

We are now only at the beginning of experimental analysis of the mechanisms responsible for these processes. However, we believe that morphologically similar processes of LF formation in the vertebrate lens may be accompanied by different programs of cell lineage-specific protein expression, depending on the developmental stage and animal species. The suggestion that terminal LF differentiation under different conditions is achieved via different

TABLE 2

CRYSTALLIN SYNTHESIS IN CORNEAL AND IRIS EPITHELIA OF ADULT *R. temporaria, X. laevis,* and *T. vulgaris* (IMMUNOAUTORADIOGRAPHIC DATA)

Species	*R. temporaria*			*X. laevis*			*T. vulgaris*		
Crystallins	α	β	γ	α	β	γ	α	β	γ
IRIS :									
lens regeneration		no			no			yes	
crystallin synthesis	-	+	-	-	+	-	-	+	-
CORNEA :									
lens regeneration		no			no			no	
crystallin synthesis	-	+	-	-	+	-	-	+	-
CONTROL :									
(LE + LFs)									
crystallin synthesis	+	+	+	+	+	+	+	+	+

(+) positive labeling, (-) no labeling.

morphogenetic pathways appears less probable. Known morphogenetic differences in the lens organogenesis or variations in its size and shape do not affect the process of LF differentiation, at least in amphibians studied to date. For example, the early stages of lens formation in *X. laevis* significantly differ from those in *R. temporaria,* but the process of LF differentiation and dynamics of embryo-specific crystallin expression in these species are similar (see Mikhailov *et al.,* 1987).

Patterns of crystallin expression during lens transdifferentiation

Transdifferentiation, previously known as metaplasia or cell type conversion, is the process by which terminally differentiated cells change their «identity» from one cell type to another (Okada, 1986, 1991; Eguchi and Kodama, 1988; Okada and Yasuda, 1993).

In amphibians, the sources of *de novo* LF formation after lentectomy or from non-lens eye tissues isolated *in vivo* and *in vitro* may include: (1) epithelial layers of the iris in newts (Eguchi *et al.,* 1974), (2) pigmented epithelium of the retina in newts (Eguchi, 1979), and (3) corneal epithelium of tadpoles in *X. Laevis* and *R. temporaria* (see Simirskii, 1981, 1982). We will briefly discuss two aspects of lens transdifferentiation in amphibians, namely: (1) the patterns of crystallin expression during LF differentiation from the iris in the adult newts, and (2) trace expression of LF-specific markers (i.e., crystallins) in non-lens eye tissues capable of transdifferentiation into LFs.

Lens transdifferentiation from the iris (Wolffian lens regeneration) has been demonstrated for many urodele amphibians, including all species of newts studied to date (Eguchi, 1988). According to immunohistochemical data, γ- and β- crystallins are the first to appear during lens regeneration. This occurs in slightly elongated cells of the posterior (inner) wall of the lens vesicle "regenerate" (i.e., in the prospective primary LFs). Thereafter, α-crystallins appear in the centrally located primary LFs of the regenerate. In the anterior (outer) layer of the lens rudiment, which develops into LE, α- and β-crystallins (but not γ-crystallins) are first detected when the formation of the secondary LFs begins in the regenerate (McDevitt and Brahma, 1981b). Note that β- and γ-crystallins appear in the lens earlier than α-crystallins during embryonic development in the newt. Consequently, the pattern of γ-, β-, and α-crystallins' appearance during lens regeneration is similar to that in the newt embryonic LFs, although the lens regenerates from the adult iris cells.

Some data indicate, however, that such a conclusion may be oversimplified. First, α-crystallins have not been detected in the larval newt lens until metamorphosis, whereas in the regenerating lens they appear long before regeneration is completed (McDevitt, 1982). Furthermore, the primary structure of γ-crystallins of the regenerated and normal newt lens proved to be slightly different (McDevitt and Brahma, 1982). Finally, the γ-crystallin content in the regenerating lens is higher than in the normal lens. Unfortunately, the attempts to use molecular probes for identifying spatial-temporal patterns of crystallin expression in either embryonic or transdifferentiating newt lens resulted in several short reports providing a limited information (see, Agata *et al.,* 1987; Mitashov *et al.,* 1992; 1994).

A possible association between crystallin expression in non-lens eye tissues and their potential for transdifferentiating into LFs have been repeatedly discussed during the long history of studies on lens differentiation and transdifferentiation (Clayton *et al.,* 1986; Okada and Yasuda, 1993). To elucidate this problem, we have previously studied crystallin synthesis in the cornea and iris of different amphibian species by means of metabolic labeling *in vitro* followed by immunoautoradiography (Simirskii *et al.,* 1984).

Later, we repeated some of these experiments using antibodies against our highly purified preparations of α-, β-, and γ-crystallins. The labeled LE and LFs isolated from the equatorial region of *R. temporaria, X laevis* and *T. vulgaris* lens served as control (Table 2).

Synthesis of β-crystallins (at relatively low levels) was observed in the corneal and iris epithelia of all species studied, regardless of the potential of these tissues for transdifferentiation into the lens. An interpretation of these observations is somewhat complicated because of a proven relationship between the β/γ-crystallin superfamily and epidermis-specific proteins in the newts (Takahashi and Takeshima, 1995; Wistow *et al.,* 1995). Note that we detected neither α- nor γ- crystallins in the amphibian cornea and iris. On the other hand, these tissues of *R. temporaria* demonstrated ρ-crystallin synthesis, but at very low (housekeeping) levels. Hence, we proposed that ρ-crystallin in the cornea and iris performs an enzymatic rather than structural function. Taken together, these results testify against a direct relationship between the capacity of adult newt iris to transdifferentiate into lens and the synthesis of crystallins in it. Transdifferentiation of the iris epithelial cells into LFs requires DNA synthesis and cell proliferation. By modifying experimental conditions, e.g., the duration of *in vitro* cultivation, properties of the substrate, and the presence of soluble factors, it is possible to control proliferation, choice of fate, and lens commitment of the initial iris epithelial cells (Eguchi, 1979). Moreover, after the «genomic decision» to switch on and start a new program, several intra- and extracellular factors may further control the realization of selected pathway of lens transdifferentiation, including crystallin gene activation.

Crystallins are found at advanced stages of iris transdifferentiation into the lens, and the dynamics of their appearance in the regenerate reminds that in the embryonic lens. Hence, we regard as ineffectual the attempts to reveal molecular mechanisms of iris transdifferentiation into lens by studying crystallin expression in the lens regenerate. Analyzing other types of regeneration-responsive molecules (Imokawa and Eguchi, 1992), as well as the expression patterns of master genes for eye morphogenesis (Del Rio-Tsonis *et al.,* 1995; Cvekl and Piatigorsky, 1996; Gehring, 1996) in the iris and regenerating lens, appears to be a more productive approach to this problem.

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