

Ontogeny and Localization of Gamma-Crystallins in *Rana temporaria*, *Ambystoma mexicanum* and *Pleurodeles waltlii* Normal Lens Development

SAMIR K. BRAHMA AND DAVID S. McDEVITT

*Department of Medical Anatomy and Embryology, The State University,
Janskerkhof 3A, Utrecht, The Netherlands, and*

*Department of Animal Biology, School of Veterinary Medicine,
University of Pennsylvania, Philadelphia, Penn. 19174, U.S.A.*

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Rana pipiens lens γ -crystallin antibodies were used in the indirect immunofluorescence staining method to investigate the role of γ -crystallins in the normal lens development of the amphibians *Rana temporaria*, *Ambystoma mexicanum* and *Pleurodeles waltlii* Michah.

In each case, the fluorescence was first localized in a number of cells in the inner wall of the lens, which was in the vesicle stage. With further differentiation, the intensity of immunofluorescence gradually increased and was restricted only to the fibre cells.

These results support the concept that, though the γ -crystallins of different amphibian species studied so far show different numbers of protein components by thin layer isoelectric focusing on polyacrylamide gel, they all have similar immunological properties, and are specific for the lens fibre differentiation.

1. Introduction

The γ -crystallins are known to have the lowest molecular weight, lowest electrophoretic mobility, and highest isoelectric points of the lens crystallins. Initiation of synthesis of this crystallin group is accompanied by a differentiation of the lens epithelial cells from the "germinative" region into fibres (Papaconstantinou, 1965, 1967; Takata, Albright and Yamada, 1966). In urodele lens regeneration, the fibre cells show a strict temporal relationship between terminal S phase and initiation of γ -crystallin synthesis (Yamada, 1967).

The rate of differentiation of the epithelial cells into fibres is gradually slowed down with the growth of the lens, and in a fully developed lens, the fibre cells are in an irreversible stationary phase (Papaconstantinou, 1967), while the epithelial cells are in reversible G_0 phase (Harding, Reddan, Unakar and Bagchi, 1971).

Biochemical evidence shows that initiation of γ -crystallin synthesis in the region of cellular elongation is sensitive to actinomycin D treatment (Papaconstantinou, Stewart and Koehn, 1966; Papaconstantinou, 1967; Stewart and Papaconstantinou, 1967a), and as the maturation process continues, γ -crystallin synthesis in these cells becomes insensitive to such a treatment (Stewart and Papaconstantinou, 1967a,b).

It has been shown that though γ -crystallin fractions form a major part of the soluble lens crystallins in the early stage of *R. catesbeiana* and *Xenopus laevis*, they show a decrease in concentration in relation to total soluble protein content with aging (Polansky and Bennett, 1970, 1973; Brahma and Bours, 1972).

The time of first appearance of γ -crystallins reported so far in the amphibian lens is unclear. According to Yamada (1967), in the regenerating newt lens α -, and β -crystallins appear in the fibre cells before the γ -crystallins; Yamada (1966), however,

stated that the results obtained with anti- α - and anti- β -crystallin antisera should be evaluated with reservation because of the antigen contamination. Ogawa (1965) also reported that in normal lens development of the Japanese newt *Triturus pyrrhogaster*, α - and β -crystallins appear first, and his results have been discussed by McDevitt, Meza and Yamada (1969). McDevitt (1972), and McDevitt and Brahma (1973) reported that γ -crystallins are one of the first of the lens crystallins to appear during normal lens development in *R. pipiens* and *X. laevis* embryos.

Rana pipiens anti- γ crystallin antibody has been utilized to detect γ -crystallins in *Nothophthalmus* (*Triturus*) *viridescens* lens, both in vivo, and in vitro (Nöthiger, McDevitt and Yamada, 1971; Yamada, Reese and McDevitt, 1973).

The investigation of the role of γ -crystallins in normal lens development was extended to one anuran and two urodele species, i.e. *Rana temporaria*, *Ambystoma mexicanum*, and *Pleurodeles waltlii* Michah using antibodies to purified adult *R. pipiens* γ -crystallins in the indirect immunofluorescence staining method.

2. Materials and Methods

Eggs of *A. mexicanum* and *P. waltlii* Michah were obtained from the Hubrecht Laboratory, Uithof, Utrecht, and *R. temporaria* eggs were collected during the breeding season from ditches near Uithof. These were reared at room temperature ($18^{\circ}\text{C} \pm 2$) and the embryos were fixed at regular intervals in precooled 95% alcohol at 4°C , processed and sectioned at $5\text{ }\mu\text{m}$ thickness, according to McDevitt et al. (1969). Staining of the sections was carried out according to Brahma and van Doorenmaalen (1971).

For normal histology of the lens, materials were fixed in parallel in Bouin's, processed, sectioned at $7\text{ }\mu\text{m}$ thickness and stained with hematoxylin and eosin according to standard histological procedures.

The stages of normal external development of *R. temporaria* were determined according to Shumway (1940) for *R. pipiens*. For *A. mexicanum* and *P. waltlii* we followed the staging of Laevitt (1948) and Gallen and Drocher (1959), respectively. As the development of lenses in all these species resembled that of *R. pipiens* described by McDevitt et al. (1969) we staged the lenses accordingly.

Isolation and preparation of specific antiserum against purified γ -crystallin antigen from adult *R. pipiens* lenses have already been described (McDevitt, 1967; McDevitt et al., 1969).

The specificity of the antiserum was controlled by replacing the specific antiserum by serum from non-immunized rabbit in the immunofluorescence staining procedure.

Sections through the lens region from the experimental and control animals were examined with E. Leitz fluorescence microscope (dark field) equipped with filter systems described previously (Brahma and van Doorenmaalen, 1971; Brahma, Rabaey and van Doorenmaalen, 1972).

Total soluble lens proteins of *R. temporaria*, *R. pipiens* and *A. mexicanum* were tested against *R. pipiens* lens γ -crystallin antibody by Ouchterlony's double diffusion method (1953) at the room temperature for 48 hr. The gel was prepared with 1.5% Bacto agar (Difco Laboratories) in high resolution buffer (Aronsson and Grönwall, 1957) at pH 8.9. The gel was washed in saline, dried and stained with Amido black.

Total soluble lens proteins of *R. temporaria*, *R. pipiens* and *A. mexicanum* were submitted to thin-layer isoelectric focusing on polyacrylamide gel according to Bours (1971), and stained with Coomassie brilliant blue R-250 (Serva).

Due to a lack of materials, we could not use soluble lens proteins from *P. waltlii* in the immunodiffusion and isofocusing experiments.

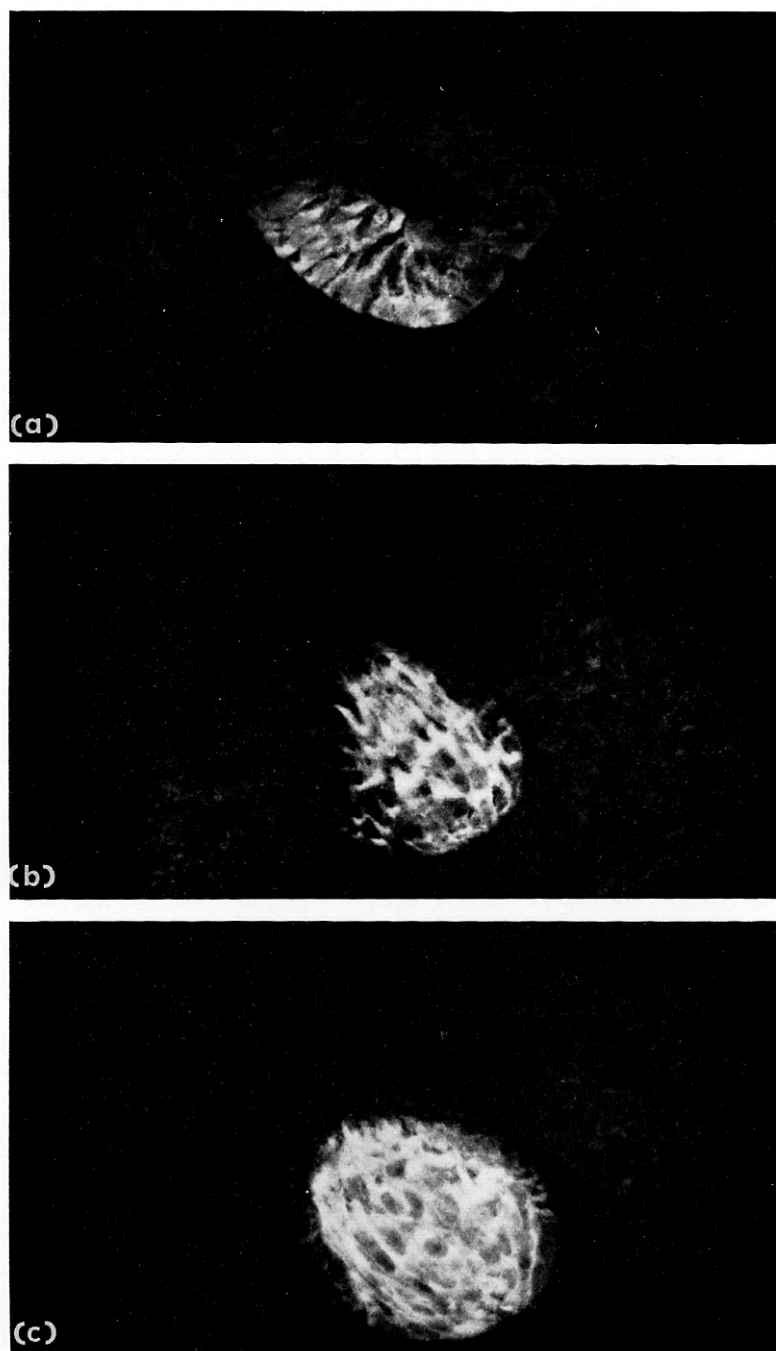


FIG. 1. Dark-field immunofluorescence photomicrographs of sections through the eye regions of embryonic *R. temporaria* treated with *R. pipiens* anti- γ crystallin antibody. (a) Stage VI. (b) Stage IX. (c) Stage X.

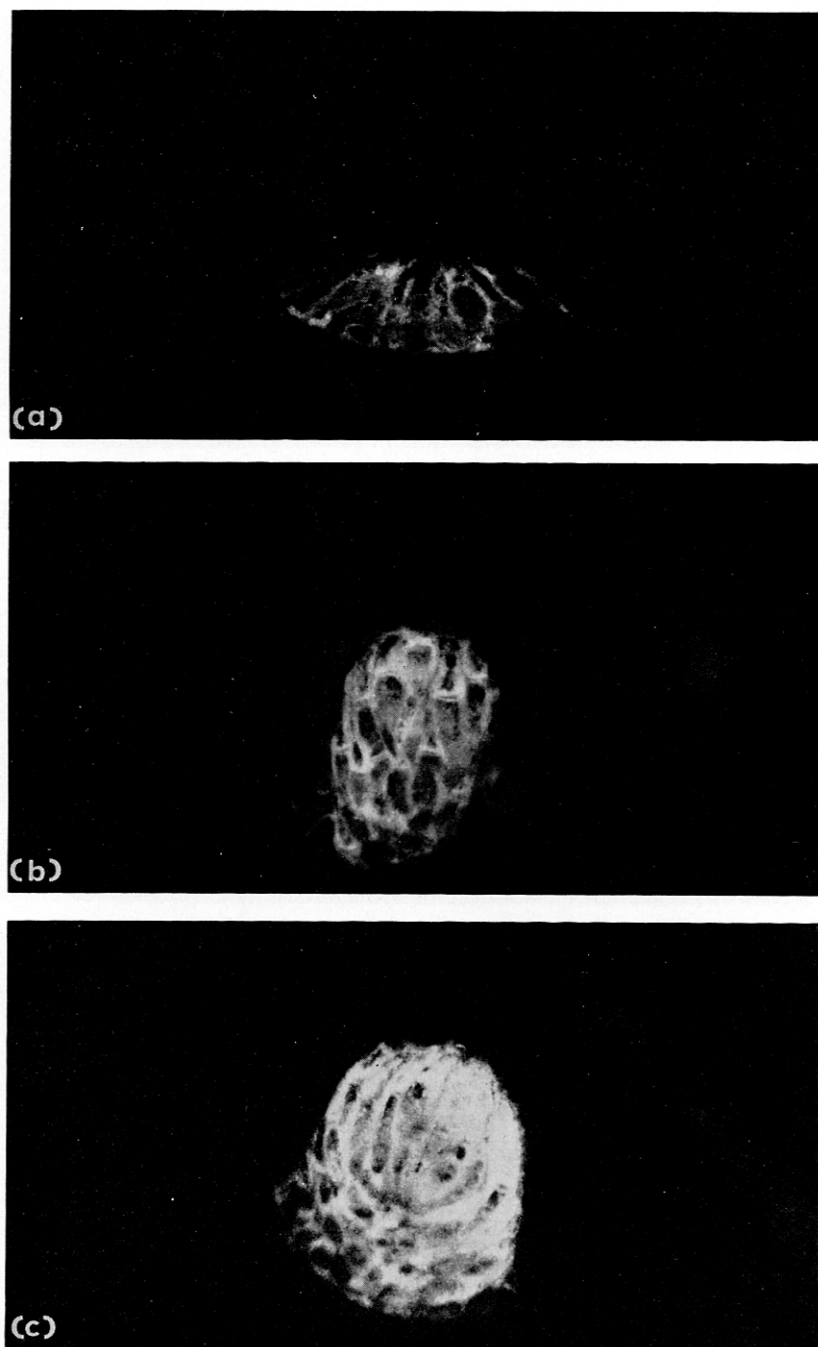


FIG. 2. Dark-field immunofluorescence photomicrographs of sections through the eye regions of embryonic *A. mexicanum* treated with *R. pipiens* anti- γ crystallin antibody. (a) Early stage VI. (b) Stage IX. (c) Stage X.

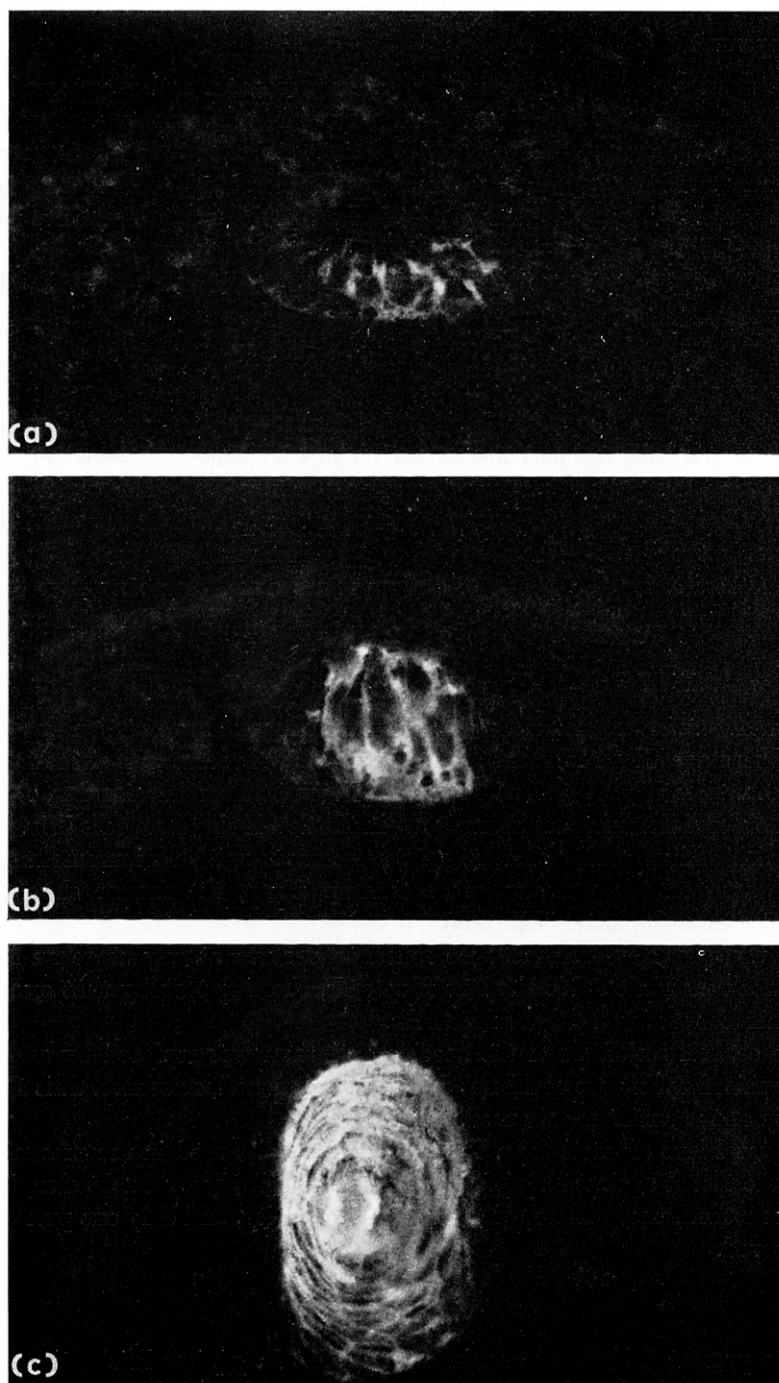


FIG. 3. Dark-field immunofluorescence photomicrographs of sections through the eye regions of embryonic *P. waltii* treated with *R. pipiens* anti- γ crystallin antibody. (a) Early stage VI. (b) Stage VIII. (c) Stage X.

3. Results

Rana temporaria

In this species, the first positive immunofluorescence reaction appeared at lens stage VI (Shumway, stage 21) in the cells comprising the inner wall of the lens vesicle [Fig. 1(a)]. In the lens stage IX (Shumway, stage 24) the immunofluorescence reaction was found to be restricted to the fibre cells [Fig. 1(b)], and in the lens stage X (Shumway, stage 25) the secondary fibres also started to show positive immunofluorescence [Fig. 1(c)].

Initially, the intensity of reaction was weak, but became stronger with the development of the lens.

Ambystoma mexicanum

In this species the first positive immunofluorescence reaction appeared earlier than in *R. temporaria*, and the lens was at early stage VI (Laevitt, stage 37). Like the previous case, the immunofluorescence reaction was found to be localized in a number of cells of the inner layer of the lens vesicle [Fig. 2(a)]. At lens stage IX (Laevitt, stage 38) only the primary fibres showed positive immunofluorescence [Fig. 2(b)]. At stage X (Laevitt, stage 39) the secondary fibres also became positive but their intensity of immunofluorescence was less than that of primary fibres [Fig. 2(c)].

Pleurodeles waltlii Michah

As with *A. mexicanum*, the first positive immunofluorescence reaction was observed at early lens stage VI (Gallien and Drocher, stage 33a) in some cells of the inner layer of the lens vesicle [Fig. 3(a)]. In the stage VIII lens (Gallien and Drocher, stage 33b), we could observe immunofluorescence only in the developing fibre cells [Fig. 3(b)]. At lens stage X (Gallien and Drocher, stage 34) there was transition of immunofluorescence intensity between the primary and secondary fibres, the former showing more fluorescence than the latter [Fig. 3(c)].

In all these species examined, we failed to observe at any stage an immunofluorescence reaction outside the lens fibre cells, showing thereby that synthesis of γ -crystallins is restricted only to fibre cells.

In the control series, when the specific antiserum was replaced by serum from non-immunized rabbit, we could not detect any immunofluorescence. This shows the specificity of the antiserum we used.

When total lens extracts from these different species used in the present experiments were tested against *R. pipiens* γ -crystallin antibody by Ouchterlony's double diffusion method, they all showed a reaction of identity, and only one precipitin line was observed.

4. Discussion

Results presented here once again confirm that *R. pipiens* lens anti- γ crystallin antibody can successfully be used to study the ontogeny of this crystallin in other amphibian species, even in those belonging to different orders.

In each case investigated, we found that an immunofluorescence reaction first appeared in a number of cells forming the inner layer of the lens vesicle. In *A. mexicanum* and *P. waltlii* it appeared earlier than in *R. temporaria*, and this could be due to a difference in the rate of lens development in these species.

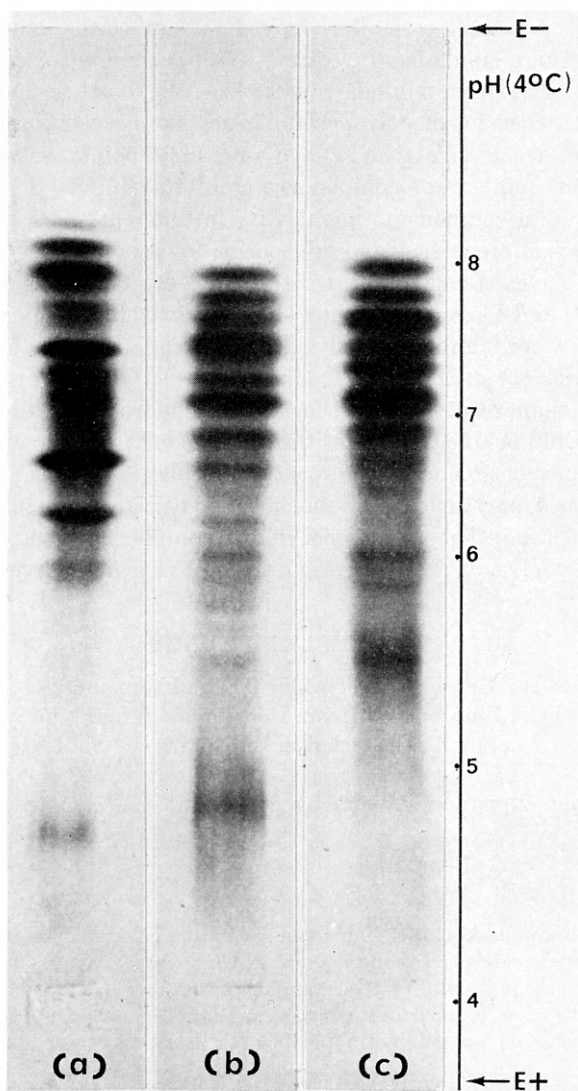


FIG. 4. Thin layer isoelectric focusing on polyacrylamide gel of total soluble lens proteins of *A. mexicanum*, *R. pipiens* and *R. temporaria*. The gel was prepared according to Bours (1971). Five per cent polyacrylamide gel was prepared as a final concentration of 2% "Ampholine" carrier ampholytes (LKB), pH range 3-10, and this was obtained by mixing ampholines having pH ranges from 3-5, 5-7 and 7-10 in equal proportions. Ten μ l of 2% solutions of the lyophilized extracts were used for each sample and the electrodes were placed at a distance of 13.5 cm from each other. The run was for 24 hr at 4°C with initial current of 3.5 mA and 50 V.

The pH was measured at 4°C with flat membrane glass electrode (Radiometer G 242C) on right and left sides of the gel along the sample migration axis.

The proteins were fixed with 14% trichloroacetic acid (TCA) washed extensively in different concentrations of TCA, and stained with Coomassie brilliant blue R-250 (serva) and then photographed.

(a) *A. mexicanum*. (b) *R. pipiens*. (c) *R. temporaria*.

Previously, Nöthiger et al. (1971) reported immunochemical identity between the γ -crystallins of *R. pipiens* and *N. viridescens*. These authors also reported that in agar electrophoresis, *R. pipiens* γ -crystallins moved in four bands, while *N. viridescens* γ -crystallin moved in a single band. McDevitt (1967) reported the existence of four fractions of γ -crystallins from adult *R. pipiens* lens. We, on the other hand, found by thin-layer isoelectric focusing on polyacrylamide gel that γ -crystallins of *A. mexicanum*, *R. pipiens* and *R. temporaria* which have isoelectric points between pH 7.0 and 8.0, show different numbers of components [Fig. 4(a),(b),(c)]. Thus, although the number of γ -crystallin components might vary in different species, they all possess similar immunological properties, and are specific for lens fibre differentiation.

In bovine lens, the existence of four fractions of γ -crystallins has been reported by Björk (1961, 1964) and Papaconstantinou (1965). Björk (1964) reported that three of the four fractions were immunologically identical, while the fourth fraction showed partial identity with the other three.

Immunological similarities between the γ -crystallins from amphibians belonging to different genera could be due to the fact that the urodeles and the anurans originated from a common ancestry (*Order Phyllospondyli*, Noble, 1931). The γ -crystallins have apparently remained unchanged immunologically, i.e. at their reactive site, during the course of evolution. Their physicochemical complexity could be due to slight changes in amino acid sequence occurring during phylogenetic divergence.

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