

Ontogeny of α A and α B Crystallin Polypeptides During *Rana temporaria* Lens Development

S. K. BRAHMA, D. S. McDEVITT* AND L. H. K. DEFIZE†

Department of Medical Anatomy and Embryology, The State University, Janskerkhof 3A, 3512 BK, Utrecht, The Netherlands, * Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104, U.S.A. and † Hubrecht Laboratory, International Embryological Institute, Uppsalalaan, 3584 CT Utrecht, The Netherlands

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The ontogeny and localization of α A and α B polypeptide chains of α -crystallin were investigated in the developing lens of *Rana temporaria*, an anuran amphibian, using the indirect immunofluorescence staining method with heterologous antibodies directed against these two polypeptides. α A and α B crystallins are primary gene products and are translated by different mRNAs in mammals. Although they show about 6000 amino-acid sequence homology (Bloemendal, 1977), the α A cDNA of rat and mouse does not hybridize to α B mRNA (Dodemont et al., 1981; King and Piatigorsky, 1983). Antigenically too, α A and α B polypeptides have been shown to be different.

These two polypeptides were isolated from mouse lens native α -crystallin by SDS-gel electrophoresis and were injected into young rabbits to raise antibodies. These antibodies were tested by immunoblotting against *R. temporaria* total lens soluble proteins before their use in the present investigation.

Results presented here show that in the developing lens of *R. temporaria*, α A appears earlier than α B, suggesting a differential gene activation. In addition, these two polypeptides could not be detected either in the developing lens epithelium or in the epithelium of young froglets (2–3 weeks post-metamorphosis).

Key words: α -crystallin; α A polypeptide; α B polypeptide; frog (*Rana temporaria*); lens; antibodies; immunofluorescence; immunoblot.

1. Introduction

Vertebrate eye lens soluble proteins are classified into α -, β -, γ - and δ -crystallin families. α -crystallin is an oligomeric protein and is characterized by high molecular weight and low isoelectric point. It is present in all vertebrate lenses with the exception of some bony fishes (Cobb, Carter and Koenig, 1968; de Jong, Terwindt and Groenewoud, 1976). Most of our knowledge regarding the physical properties of α -crystallin comes from bovine lens, where it forms about 3000 of water soluble proteins (de Jong et al., 1976).

α -crystallin is composed of two major acidic and two major basic polypeptides, namely, α A₂, α B₂, α A₁ and α B₁ which can be separated completely in the presence of urea. In mammals it has been shown that α A₂ and α B₂ are primary gene products and are translated from 14S and 10S mRNAs, respectively, while α A₁ and α B₁ have been found to be post-translational modifications of the respective polypeptides (Harding and Dilley, 1976; Bloemendal, 1977). This modification was suggested to be due to non-enzymatic deamidation (Bloemendal, Berns, van der Ouderaa and de Jong, 1972; van Venrooij, de Jong, Janssen and Bloemendal, 1974; van Kleef, de Jong and

Please send correspondence to Dr S. K. Brahma, Department of Medical Anatomy and Embryology, The State University, Janskerkhof 3A, 3512BK Utrecht, The Netherlands.

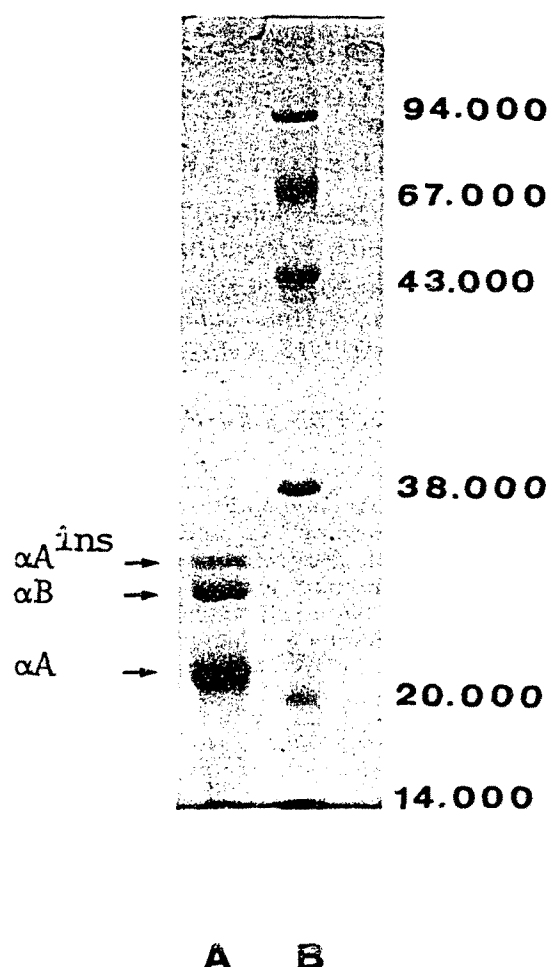


FIG. 1. SDS-gel electrophoresis of purified mouse lens α -crystallin showing αA , αB and αA^{ins} polypeptides (lane A); molecular-weight marker proteins (lane B).

Hoenders, 1975). Recently, however, it has been reported that αA_1 and αB_1 could arise by direct phosphorylation of αA_2 and αB_2 respectively (Spector, Chiesa, Sredy and Garner, 1985). In the frog *Rana temporaria* it has been shown that αA mRNA is much smaller than the corresponding mammalian mRNA (Tomarev et al. 1983). The 3' non-coding sequence of the mRNA is only 130 nucleotides in length, over 520* in most examined. Because of this and other internal differences in this region, it does not appear necessary for proper expression.

The proportion of α -crystallin varies in different vertebrates (de Jong et al., 1976); according to Puri, Augusteyn, Owen and Siezen (1983). However, there is no immunologically detectable phylogenetic specificity of vertebrate α -crystallin, thus permitting the use of heterologous antibodies in this report. de Jong et al. (1976) studied the subunit composition of α -crystallin from three anuran amphibians along with other vertebrate lens α -crystallins and reported that *R. temporaria* and *Bufo bufo* α -crystallins showed essentially similar patterns, and found that electrophoretic mobilities of α_2 chains of *Rana*, *Bufo* and cow are identical. Tomarev et al. (1983) have determined αA_2 sequence of *R. temporaria* derived from the cDNA sequence. It confirms the resistance to change in evolution of α -crystallin primary structure.

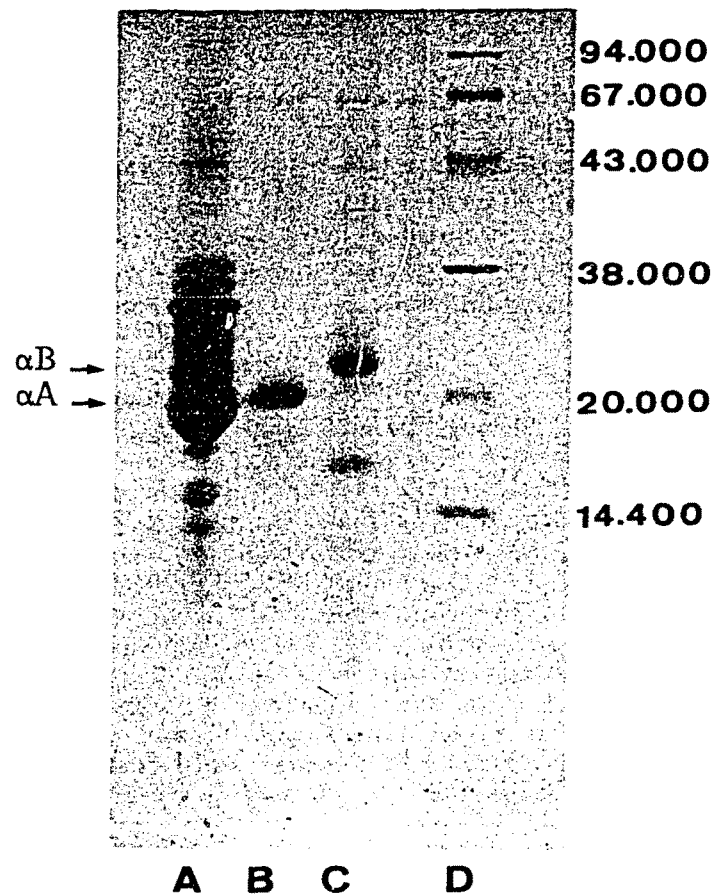


FIG. 2. SDS-gel electrophoresis of mouse lens total soluble proteins (lane A); isolated α A and α B polypeptides (lanes B and C) and molecular-weight marker proteins (lane D).

Biochemically, it has been shown that αA_2 and αB_2 are present in the adult bovine lens epithelial cells, while in the fibre cells all four polypeptides are present. The amount of αA_1 and αB_1 increases, correlated with the process of cellular growth and differentiation (Delcour and Papaconstantinou, 1970). Palmer and Papaconstantinou (1969) have shown that the αA_1 subunit is not detectable in bovine foetal lens extract but appear gradually during the development of the lens. The ratio of αA_2 and αB_2 increases significantly in highly differentiated fibre cells as compared with the epithelial cells (Delcour and Papaconstantinou, 1974). Vermorken, Hilderink, van de Ven and Bloemendal (1978a) have shown that in the cells of the central epithelium more αB_2 is synthesized than αA_2 , suggesting a differential gene activation, while a marked increase in the αA_2 chain accompanies the transition from epithelium to fibre cells (Vermorken and Bloemendal, 1978b).

It has been suggested that the α A and α B genes originated by gene duplication (de Jong et al., 1976; Wistow, 1985), α -crystallin is highly conserved evolutionarily (de Jong, 1981); however, there is, for example, only a single amino-acid difference evident as deduced from the coding sequence in exon 1 of the human αA_2 gene, as compared with that of mouse (McDevitt, Hawkins, Jaworski and Piatigorsky, 1986). Although there is about 60% sequence homology between αA_2 and αB_2 chains (van der Ouderaa, de Jong and Bloemendal, 1973; van der Ouderaa, de Jong, Hilderink

and Bloemendal, 1974), the αA_2 cDNA of rat and mouse does not hybridize to the αB_2 mRNA (Dodemont, Andreoli, Moormann, Ramaekers, Schoenmakers and Bloemendal, 1981; King, Shinohara and Piatigorsky, 1982). These two chains are also known to be antigenically different (Manski and Spector, 1972; van Kamp, 1973; Manski and Malinowski, 1980).

The present communication deals with the ontogeny and localization of αA and αB crystallin polypeptides during lens development of *Rana temporaria*, an anuran amphibian. The indirect immunofluorescence staining method, with antibodies directed against these two polypeptides from mouse lens was employed. This is the first reported use of specific antibodies against these two polypeptides in studies of lens development.

2. Materials and Methods

Isolation of αA and αB polypeptides

The starting material was mouse lens native α -crystallin, which was isolated and purified by repeated gel filtration over Bio Gel A 0.5 m (200–400 mesh, Bio Rad) using Tris-EDTA-KCl buffer at pH 7.4 (50 mM Tris, 0.1 mM EDTA, 0.1 M KCl). The isolated fraction was dialysed extensively against deionized water at 4°C, lyophilized and then subjected to SDS-gel electrophoresis (Laemmli, 1970). This method does not distinguish between the subunits of αA and of αB : only a fast-moving band representing the αA chain and a slower moving band representing the αB chain are visible after staining (Bloemendal, 1977). With mouse lens α -crystallin three polypeptides, namely, αA , αB and αA^{ins} are detectable (Fig. 1, Lane A). The αA^{ins} is similar to αA chain but has an additional insert of 23 amino acids between residues 63 and 64; the mRNA of this polypeptide is derived from the same gene as αA (King and Piatigorsky, 1983).

We excised the αA and αB polypeptides from a number of stained gels, pooled and extracted the respective polypeptides and lyophilized them according to Sreekrishna, Jones, Geutzwil, Prasad and Joshi (1980).

The isolated αA and αB polypeptides were examined by SDS re-electrophoresis for homogeneity (Fig. 2, lanes B and C), and then injected into young rabbits to raise antibodies (Harboe and Ingild, 1973).

Tissue preparation

Various developmental stages of *Rana temporaria* reared in a laboratory aquarium from freshly fertilized eggs were fixed, processed and sectioned at 6 μ m thickness through the eye lens region according to McDevitt, Meza and Yamada (1969). Staging of the lens development as described by McDevitt et al. (1969) for *Rana pipiens* was used in the present investigation.

Immunoblotting of the antibodies

R. temporaria total lens soluble proteins were translated to nitrocellulose membranes (Towbin, Staehlin and Gordon, 1979) and were immune-reacted with the two antibodies. The color was developed with 3-3', 5-5'tetramethyl benzidine (Fig. 3, lanes C and D).

Immunofluorescence staining

The indirect immunofluorescence staining method was used with fluorescein (FITC)-conjugated goat anti-rabbit γ -globulin as the secondary antibody. The sections were first treated with 5000 goat serum to remove any non-specific activity of the second layer of anti-immunoglobulin (Farr and Nakane, 1981).

In the control series the immune sera were replaced by serum from a non-immunized rabbit.

Stained sections were examined with a Leitz Dialux microscope fitted with a Ploemopak 2.4, and photographs were taken with a Wild MPS 45 automatic camera using Tri-X film from Kodak.

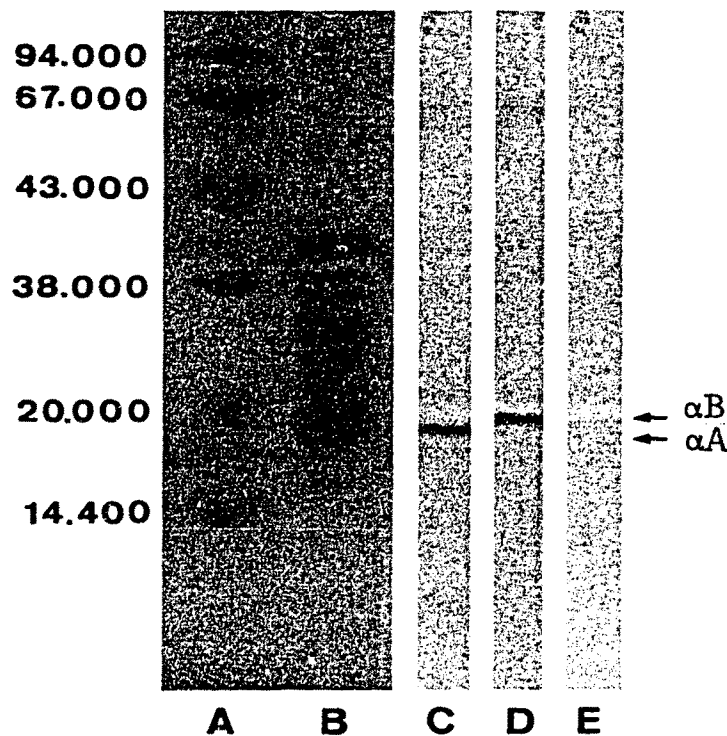


FIG. 3. *Rana temporaria* total soluble proteins were electroblotted to nitrocellulose (NC) membrane and immunoreacted with specific mouse lens α A and α B antibodies followed by staining with horseradish peroxidase (HRP)-conjugated rabbit anti mouse γ -immunoglobulin (RAM-IgG) (lanes C and D); molecular-weight marker proteins (lane A); *R. temporaria* total soluble proteins stained with Amido Black (lane B) and a control staining of the NC membrane with HRP-conjugated GAR-IgG. Here the primary antibody was omitted (lane E).

3. Results and Discussion

SDS-gel re-electrophoresis of the isolated α A and α B polypeptides from mouse lens native α -crystallin is shown in Fig. 2 (lanes B and C). (Some degradation product to which antibody was not made was visible at about the 16000 MW region in the α B sample) Immunoblotting results clearly demonstrated that the two antibodies reacted only with the respective polypeptide antigen of *R. temporaria* lens without cross reaction (Fig. 3, lanes C and D).

The first positive reaction with α A antibody was observed in a few, centrally located, elongating primary fibre cells of the late stage VI lens (Fig. 4A). In lenses between stages VI and VIII the reaction extended to additional elongating fibres, while the outer wall of the lens (external layer) facing the cornea that would ultimately form the epithelium did not show any reaction (Figure 4B). By stage IX the lens cavity was completely obliterated by the elongating primary fibres and the reaction extended to the entire mass of fibre cells, but not to the epithelium (Fig. 4C). There was no change in the staining profile in lenses from later stages of development (Fig. 4D). We also examined lenses from 2-3 weeks post-metamorphosis froglets but could not detect any positive reaction in the epithelium with α A antibody (Fig. 4E).

A lens identical to late stage VI which showed first positive reaction when treated with α A antibody did not reveal any reaction when treated with α B antibody (Fig. 5A). The first positive reaction with this antibody was not observed until stage VIII

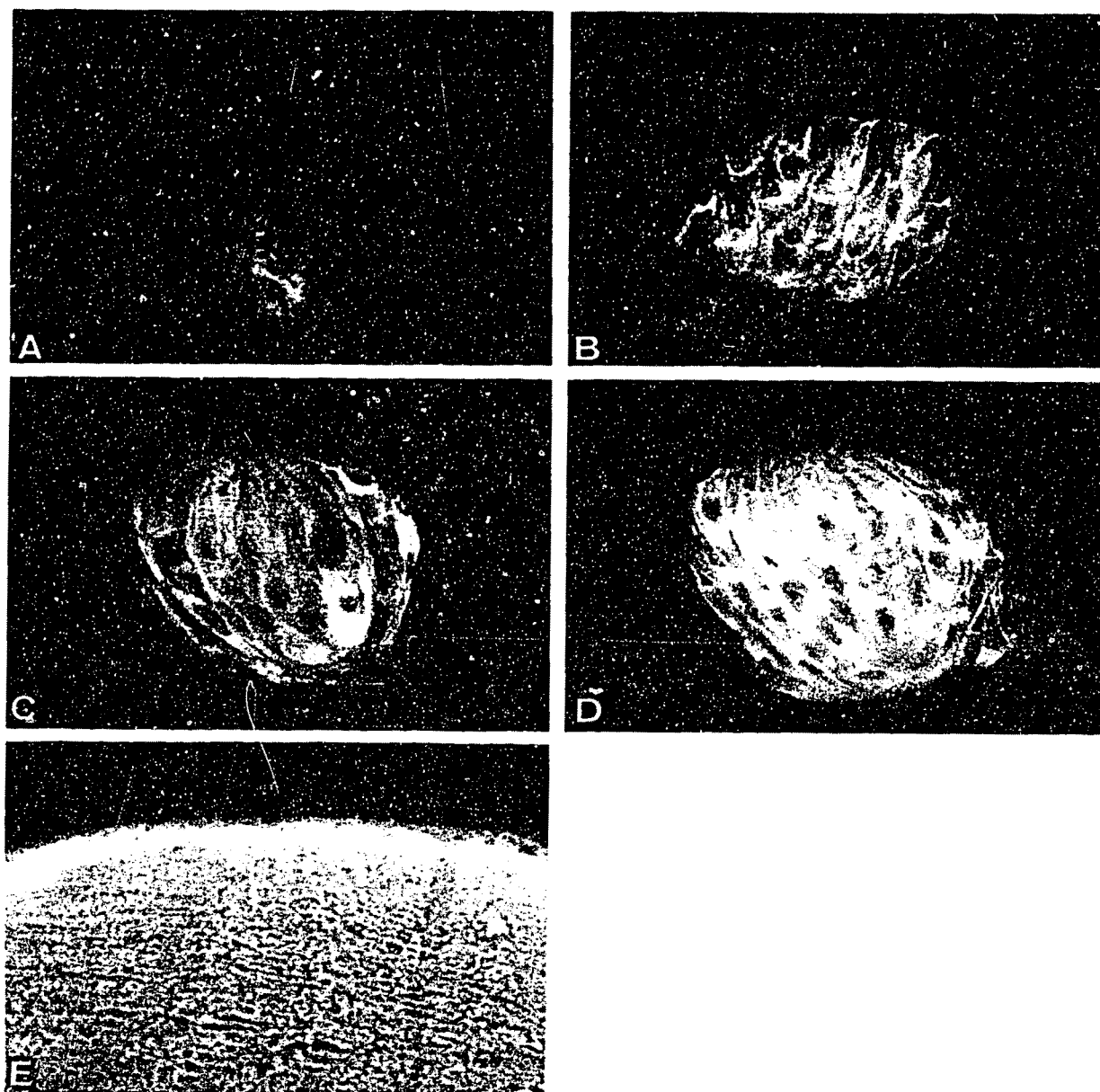


FIG. 4. A-E. Lens developmental stages of late VI, late VII, IX, X and 2-3 week-old froglet. Dark field immunofluorescence photomicrographs of developing eye lens from *R. temporaria* exposed to mouse lens α A antibody. Fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit γ -globulin was used as secondary antibody. See text for description.

(Fig. 5B). Albeit delayed, subsequent localization with this antibody was similar to that obtained with antibody to α A polypeptide in succeeding stages of lens development, including froglet lens i.e. the external layer-epithelium did not show any positive reaction (Fig. 5C-E).

Using previously prepared antibodies directed against *Rana esculenta* native α -crystallin, we also could not detect any positive reaction in the lens epithelium of *R. temporaria* at any stage of lens development, including froglets (Fig. 6A, B). In addition, the control series were negative.

From the results presented in this communication it appears that in the developing

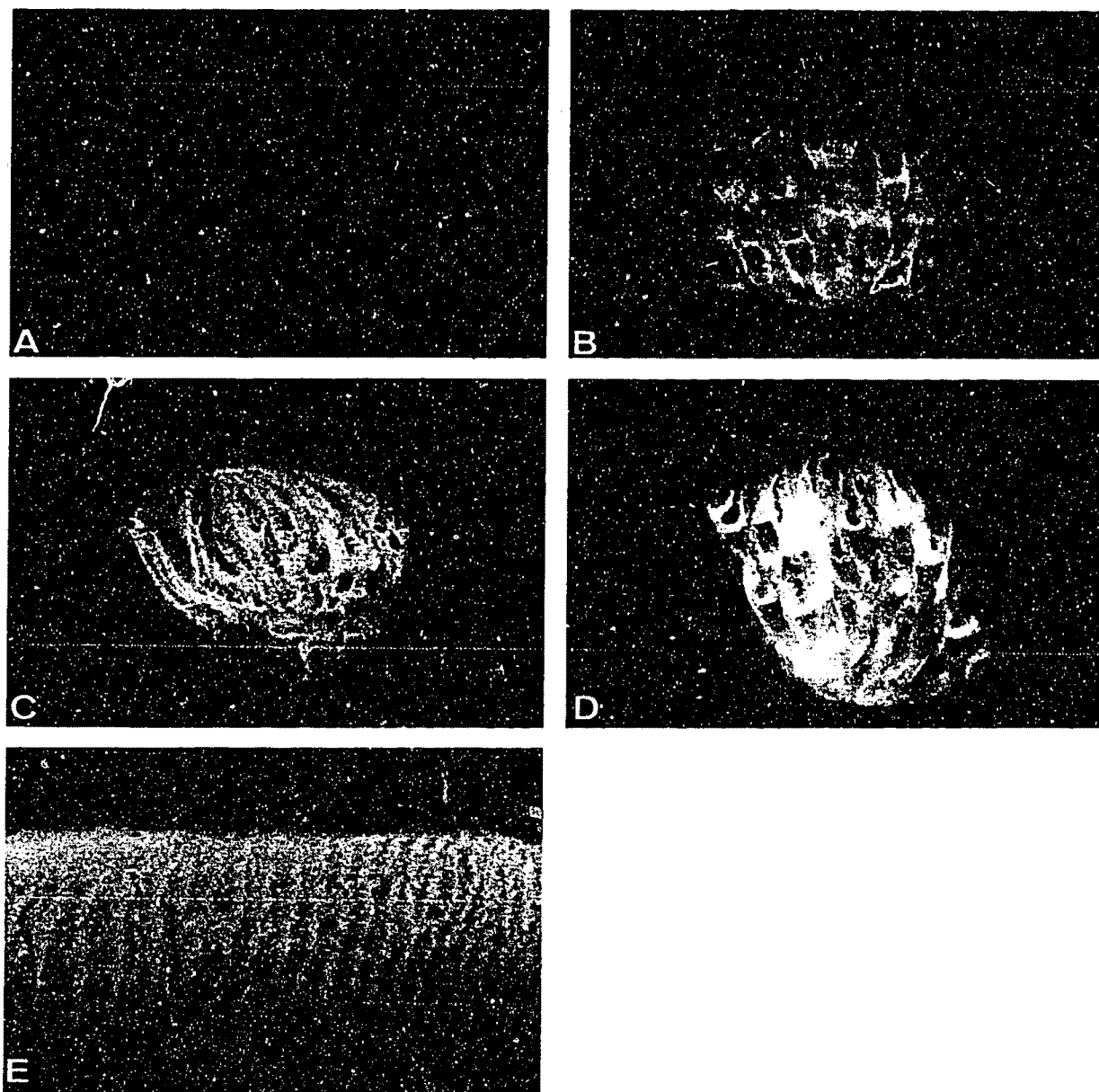


FIG. 5. A-E. Lens developmental stages of late VI, late VII, IX, X and 2-3 week-old froglet. Dark-field immunofluorescence photomicrographs of developing eye lens from *R. temporaria* exposed to mouse lens α B antibody. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit γ -globulin was used as secondary antibody (A-E). See test for description.

R. temporaria lens, α A is detectable earlier than the α B suggesting differential gene activation. It also appears that *R. temporaria* lens epithelium does not contain α -crystallin.

Using homologous antibodies to native α -crystallin, we reported earlier that in normally developing newt lens, α -crystallin is absent in the epithelium, while in the regenerating lens from the same species (*Notophthalmus viridescens*) α -crystallin can be detected in the epithelium from stage VIII of lens regeneration (McDevitt and Brahma, 1981, 1982a).

These results suggest that α -crystallin gene expression in the epithelium of

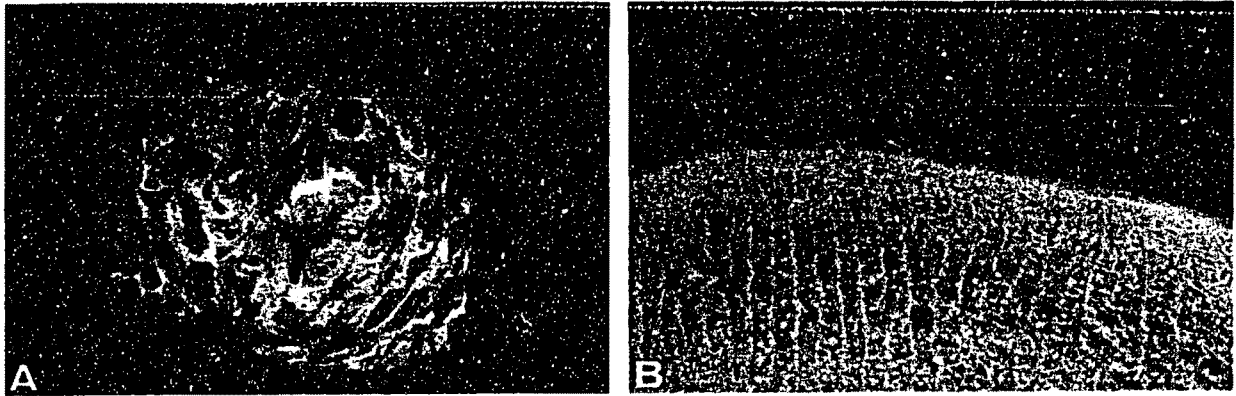


FIG. 6. A, B. Lens developmental stages of stage X and 2-3-week froglet. Dark-field immunofluorescence photomicrographs of developing eye lens from *Rana temporaria* exposed to *R. esculenta* native α -crystallin antibody. Fluorescent isothiocyanate (FITC)-conjugated goat anti-rabbit γ -globulin was used as secondary antibody. The epithelium does not show any reaction.

normally developing (*R. temporaria* and *N. viridescens*) and regenerating lenses (*N. viridescens*) does not follow the same course. The differing origin of embryonic and regenerating lenses, e.g. epithelial ectoderm vs. neural ectoderm, and embryonic vs. adult tissue (for review, see McDevitt and Brahma, 1982b) may account for this variation.

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