Int. J. Dev. Biol. 36: 363-372 (1992)



*Original Article*

# Localization of δ-crystallin RNA during lens morphogenesis and differentiation in the normal and *Talpid*$^3$ chick embryo

MARK W. HEAD#, EDWARD L. TRIPLETT#, DONALD A. EDE# and RUTH M. CLAYTON*

*Institute of Cell, Animal and Population Biology, University of Edinburgh, Edinburgh, United Kingdom*

**ABSTRACT** **Embryonic lens fiber cell differentiation in the chick is marked by the accumulation of δ-crystallin protein. The levels of δ-crystallin RNA are shown here to rise dramatically in the cells of the posterior lens pit prior to their elongation and differentiation as lens fibers. This increase correlates with regional proximity to the underlying optic cup (future retina). This accumulation of δ-crystallin RNA during lens induction operates selectively on the δ1-crystallin transcripts whereas δ2-crystallin/argininsosuccinate lyase RNA is detectable at lower levels in all developing ocular tissues throughout this period. The *talpid*$^3$ mutant forms a flat «bridge» of thickened placode-like cells in the head epithelium between the two lens placodes, and this bridge also accumulates δ1-crystallin RNA, suggesting that the selective increase in δ1-crystallin RNA levels over those of δ2-crystallin represents an early event in cellular commitment to lens fiber differentiation in the chick. The significance of the sequence of temporal changes in inductive sources for lens fiber formation is discussed, and we propose that the role of the optic cup is to provide, bound to its extra-cellular material (ECM), a high local concentration of the same growth factors which act as fiber inducers in the older eye.**

KEY WORDS: *δ1- and δ2-crystallin RNA, lens induction, lens fiber differentiation, in situ hybridization, chick mutant*

## Introduction

Morphogenesis and cell differentiation proceed concurrently during embryonic development and are effected, at least in part, by a temporal hierarchy of inductive interactions which progressively restrict and eventually determine the cellular and molecular characteristics subsequently expressed.

The transparent vertebrate lens is derived from competent head ectoderm after an inductive interaction with the evaginating neuroectoderm (optic vesicle). The lens placode cells thus formed invaginate into the neuroectodermal optic cup (future retina) and form a vesicle, and the primary fibers which form by elongation of the cells of the posterior vesicle project into its lumen. All subsequent lens fibers (secondary fibers) are formed from the equatorial region of the lens epithelium. In birds and reptiles, these equatorial cells acquire a columnar shape and constitute the annular pad. The cellular and molecular features of lens induction and differentiation are reviewed by McAvoy (1980) and Piatigorsky (1981).

The area of head ectoderm competent to form lens is much larger than that which normally comes into contact with, and is induced by the optic vesicle (see, for example, Barabanov and Fedtsova 1982), and this competence is thought to result from earlier inductive interactions during gastrulation (reviewed by Saha *et al.*, 1989).

Although a latent competence for lens differentiation is maintained in a number of embryonic chick tissues, including retina, as evidenced by their capacity to transdifferentiate to the lens phenotype *in vitro* (reviewed by Clayton, 1990), reports of ectopic lens formation *in vivo* are restricted to three independent autosomal recessive *talpid* mutations in the domestic fowl (see Ede and Kelly, 1964).

The effects of the homozygous condition in *talpid*$^3$ are highly pleiotropic, including abnormal limb development and a gross distortion of the morphology in the head region which includes a failure of pituitary differentiation from the hypophysis, the formation of multiple epiphyses (future pineal) as well as the formation of a palisaded ventral ectodermal bridge connecting the eye lenses, which because of a ventral displacement of the optic cups, are closer together than normal. There is also a variable production of small and large ectopic lenses in the mid-line head mesenchyme (Ede and Kelly, 1964).

The occurrence of ectopic lenses and a lens bridge in the *talpid*$^3$ mutant provide a unique opportunity not only to investigate the mechanisms involved in lens induction but also to test the specificity

***Address for reprints**: Institute of Cell, Animal and Population Biology (Crew Building), University of Edinburgh, King's Buildings, West Mains Road, Edinburgh EH9 3JN, Scotland, United Kingdom. FAX: 31-667-4507.

**#Addresses:** Mark W. Head is at the Department of Pathology, College of Physicians and Surgeons of Columbia University, 630 West 168th Street, New York, USA; Edward L. Triplett is at the Department of Biological Sciences, University of California, Santa Barbara, CA 93106, USA and Donald A. Ede was formerly at the Department of Zoology, University of Glasgow, Glasgow G12 8QQ, United Kingdom.

0214-6282/92/$03.00

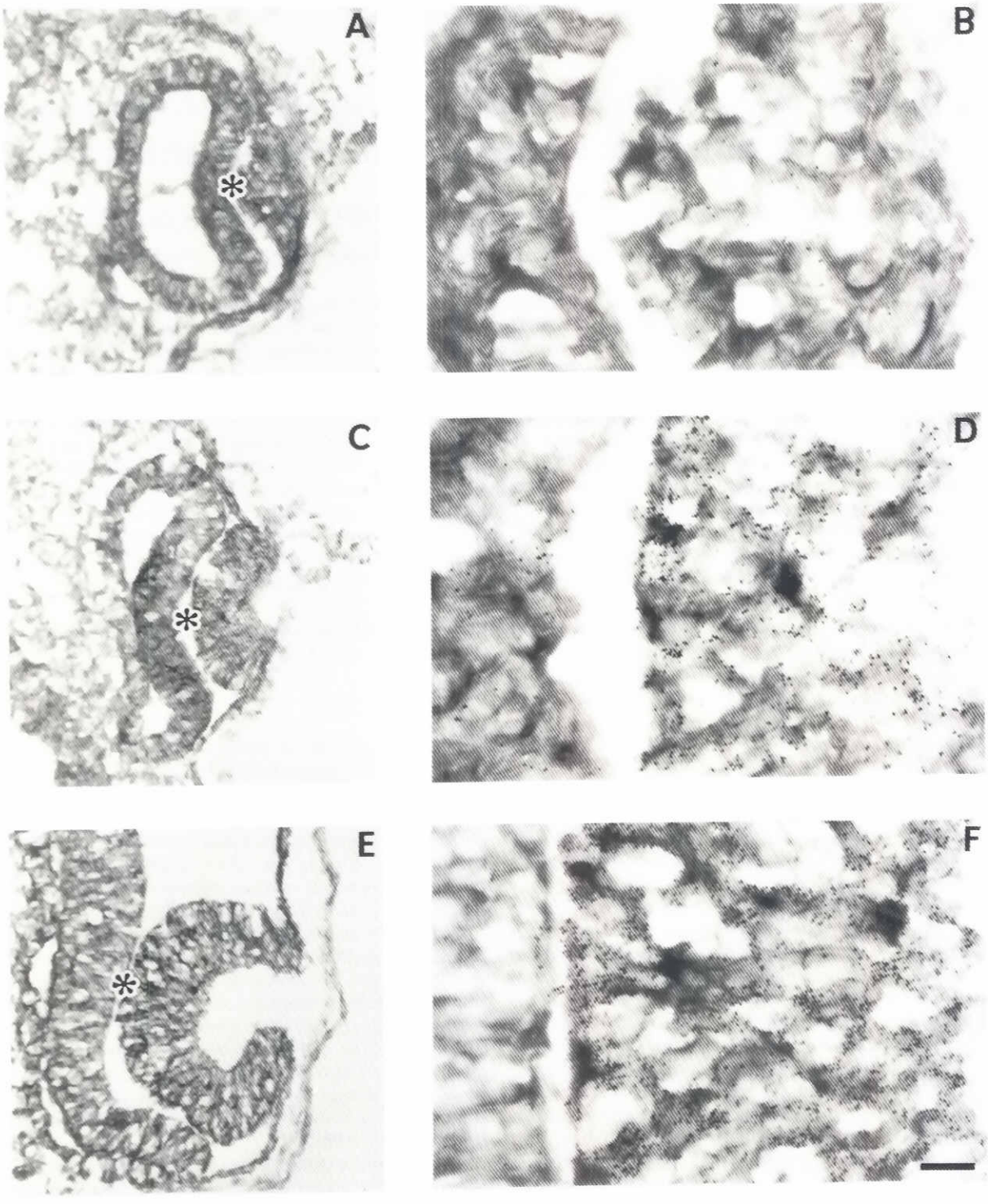


**Fig. 1. Localization of δ-crystallin RNA by *in situ* hybridization in transverse sections of chick embryos at stage 11.5 (A, B)**, *stage 12* **(C, D)** *and stage 15* **(E, F).** *The regions of A, C and E shown at a higher magnification in B, D and F are indicated. Scale bar for B, D and F represents 10 μm.*

of cellular response in terms of the activation of crystallin gene expression and its relationship to proximity to the optic cup (future retina).

The relationship between lens induction and biochemical differentiation in the chick has focused primarily on the expression of δ-crystallin, which is both the first crystallin to appear and the most

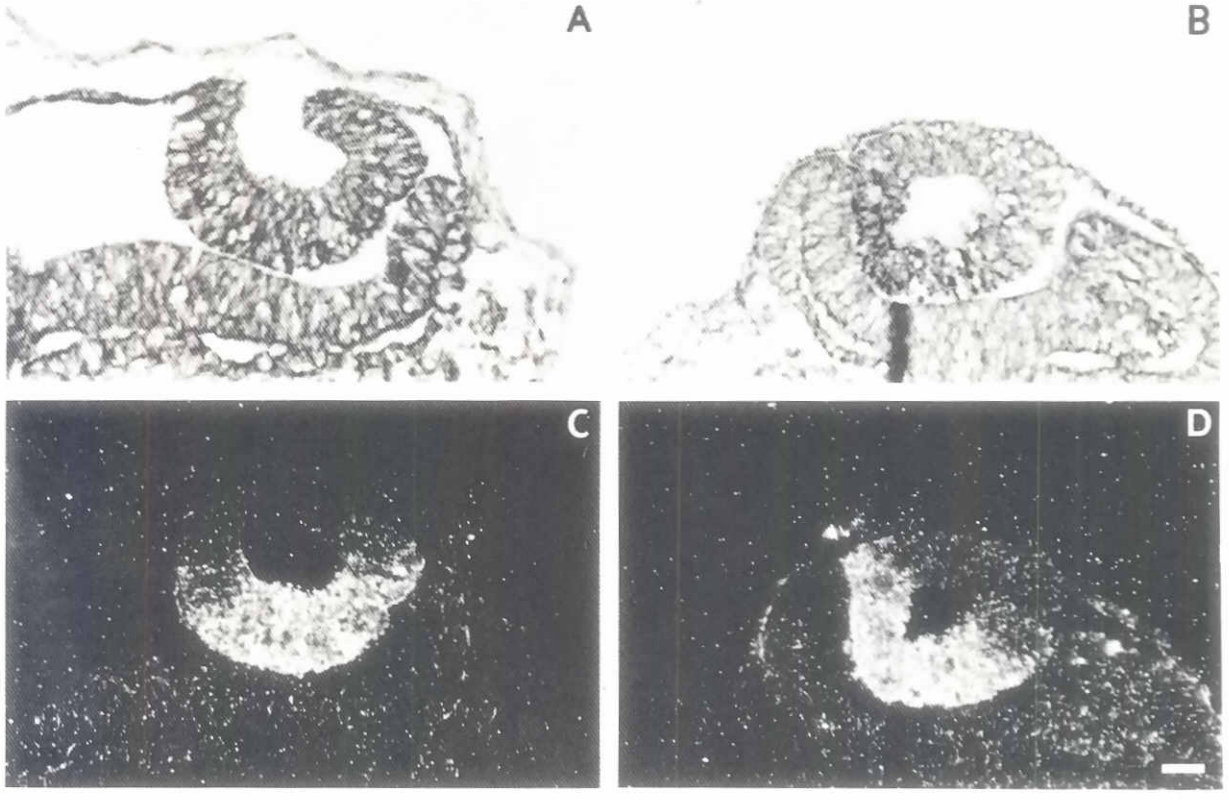


**Fig. 2. Localization of δ-crystallin RNA by *in situ* hybridization in transverse sections of a stage 15 chick embryo. (A and B)** *Photographed under transmitted light;* **(C and D)** *show the corresponding dark-field illumination micrographs. The section shown in B does not include the optic pore and therefore appears to show a vesicle. Scale bar represents 30 μm.*

abundant soluble protein throughout the embryonic period (Rabaey, 1962). The level of δ-crystallin synthesis rises dramatically as the lens pit is formed (Katoh and Yoshida, 1973) and the protein localizes to the most central and posterior cells in this structure (Zwaan and Ikeda, 1968; Brahma and van Dooremaalen, 1971). Quantitative solution hybridization using embryo head RNA shows that δ-crystallin RNA accumulates steadily before the rise in synthesis of δ-crystallin protein and extrapolation of these results backwards in time pointed to placode formation as the point at which δ-crystallin RNA accumulation is initiated (Shinohara and Piatigorsky, 1976). However, recent evidence shows that the initiation of δ-crystallin transcription must occur very much earlier than previously thought, at a time prior to any overt lens differentiation, since very low levels of δ-crystallin RNA are present (<10 transcripts/cell) not only in competent head ectoderm but also in head mesoderm, neural tube and trunk ectoderm and mesoderm (Sullivan *et al.*, 1991). The level of δ-crystallin RNA and protein in the lens, once formed, remains high throughout the embryonic period and both the protein and RNA levels are over ten times higher in the lens fiber mass than in the central or equatorial epithelium (Pal and Modak, 1984; Hejtmancik *et al.*, 1985).

*In situ* hybridization has been used to study the localization of α-, ß- and γ-crystallin RNAs during lens development in rats (van Leen *et al.*, 1987) and mice (Treton *et al.*, 1991), but there has been no study in birds, and mammals lack both δ-crystallin as a major lens protein and an annular pad region as a distinct morphological structure.

The chick genome contains two non-allelic δ-crystallin genes which exhibit a very high degree of sequence homology (Nickerson *et al.*, 1986). Transcripts of both δ-crystallin genes are found in the lens but the level of δ1-crystallin RNA is higher than that of δ2-crystallin RNA in the lens (Parker *et al.*, 1988; Head *et al.*, 1991b) and is especially high in the lens fiber mass (Thomas *et al.*, 1990; Head *et al.*, 1991b).

The lower levels of δ-crystallin RNA previously detected in non-lens tissues, including embryonic retina and brain (Agata *et al.*, 1983; Bower *et al.*, 1983), are largely but not exclusively those of δ2-crystallin (Thomas *et al.*, 1990; Head *et al.*, 1991b). It seems increasingly likely that the function of δ2-crystallin in non-lens tissues is that of the urea cycle enzyme argininosuccinate lyase (ASL) (Piatigorsky *et al.*, 1988; de Pomerai *et al.*, 1991). However, unlike δ2-crystallin, δ1-crystallin expressed in mouse or yeast cells has no ASL activity (Barbosa *et al.*, 1991; Kondoh *et al.*, 1991) so that any role it may play in extralenticular sites must be non-enzymic (Head *et al.*, 1991b).

We have studied the ontogeny and inter- and intracellular localization of δ-crystallin RNA during lens morphogenesis in the normal and in the *talpid*$^3$ chick in order to examine the relationship between lens induction, fiber formation and the accumulation of δ-crystallin RNA.

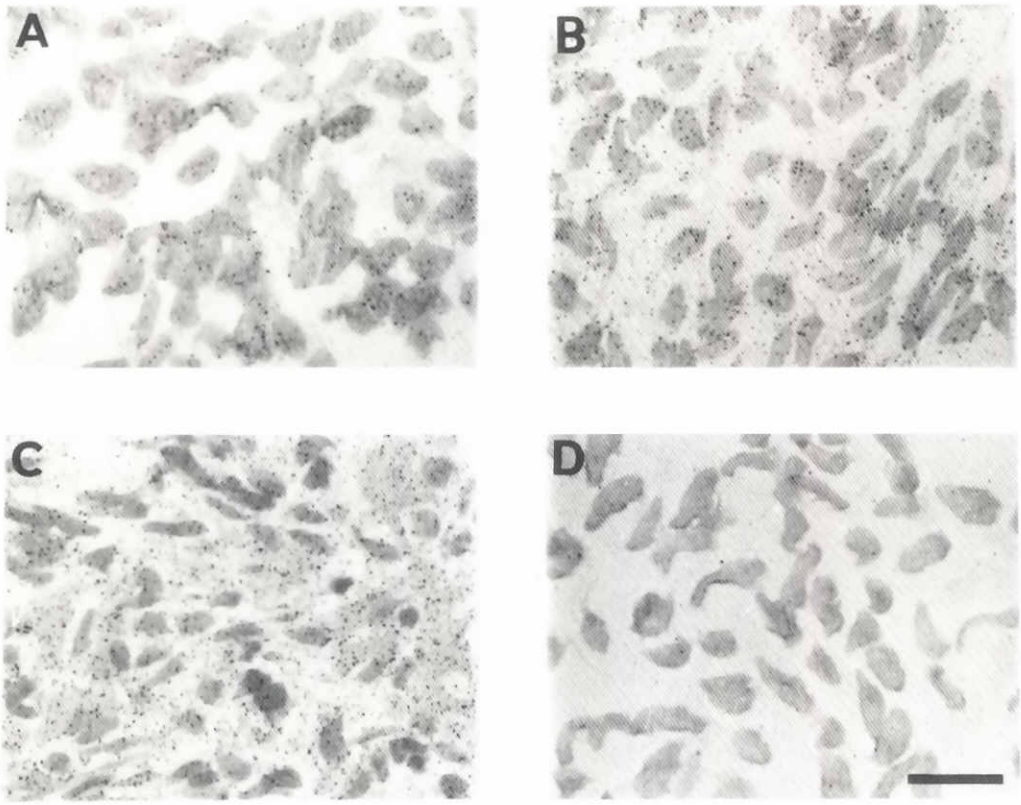


**Fig. 3. Localization of δ-crystallin RNA in tissue squashes of dissected lens placode (A), lens vesicle (B) and a 3.5 day embryo lens (C).** *Hybridization using a ß-crystallin probe and the 3.5 day embryo lens is shown for comparison* **(D)**. *Scale bar represents 30 μm.*

In order to discriminate between the transcripts of the δ1- and δ2-crystallin genes in developing ocular tissues, we have employed the gene-specific oligonucleotide probes described previously (Parker *et al.*, 1988; Head *et al.*, 1991b).

## Results

The localization and time course of accumulation of δ-crystallin transcripts during lens morphogenesis were assessed by *in situ* hybridization of a δ-crystallin probe to sectioned embryos of selected developmental stages. At stage 11.5 the number of grains over cells of the lens placode is low and similar to the number of grains seen over cells of the surrounding head ectoderm and optic vesicle (Fig. 1A, B). At stage 12 cells of the invaginating lens placode are moderately heavily labeled (Fig. 1C, D) and by stage 15 the intensity of the labeling has again increased, particularly in the most central and posterior region of the lens pit which is in the closest contact with the optic cup (Fig. 1E, F). The association of proximity to the optic cup and δ-crystallin expression is seen most clearly at stage 15 when transmitted light and dark-field photomicrographs are compared (Fig. 2). Both in a section containing the optic pore (Fig. 2A) and in a section from this same stage (which does not include the optic pore and therefore appears to show a lens vesicle, Fig. 2B), only the areas around the circumference of the lens pit which are in closest apposition to the optic cup are labeled intensely (Fig. 2C, D). Hybridization with the α- and ß-crystallin probes to neighboring sections at these developmental stages gave negative results (data not shown).

The intracellular localization and accumulation of δ-crystallin transcripts was assessed in squashes of dissected lens rudiments (Fig. 3). The cells of the flat placode show light nuclear labeling with no cytoplasmic label (Fig. 3A). Both the degree of labeling and the proportion of grains found in the cytoplasm of the cells increases in the lens vesicle stage (Fig. 3B) and the 3.5 day lens stage (stage 21) (Fig. 3C). Hybridization using a ß-crystallin probe cDNA probe shows a virtual absence of label in cells of the 3.5 day stage lens (Fig. 3D).

At 14 days of development, δ-crystallin RNA can be seen in both the nuclear and inner cortical fiber cells, the level being relatively higher in the inner cortical fibers, and a proportion of these cells have heavily labeled nuclei (Fig. 4 C, D). The highest density of labeling evident at this stage is in the transitional region between cells of the annular pad and the most recently formed outer cortical fibers (Fig. 4B). This pattern can be clearly seen at a lower magnification and dark-field illumination (Fig. 5B), but a different pattern is seen when the αA-crystallin probe is used (Fig 5A). αA-crystallin RNA is abundantly expressed in the annular pad and the outer cortical fibers but is below detectable levels in the inner cortical and nuclear fibers.

At stage 15 of embryonic development in normal chick embryos the most posterior cells of the lens pit are selectively labeled by the δ1-crystallin probe (Fig. 6A) as compared to the labeling pattern seen when the δ2-crystallin probe is used (Fig. 6B). The primary lens fibers seen at stage 18, and at stage 21 (3.5 days) are uniformly and heavily labeled by the δ1-crystallin probe whereas the corresponding lens epithelium is only lightly labeled (Fig. 6C, E). Only low or background levels of silver grains are seen over tissues other than lens with the δ1-crystallin probe (Fig. 6A, C, E) but above

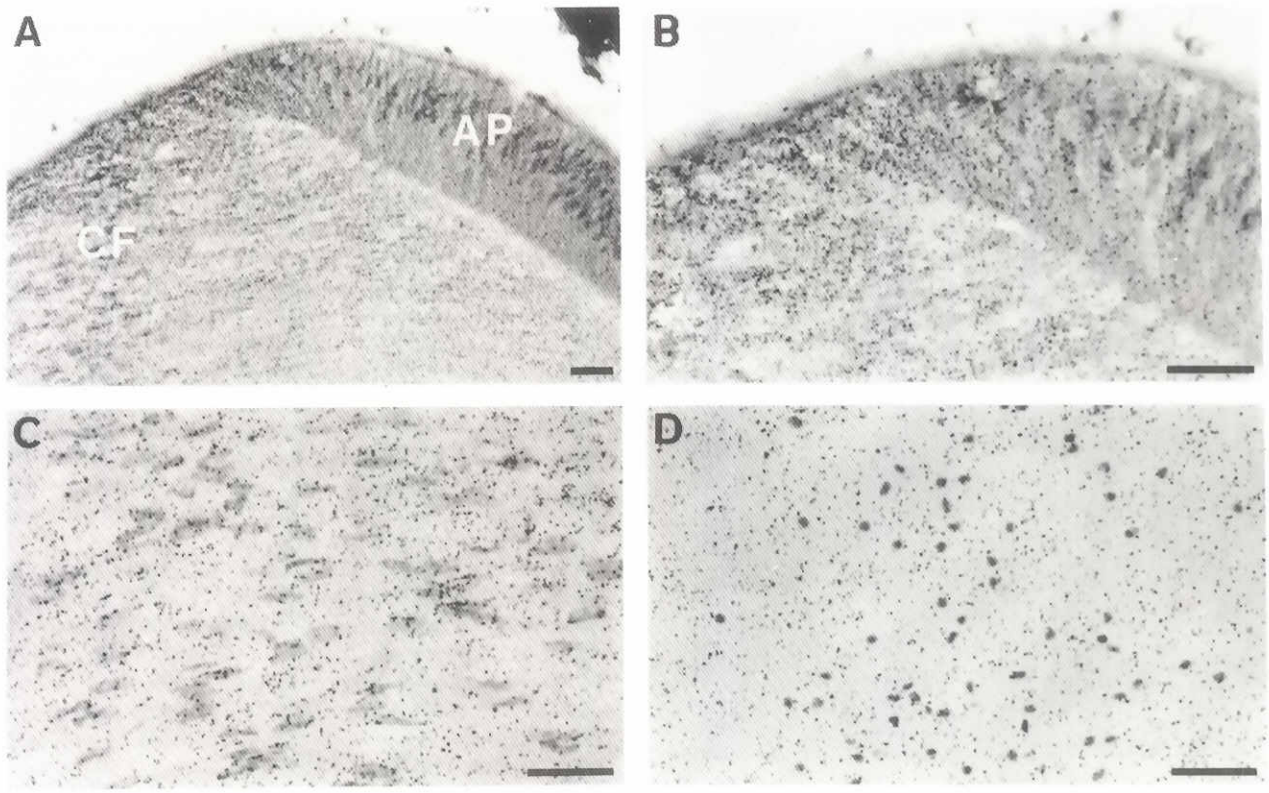


**Fig. 4. Localization of δ-crystallin RNA by *in situ* hybridization to median sagittal sections of 14 day chick embryo eye showing:** *lens bow region* **(A)** *including the cortical fibers (CF) and the annular pad (AP), annular pad/cortical lens fiber boundary* **(B)**, *inner cortical lens fibers* **(C)** *and nuclear lens fibers* **(D)**. *Scale bars represent 30 μm.*

background levels of label are seen over all tissues, including lens, at each stage examined using the δ2-crystallin probe (Fig. 6B, D, F).

Transverse sections through the head region of a *talpid*[3] homozygote at 3.5 days of embryonic development (stage 21) show the eyes displaced ventrally (Fig, 7A, B) and the eye lenses (L) connected by a lens bridge (LB) composed of palisaded cells (Fig. 7A). *In situ* hybridization with the δ1-crystallin probe and photography under dark field illumination shows heavy labeling over the eye lenses and the lens bridge but only light or background levels over other tissues (Fig. 7C, E). Hybridization to the same region of a neighboring section, with the δ2-crystallin probe, results in moderate labeling over optic cup, head mesenchyme, and the lens bridge (Fig. 7F). Transverse sections in a more posterior plane do not include the lens bridge but do show a vesicle-like structure in ventral head mesenchyme (arrowed in Fig. 7B). Hybridization with the δ1-crystallin probe labels the eye lenses (Fig. 7D), whereas the δ2-crystallin probe labels all cellular structures (Fig. 7G).

## Discussion

The ontogeny of δ-crystallin expression during lens morphogenesis in the chick has been studied previously *in situ*, at the level of accumulated protein by immunofluorescence (Zwaan and Ikeda, 1968; Brahma and van Doorenmaalen, 1971), and, in dissected material, at the level of protein synthesis (Katoh and Yoshida, 1973) and RNA accumulation (Shinohara and Piatigorsky, 1976). *In situ* hybridization provides direct evidence that the temporal and spatial localization of crystallin RNA closely parallels the pattern of δ-crystallin accumulation as identified by immunofluorescence studies, implying that all presumptive lens cells which transcribe and accumulate δ-crystallin RNA rapidly process and translate at least some proportion of it.

In sectioned material, lens placode invagination rather than lens placode thickening is the stage at which δ-crystallin RNA can be seen to exhibit a marked accumulation, although hybridization to tissue squashes indicates that this increase occurs from a prior low level of δ-crystallin RNA already expressed in the nuclei of the uninvaginated lens placode cells. A nuclear localization of δ-crystallin RNA was not identified in the sectioned lens placode but there are differences in handling and fixation between these two techniques, and the suitability of the squash technique for the detection of nuclear RNA is documented (Jeanny *et al.*, 1985) and has been commented on by others (Van Leen *et al.*, 1987). Since we did not attempt to prepare squashes of isolated head ectoderm from this early stage, we do not know whether nuclear δ-crystallin RNA marks only the lens placode, and is accumulated in response to lens induction by the optic vesicle, or whether it extends over the entire head ectoderm and may therefore be associated with competence for lens differentiation. Indirect evidence suggests that the former is likely to be the case. Firstly the low levels of δ-crystallin transcripts found in several early embryonic tissue layers, including stage 10 head ectoderm represents <10 transcripts/cell (Sullivan *et al.*,

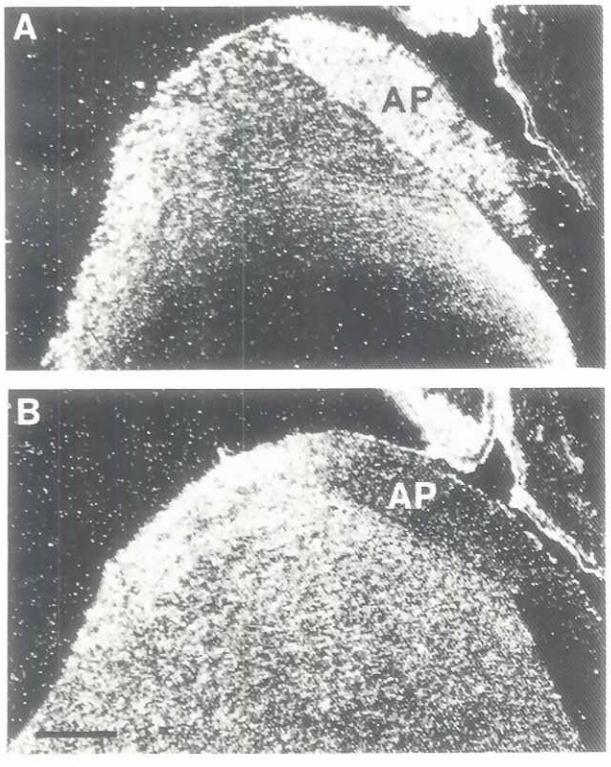


**Fig. 5. Localization of αA-crystallin RNA (A) and δ-crystallin RNA (B) by *in situ* hybridization to median sagittal sections of 14 day chick embryo eye photographed under dark-field illumination.** *Scale bar represents 100 μm.*

1991), and, if evenly distributed, would be well below the sensitivity of the *in situ* hybridization technique used here (>100 transcripts/cell, Jeanny *et al.*, 1985). Secondly, since the δ-crystallin in the placode cells is nuclear, it is unlikely to have made a detectable contribution to the poly $A^+$ δ-crystallin RNA found in whole embryo heads at the placode stage (Shinohara and Piatigorsky, 1976) nor to the fully processed δ-crystallin RNA found in head ectoderm, head mesoderm and neural tube by Sullivan *et al.* (1991). Since we find that the RNA outside of the placode is that of δ2-crystallin, it seems likely to us that these authors were detecting δ2-crystallin RNA, and that this obscured the events in the placode itself, and the finding we report here — that the accumulation of abundant cytoplasmic δ1-crystallin RNA occurs during invagination, and not during placode formation.

As lens placode invagination proceeds, δ-crystallin RNA accumulates rapidly, particularly in the presumptive primary lens fiber cells which are located in the region of the lens pit in closest contact with the optic cup, suggesting that close contact continues to play an important role in the spatial regulation of δ-crystallin expression. These events occur in the absence of any detectable αA- or ßB2-crystallin transcripts. By 3.5 days of development, when the primary lens fiber cells have formed, δ-crystallin RNA is abundant and largely cytoplasmic in location.

Our observations at 14 days of embryonic development confirms the quantitative differences in δ-crystallin RNA content of lens epithelial and fiber cell regions reported by Hejtmancik *et al.* (1985). Nuclear fibers retain some evenly distributed hybridizable δ-crystallin RNA. Cells in the inner cortical region are still transcribing δ-crystallin RNA at this stage, as judged by the dense nuclear labeling seen in some of the cells, but the annular pad/cortical fiber region has even higher levels of δ-crystallin RNA. The increase in the steady state level of δ-crystallin RNA is relatively abrupt and occurs in the annular pad/cortical fiber boundary just before the zone of cell elongation. These observations suggest that the initiation of high level δ-crystallin expression is associated with cell elongation, just as it is in the cells of the posterior lens pit. This association does not hold true for αA-crystallin gene expression since αA-crystallin RNA is present at high steady state levels in the cells of the annular pad, and the spatial distribution shown here by *in situ* hybridization closely parallels, and presumably accounts for, the pattern of αA-crystallin accumulation identified by immunofluorescence at this same stage of development (Ueda, 1989). The gradient of decreasing δ-crystallin RNA levels from the lens cortex to the lens nucleus should be interpreted with caution, since there is a corresponding increase in cell length and cell volume (Beebe *et al.*, 1982).

The accumulation of high levels of δ-crystallin protein and RNA (Pal and Modak 1984; Hejtmancik *et al.*, 1985) is due to a selective increase in the level of δ1-crystallin transcripts compared to those of the δ2-crystallin gene (Thomas *et al.*, 1990; Head *et al.*, 1991b). The results presented here clearly demonstrate that the increase in δ-crystallin RNA abundance during lens placode invagination and primary lens fiber formation is also due to a selective increase in δ1-crystallin RNA, with no corresponding increase in the level of δ2-crystallin RNA. However, the extralenticular δ-crystallin transcripts previously detected in these very early embryos (Agata *et al.*, 1983; Bower *et al.*, 1983; Sullivan *et al.*, 1991) are evidently mainly those of δ2-crystallin. These findings are consistent with our previous report on the relative levels of δ1- and δ2-crystallin RNA in lens and retina by Northern transfer and dot blot hybridization at later stages of development (Head *et al.*, 1991b). Our observation that δ2-crystallin RNA is relatively poorly processed irrespective of tissue of origin strongly indicates that δ1- and δ2-crystallin polypeptide levels in non-lens tissues may not directly reflect the relative levels of their respective RNAs (see Head *et al.*, 1991b) Indeed, the δ-crystallin detectable in post-hatch chick cornea by Western blotting (Head *et al.*, 1991a) has recently been reported to be the δ1-crystallin gene product (Li *et al.*, 1992).

Here we show that the cells of the lens and the lens bridge of the *talpid*$^3$ mutant both accumulate high levels of δ1-crystallin RNA, while the other extralenticular tissues of *talpid*$^3$ , as in normal chicks, express low levels of δ2-crystallin RNA. We cannot be certain whether the the vesicle-like structure which expresses low levels of δ2-crystallin RNA and not δ1-crystallin RNA seen in Fig. 7B is a small ectopic lens or represents the abnormal Rathke's pouch found in *talpid*$^3$ (see Ede and Kelly, 1964). The cells of the lens bridge are palisaded but have not acquired a lens fiber cell morphology. The combination of a columnar morphology and the expression of δ1-crystallin RNA suggests that the lens bridge may be analogous to the invaginating lens placode seen during normal development and to the columnar cells of the annular pad of the later lens. The larger scale morphology of the *ta*$^3$ lens bridge suggests a particularly wide area of early invagination into the mesenchyme in the ventral region between the eyes.

δ1-crystallin expression appears to be regulated during lens development by two successive mechanisms. Firstly, in presumptive primary (nuclear) fibers during lens induction, by intimate

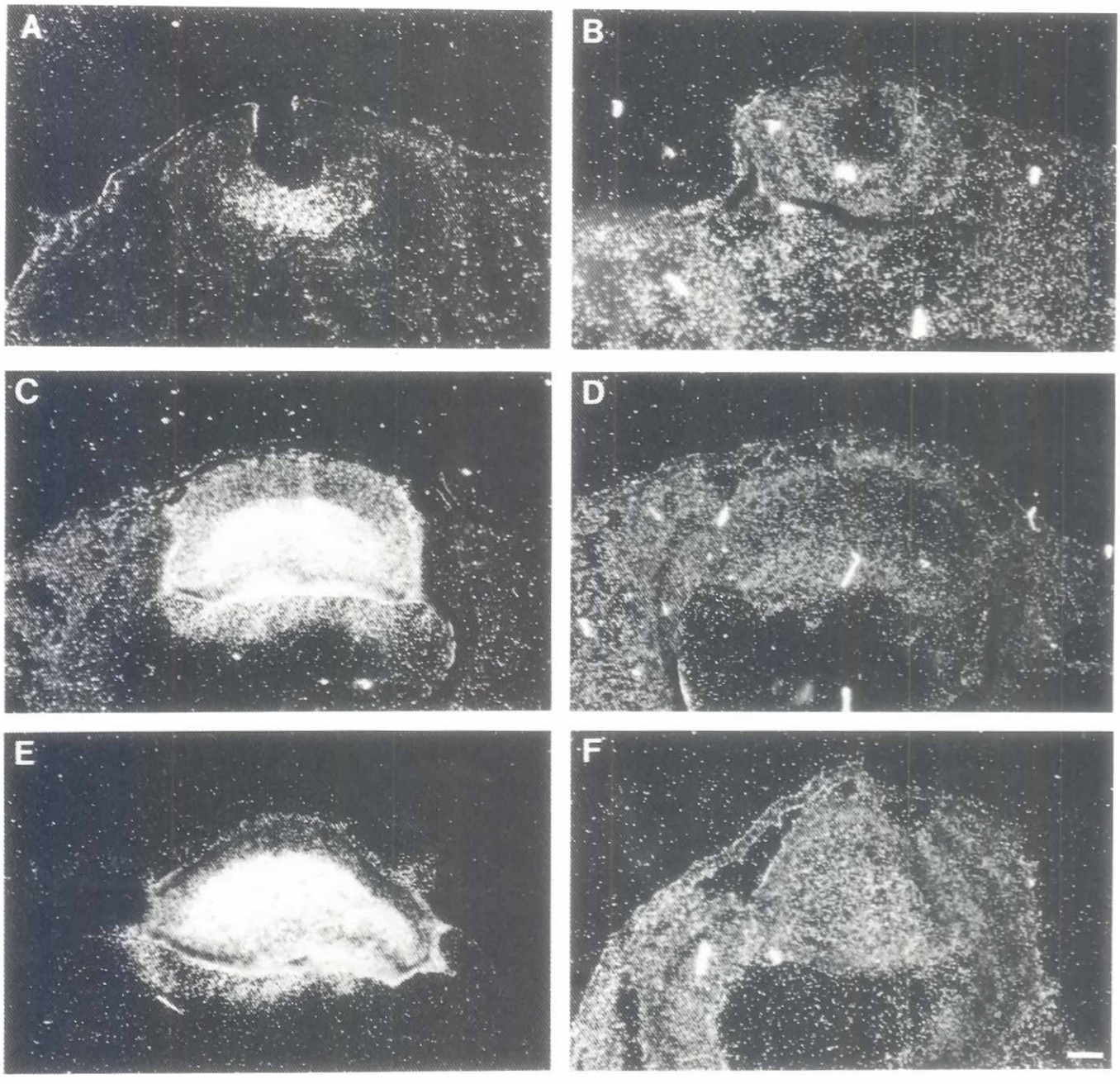


**Fig. 6. Localization of δ1-crystallin RNA (A, C, E) and δ2-crystallin RNA (B, D, F) in the developing eye by *in situ* hybridization in transverse section through the head region of normal embryos** *at stage 15* **(A, B)**, *stage 18* **(C, D)** *and stage 21, 3.5 days of incubation,* **(E, F).** *Scale bar represents 100 μm.*

contact with the optic cup, and secondly during secondary (cortical) fiber formation, when any retinal influence could only be indirect, via the vitreous humor. The *talpid*[3] lens bridge, however falls into neither category.

A number of growth and differentiation factors, including lentropin, insulin, IGF-I, FGF, and retinoic acid, have been shown to affect chick lens fiber formation and δ-crystallin expression *in vitro*, and in some cases, such as that of FGF, are known to be synthesized in the retina, to be present in the vitreous humor and to have binding sites in the lens (Beebe *et al.*, 1980, 1987; Mascarelli *et al.*, 1986, 1987; Bassas *et al.*, 1987; Alemany *et al.*, 1989; Bassnett and Beebe, 1990; Patek and Clayton, 1990; Peralta Soler *et al.*, 1990).

In normal embryos the regional specification of lens placode formation from competent head ectoderm results from intimate contact from the evaginating neuroectodermal optic vesicle, and the interfacial matrix during induction is rich in glycoprotein and

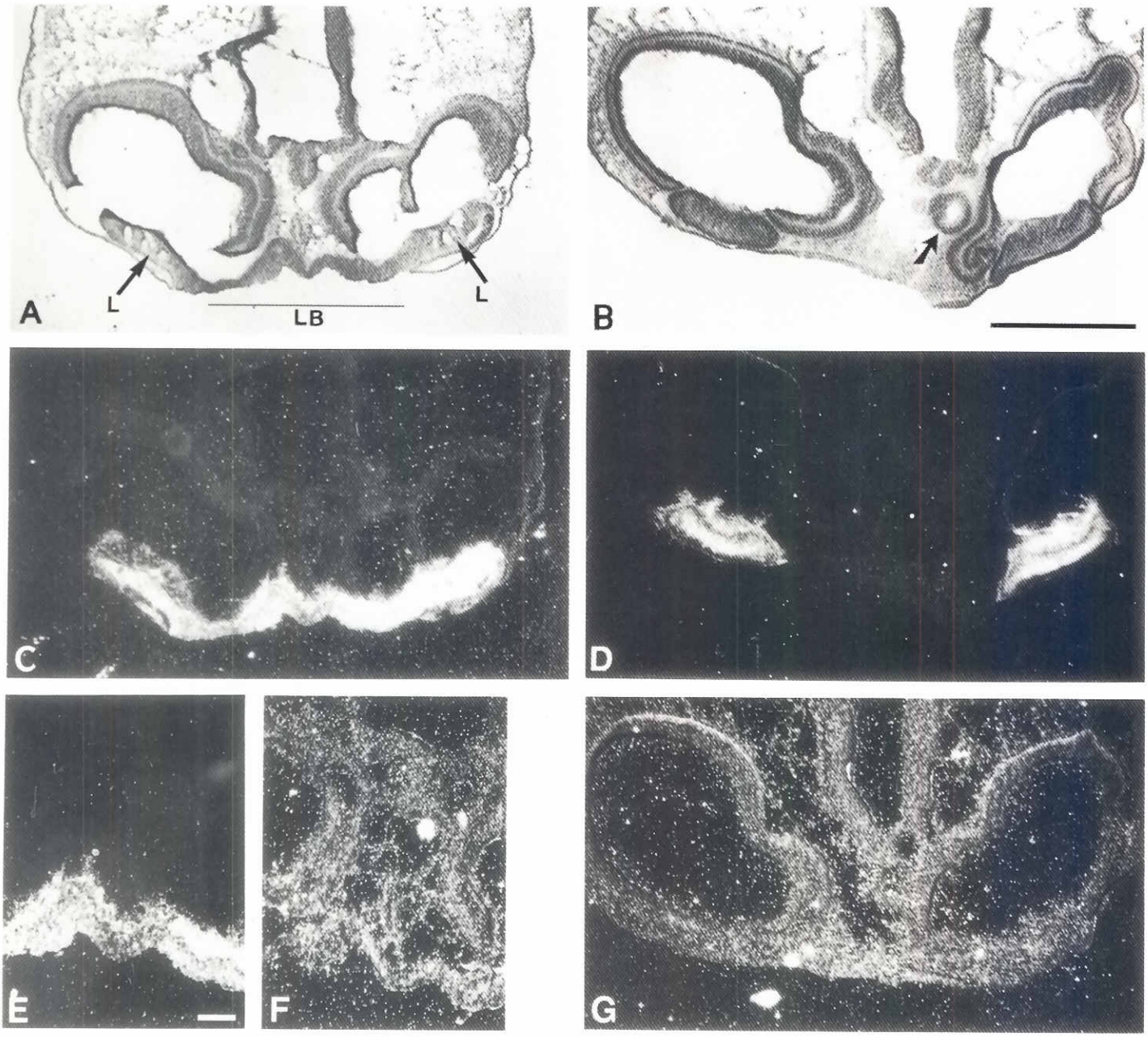


**Fig. 7. Localization of δ1-crystallin RNA (A-E) and δ2-crystallin RNA (F, G) by *in situ* hybridization in transverse sections through the head region of a *talpid*$^3$ homozygote at 3.5 days of embryonic development** *photographed under transmitted light* **(A, B)** *or dark-field illumination* **(C-G).** *The eye lens, L, and lens bridge, LB, can be seen in A and a vesicle-like structure is arrowed in B. Scale for A, B, C, D and G is shown in B by a 500 μm scale bar. The scale of E and F is shown in E by a 100 μm scale bar.*

proteoglycans (Hendrix and Zwaan, 1974, 1975), including bFGF-binding heparan sulfate proteoglycan (Fayein *et al.*, 1990). It may be, therefore, that the restriction of lens induction to the eye cup contact zone is because the extracellular matrix provides a strictly localized concentration of bFGF or other HBGFs above the necessary threshold required for induction. The closeness of the two eye cups to each other in the *talpid*$^3$ embryo may, similarly, make possible a sufficiently high concentration of soluble inducer in the area between them.

bFGF is present in vitreous humor of later stages and binds to the heparan sulfate proteoglycan of the lens capsule (Cirillo *et al.*, 1990). It is a good candidate inducer of secondary fiber formation (Chamberlain and McAvoy, 1987; McAvoy and Chamberlain, 1989). However, since lens epithelial cells express bFGF (Schwegerer *et*

*al.*, 1988) as well as IGF-I (Caldés *et al.*, 1991), autocrine factors may also be relevant.

## Materials and Methods

### *In situ* hybridization with cloned probes

The Pst1 inserts from the δ-crystallin cDNA clone M56 (Bower *et al.*, 1983), the ß-crystallin clone 026 (Errington *et al.*, 1986) and the EcoR1 fragment of the αA-crystallin genomic clone L21a (Errington *et al.*, 1985) were labeled with $^3$H-dCTP (>1.85TBq/mmol, Amersham, UK) by random primed synthesis (Feinberg and Vogelstein 1983). Fertile eggs of the N-J genotype (described in Patek and Clayton, 1988) were incubated for defined periods and the stage of development confirmed by observation according to Hamburger and Hamilton (1951). *In situ* hybridization with cDNA probes was by the method of Hafen *et al.* (1983) for sectioned material and Jeanny *et al.* (1985) for tissue squashes, employing, in both cases, a modified hybridization buffer consisting of 50% formamide, 0.6M NaCl, 10 mM Tris-HCl. pH 7.0, 1 mM EDTA, 1x Denhardt's solution, 1% dextran sulfate, 250 µg/ml heat sheared herring sperm DNA, 500 µg/ml yeast tRNA and $^3$H-labeled probe present at 1 µg/ml. All other steps were as described in Bower *et al.* (1983). All *in situ* hybridizations to sectioned material presented here were hybridized with the same δ-crystallin cDNA probe preparation, and exposed for an identical length of time thus allowing direct comparison of local silver grain intensity as a measure of relative δ-crystallin RNA abundance between stages.

### *In situ* hybridization with oligonucleotide probes

After designated periods of egg incubation, chick embryos were examined and classified as affected *talpid*$^3$ homozygotes or unaffected normal embryos according to the criteria of Ede and Kelly (1964). Chick embryos of an unrelated commercial egg laying strain (N-J) were also examined and their stage of development classified according to Hamburger and Hamilton (1951). Embryo heads were embedded, sectioned and fixed for *in situ* hybridization as previously described (Bower *et al.*, 1983). The oligonucleotide probes specific for the δ1- and δ2-crystallin transcripts were labeled with $^3$H-dCTP (>1.85TBq/m mol, Amersham, UK) and purified as according to Head *et al.* (1991b). Hybridization was carried out with $^3$H-labeled probe present at 0.1 µg/ml in 0.9M NaCl, 90 mM Tris-HCl pH 7.5, 9 mM EDTA, 5X Denhardt's solution, 1% dextran sulfate, 250 µg/ml heat sheared herring sperm DNA and 500 µg/ml yeast tRNA for 18 h at 37°C. Washing included two 30 minute washes in 3 x SSC at 37°C and autoradiography and staining were as described in Bower *et al.* (1983). Hybridization slides were photographed under transmitted light- and dark-field illumination using an Olympus Vanox microscope.

*Acknowledgments*

*We thank AFRC (Roslin) for the provision of fertile N-J eggs, C. Nicolson for technical assistance, F. Johnstone and G. Brown for their photographic skills and M. Ramsay and P. Robinson for preparing the manuscript. M.W. Head gratefully acknowledges the support of EURAGE in funding a visit to the laboratory of Dr. Y. Courtois to study in situ hybridization techniques with Dr. J-C Jeanny.*

## References

AGATA, K., YASUDA, K. and OKADA, T.S. (1983). Gene coding for a lens specific protein, δ-crystallin, is transcribed in nonlens tissues in chick embryos. *Dev. Biol. 100:* 222-226.

ALEMANY, J., ZELENKA, P., SERRANO, J. and DE PABLO, F. (1989). Insulin-like growth factor 1 and insulin regulate delta crystallin gene expression in developing lens. *J. Biol. Chem. 264:* 17559-17563.

BARABANOV, V.M. and FEDTSOVA, N.G. (1982). The distribution of lens differentiation capacity in the head ectoderm of chick embryos. *Differentiation 21:* 183-190.

BARBOSA, P., WISTOW, G.J., CIALKOWSKI, M., PIATIGORSKY, J. and O'BRIEN, W.E. (1991). Expression of duck lens δ-crystallin cDNAs in yeast and bacterial hosts. *J. Biol. Chem. 266:* 22319-23322.

BASSAS, L., ZELENKA, P.S., SERRANO, J. and DE PABLO, F. (1987). Insulin and IGF receptors are developmentally regulated in the chick embryo eye lens. *Exp. Cell Res. 168:* 561-566.

BASSNETT, S. and BEEBE, D.C. (1990). Localization of insulin-like growth factor-1 binding sites in the embryonic chicken eye. *Invest. Ophthalmol. Vis. Sci. 31:* 1637-1643.

BEEBE, D.C., COMPART, P.J., JOHNSON, M.C., FEAGANS, D.E. and FEINBERG, R.N. (1982). The mechanism of cell elongation during lens fiber cell elongation. *Dev. Biol. 92:* 54-59.

BEEBE, D.C., FEAGANS, D.E. and JEBENS, H.A.H. (1980). Lentropin. A factor in vitreous humor that promotes lens fiber cell differentiation. *Proc. Natl. Acad. Sci. USA 77:* 490-493.

BEEBE, D.C., SILVER, M.H., BELCHER, K.S., VON WYK, J.J., SVABODA, M.E. and ZELENKA, P.S. (1987). Lentropin, a protein that controls lens fiber formation, is related functionally and immunologically to the insulin-like growth factors. *Proc. Natl. Acad. Sci. USA 84:* 2327-2330.

BOWER, D.J., ERRINGTON, L.H., POLLOCK, B.J., MORRIS, S. and CLAYTON, R.M. (1983). The pattern of expression of chick δ-crystallin genes in lens differentiation and transdifferentiating cultured tissues. *EMBO J. 2:* 333-338.

BRAHMA, S.K. and VAN DOORENMAALEN, W.J. (1971). Immunofluorescence studies of chick lens FISC and δ-crystallin antigens during lens morphogenesis and development. *Ophthalmic Res. 2:* 344-357.

CALDES, T., ALEMANY, J., ROBCIS, H.L. and DE PABLO, F. (1991). Expression of insulin-like growth factor I in developing lens is compartmentalized. *J. Biol. Chem. 266:* 20786-20790.

CHAMBERLAIN, C.G. and McAVOY, J.W. (1987). Evidence that fibroblast growth factor promotes lens fiber differentiation. *Curr. Eye Res. 6:* 1165-1168.

CIRILLO, A., ARRUTI, C., COURTOIS, Y. and JEANNY, J-C. (1990). Localization of basic fibroblast growth factor binding sites in the chick embryonic neural retina. *Differentiation. 45*: 161-167.

CLAYTON, R.M. (1990). Transdifferentiation. In *Stochastic Modelling in Biology: Relevant Mathematical Concepts and Recent Applications*. (Ed P. Tautu). World Scientific Press, Singapore, pp. 15-29.

DE POMERAI, D.I., IP. W.K., McLAUGHLIN, M. and PERRY, K.C. (1991). Expression in non-lens tissues of an enzyme activity related to the 'lens specific' protein, δ-crystallin. *Development 111:* 181-190.

EDE, D.A. and KELLY, W.A. (1964). Developmental abnormalities in the head region of the *talpid*$^3$ mutant of the fowl. *J. Embryol. Exp. Morphol. 12:* 161-182.

ERRINGTON, L.H., BOWER, J., CUTHBERT, J. and CLAYTON, R.M. (1985). The expression of chick αA2-crystallin RNA during lens development and transdifferentiation. *Biol. Cell 54:* 101-108.

ERRINGTON, L.H., BOWER, D.J. and CLAYTON, R.M. (1986). Identification and characterization of a chick α-crystallin genomic clone and preliminary identification of a chick ß-crystallin cDNA clone. In *Coordinated Regulation of Gene Expression* (Eds. R.M. Clayton and D.E.S. Truman). Plenum Press, New York and London, pp. 371-375.

FAYEIN, N.A., COURTOIS, Y. and JEANNY, J-C. (1990). Ontogeny of basic fibroblast growth factor binding sites in mouse ocular tissues. *Differentiation 188:* 75-88.

FEINBERG, A.P. and VOGELSTEIN, B. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Ann. Biochem. 132:* 6-13.

HAFEN, E., LEVINE, M., GARBER, R.L. and GEHRIN, W.J. (1983). An improved *in situ* hybridisation method for the detection of cellular RNAs in *Drosophila* tissue sections and its application for localizing transcripts of the homeotic antennapedia complex. *EMBO J. 2:* 617-623.

HAMBURGER, V. and HAMILTON, H.L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol. 88:* 49-92.

HEAD, M.W., PETER, A. and CLAYTON, R.M. (1991a). Evidence for the extralenticular expression of members of the ß-crystallin gene family in the chick and a comparison with δ-crystallin during differentiation and transdifferentiation. *Differentiation 48:* 147-156.

HEAD. M.W., TRIPLETT, E.L. and CLAYTON, R.M. (1991b). Independent regulation of two co-expressed δ-crystallin genes in chick lens and nonlens tissues. *Exp. Cell Res. 193:* 370-374.

HEJTMANCIK, J.F., BEEBE, D.C., OSTRER, H. and PIATIGORSKY, J. (1985). α- and ß-crystallin RNA levels in the embryonic and post-hatched chicken lens: temporal and spatial changes during development. *Dev. Biol. 109:* 72-81.

HENDRIX, R.W. and ZWAAN, J. (1974). Changes in the glycoprotein concentration of the extracellular matrix between lens and optic vesicle associated with early lens differentiation. *Differentiation 2:* 357-362.

HENDRIX, R.W. and ZWAAN, J. (1975). The matrix of the optic vesicle-presumptive lens interface during induction in the chick embryo. *J. Embryol. Exp. Morphol. 33:* 1023-1049.

JEANNY, J-C., BOWER, D.J., ERRINGTON, L.H., MORRIS, S. and CLAYTON, R.M. (1985). Cellular heterogeneity in the expression of the δ-crystallin gene in non-lens tissues. *Dev. Biol. 112:* 94-99.

KATOH A. and YOSHIDA, K. (1973). Delta crystallin synthesis during chick lens differentiation. *Exp. Eye Res. 15:* 353-360.

KONDOH, H., ARAKI, I., YASUDA, K., MATSUBATA, T. and MORI, M. (1991). Expression of the chicken «δ2-crystallin» gene in mouse cells: evidence for encoding for argininosuccinate lyase. *Gene 99:* 267-271.

LI, X., CUTHBERTSON, A., ZELENDA, P.S., NORMAN, B. and PIATIGORSKY, J. (1992). Expression of δ1- and δ2-crystallin in lens, retina and cornea of the chicken eye. *Invest. Ophthalmol. Vis. Sci. 33 (Suppl.):* 1043.

MASCARELLI, F., ARRUTI, C., COUNIS, M.F., CHAUDUN, E. and COURTOIS, Y. (1986). *In vitro* stimulation of protein synthesis in adult bovine lens and in embryonic chick lens by eye-derived growth factors (EDGFs). In *Modern Trends in Aging Research* Vol. 147 (Eds. Y. Courtois, B. Faucheux, B. Forche, D.J. Knook and J.A. Treton). John Libbey, Eurotext-INSERM, Paris, pp. 293-301.

MASCARELLI, F., RAULAIS, D., COUNIS, M.F. and COURTOIS, Y. (1987). Characterization of acidic and basic fibroblast growth factors in brain, retina and vitreous of the chick embryo. *Biochem. Biophys. Res. Commun. 146:* 478-486.

McAVOY, J.W. (1980). Induction of the eye lens. *Differentiation 17:* 137-149.

McAVOY, J.W. and CHAMBERLAIN, C.G. (1989). Fibroblast growth factor (FGF) induces different responses in lens epithelial cells depending on its concentration. *Development 107:* 221-228.

NICKERSON, J.M., WAWROUSEK, E.F., BORRAS, T., HAWKINS, J.W., NORMAN, B.L., FILPULA, D.R., NAGLE, J.W., ALLY, A.H. and PIATIGORSKY, J. (1986). Sequence of the chicken δ2-crystallin gene and its intragenic spacer: extreme homology with the δ1-crystallin gene. *J. Biol. Chem. 261:* 552-557.

PAL, J.K. and MODAK, S.P. (1984). Immunochemical characterization and quantitative distribution of crystallins in the epithelium and differentiating fiber cell populations of chick embryonic lens. *Exp. Eye Res. 39:* 415-434.

PARKER, D.S., WAWROUSEK, E.F. and PIATIGORSKY, J. (1988). Expression of the δ-crystallin genes in embryonic chicken lens. *Dev. Biol. 126:* 375-381.

PATEK, C.E. and CLAYTON, R.M. (1988). The influence of genotype on the process of aging of chick lens cells *in vitro*. *Exp. Cell Res. 174:* 330-343.

PATEK, C.E. and CLAYTON, R.M. (1990). Age-related changes in the response of chick lens cells during long-term culture to insulin, cyclic AMP, retinoic acid and a bovine retinal extract. *Exp. Eye Res. 50:* 345-354.

PERALTA SOLER, A., ALEMANY, J., SMITH, R.M., DE PABLO, F. and JARETT, L. (1990). The state of differentiation of embryonic chicken lens cells determines insulin-like growth factor 1 internalization. *Endocrinology 127:* 595-603.

PIATIGORSKY, J. (1981). Lens differentiation in vertebrates. *Differentiation 19:* 134-154.

PIATIGORSKY, J., O'BRIEN, W.E., NORMAN, B.L., KALUMUCK, K., WISTOW, G.J., BORASS, T., NICKERSON, J.M. and WAWROUSEK, E.F. (1988). Gene sharing by δ-crystallin and argininosuccinate lyase. *Proc. Natl. Acad. Sci. USA 84:* 3479-3483.

RABAEY, M. (1962). Electrophoretic and immunoelectrophoretic studies of the soluble proteins in the developing lens of birds. *Exp. Eye Res. 1:* 310-316.

SAHA, T.S., SPANN, C.L. and GRAINGER, R.M. (1989). Embryonic lens induction: more than meets the optic vesicle. *Cell Differ. Dev. 28:* 153-172.

SCHWEIGERER, L., FERRARA, N., HAAPARANTA, T., NEUFELD, G. and GOSPONDAROWICZ, D. (1988). Basic fibroblast growth factor: expression in cultured cells derived from corneal endothelium and lens epithelium. *Exp. Eye Res. 46:* 71-80.

SHINOHARA, T. and PIATIGORSKY, J. (1976). Quantitation of δ-crystallin messenger RNA during lens induction in chick embryos. *Proc. Natl. Acad. Sci., USA. 73:* 2808-2812.

SULLIVAN, C.H., O'FARRELL, S. and GRAINGER, R.M. (1991). δ-crystallin gene expression and patterns of methylation demonstrate two levels of regulation for the δ-crystallin genes in embryonic chick tissues. *Dev. Biol. 145:* 40-50.

THOMAS, G., ZELENKA, P.S., CUTHBERTSON, R.A., NORMAN, B.L. and PIATIGORSKY, J. (1990). Differential expression of the two δ-crystallin/argininosuccinate lyase genes in lens, heart, and brain of chicken embryos. *New Biol. 2:* 903-914.

TRETON, J.A., JACQUEMIN, E., COURTOIS, E. and JEANNY, J-C. (1991). Differential localization by *in situ* hybridization of specific crystallin transcripts during mouse lens development. *Differentiation 47:* 143-147.

UEDA, Y. (1989). Monoclonal antibodies to chick crystallins. *Exp. Eye Res. 48:* 107-115.

VAN LEEN, R., BREUER, M.L., LUBSEN, N.H. and SCHOENMAKERS, J.G.G. (1987). Developmental expression of crystallin genes: *in situ* hybridization reveals a differential localization of specific mRNAs. *Dev. Biol. 123:* 338-345.

ZWAAN, J. and IKEDA, A. (1968). Macromolecular events during differentiation of the chick lens. *Exp. Eye Res. 7:* 461-472.

*Accepted for publication: April 1992*