THE REGULATION OF CRYSTALLIN GENE EXPRESSION

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Thesis presented for the degree of

Doctor of Philosophy

Institute of Cell, Animal and Population Biology,

University of Edinburgh

1991

ABSTRACT

The crystallins are the super-abundant structural proteins of vertebrate lens fibre cells, but certain of the crystallins are found at very much lower levels in some non-lens tissues. Chick α-, ß- and δ-crystallins are each encoded by a multi-gene family and their expression is developmentally regulated resulting in the changing pattern of crystallin composition of the successively formed lens fibre cells. The presence of α-, ß- and δ-crystallins in extralenticular chick tissues, including the retina, is examined here at the level of individual crystallin RNAs by Northern, dot-blot and in situ hybridisation, and the accumulated crystallin polypeptides are examined by Western blotting and immunohistochemistry. αA-crystallin, several ß-crystallins and both δ1- and δ2-crystallins are found to be expressed in non-lens chick tissues and the quantitative balance of δ-crystallin and individual ß-crystallin polypeptides in non-lens tissues is found to be tissue-specific. The relative steady-state levels of δ1- and δ2-crystallin mRNA are found to differ between lens and non-lens tissue and to be effected by transcriptional and post-transcriptional mechanisms. Selective inhibition of αA-crystallin synthesis using antisense oligonucleotides in vitro shows that αA-crystallin appears to be required for the correct differentiation of both developing lens and retina. The pattern of α-, ß- and δ-crystallin expression in non-lens tissues is interpreted in terms of the possible status of crystallins as multifunctional proteins and is used to question current theories concerning the evolutionary origin, expression and current functions of these proteins.

DECLARATION

I declare that the composition of this thesis and the work described herein are my own, except where specifically stated otherwise. In each paper included in the main text of the thesis, the work described was carried out solely by me, or mainly by me but in conjunction with the other named workers. The respective contributions of the other authors are described in Acknowledgments. With the exception of papers 4,8 and 9, these manuscripts were prepared by me and revised, where necessary, for publication by the co-author, my PhD supervisor, R.M. Clayton. My contribution to paper 4 was significant and this is included in the main text of the thesis. My contributions to papers 8 and 9 were smaller and these papers comprise the appendix.

ACKNOWLEDGMENTS

I am deeply indebted to my two supervisors, R.M. Clayton and D.E.S. Truman, for the opportunity to pursue the studies described here and for their support and encouragement throughout. Many of the various members of R.M Clayton's research group, both past and present, have given me the benefit of their extensive knowledge and provided me with training in relevant techniques. I would particularly like to thank J. Cuthbert for instruction on protein separation techniques, S.K.A. Sedowofia for advice on tissue culture methods, J-C. Jeanny for introducing me to *in situ* hybridisation and C. Patek for his interest and criticism.

Specifically, I would like to thank R.M. Clayton for the micro-dissection and manipulation of all early embryonic chick eye rudiments for *in vitro* studies (paper 7) and E.L. Triplett and R.M. Clayton for their collaboration in the design of antisense oligonucleotides and the development of their use as hybridisation probes (papers 1 and 3) and their use in the *in vitro* experiments shown in paper 7. The *talpid*[3] chick embryos were kindly provided and identified by D.A. Ede (paper 3).

The technical assistance of A. Peter in those experiments which involved chick neural retina cell culture (paper 5) is gratefully acknowledged, as is the assistance given by C. Nicolson in the preparation of the frozen section shown in paper 3. Photographic reproduction, for publication and presentation here, has relied on the skills of F. Johnstone. I would also like to thank M. Ramsay, P. Robinson and M. Wright for typing this thesis.

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INTRODUCTION

1. Lens Differentiation and Crystallin Expression

i. Induction, morphogenesis and cell differentiation

During the early stages of development in vertebrates the spatial and temporal coordination of lens and retina differentiation is achieved by an inductive interaction between the evaginating neuroectodermal optic vesicle and the competent head ectoderm. The resultant lens placode invaginates into the optic cup (future retina) and detaches from the head ectoderm, forming a vesicle, the lumen of which is filled by primary lens fibres derived by elongation of the cells of the posterior lens vesicle. The cells of the anterior half of the lens vesicle assume a cuboidal morphology and constitute the lens epithelium which covers the anterior face of the lens. The subsequent growth of the lens occurs by the addition of concentric layers of secondary fibres derived by the terminal differentiation of the most posterior epithelial cells lying at the lens equator.

Lens fibre cells are not lost during lens growth. The primary fibres remain throughout life in the center or lens nucleus and the array of concentrically arranged cortical lens fibre cells , reflects the temporal order of their deposition, with the earliest formed fibre cells towards the center and the most recently formed at the periphery.

Lens fibre cell differentiation involves coordinated changes in cell morphology and biochemistry. These include cell elongation, loss of cytoplasmic organelles, with eventual nuclear degeneration, alterations in cell membrane composition and cytoarchitectural components and the accumulation of the water soluble proteins collectively termed, the crystallins. Specific aspects of the induction, development and differentiation of the lens can be found in McAvoy (1980a),Bloemendal (1981), Piatigorsky (1981) and Maisel (1985). The protein concentration in lens fibre cells is high,

typically between 20-60% of wet weight and the crystallins account for 80-90% of the water soluble fraction (reviewed Clayton 1974; Bloemendal 1981,1982; Harding and Crabbe 1984; Wistow and Piatigorsky 1988). Lens transparency may be accounted for, at least in part, by high protein concentration, which is itself dependent on short-range order molecular interactions of the crystallins and regular, orderly packing (Delaye and Tardieu 1983; reviewed Slingsby 1985). However the crystallin composition of successively formed lens fibres is not homogeneous (reviewed Clayton 1970; Piatigorsky 1981; McDevitt and Brahma 1982; Harding and Crabbe 1984) and the gradient of protein concentration and refractive index, both of which fall from lens nucleus to cortex may result from the differential interactive properties of the crystallins (Clayton 1974; Veretout and Tardieu 1989).

Although the protein composition of the lens is relatively simple, in that a low number of proteins comprise a large proportion of the soluble fraction, the crystallins are remarkably diverse in their structure and expression both within and between species.

ii. Crystallin diversity

The α-, ß-, γ- and δ-crystallin proteins and polypeptide subunits of the lens are well characterised and in some cases the relevant genes have been sequenced (reviewed Bloemendal 1985; Yasuda and Okada 1986; Lubsen et al 1988; Wistow and Piatigorsky 1988). α-crystallin is a high molecular weight multimeric protein composed of the two related αA- and αB-crystallin polypeptide subunits with molecular weights of approximately 20 kilodaltons (kD). Native ß-crystallin is also a hetero-oligomer but found in discrete size classes, ß-light ($ß_L$) or ß-heavy ($ß_H$) in the native state corresponding to the number, dimers or oligomers, and composition of the six or more primary gene products which vary in molecular weight from 20-35kD. The α- and ß-crystallins are represented in all vertebrate lenses so far examined The ß-crystallin genes form a super-gene family with the monomeric γ-crystallins which are found in mammals. γ-crystallins other than γs are not found in birds and reptiles where they are apparently replaced

in the ontogenic sequence by δ-crystallin. Native δ-crystallin is a tetrameric protein of approximately 200kD (reviewed by Piatigorsky 1984). The twelve native protein isoelectric variants of δ-crystallin reported by Pal and Modak (1984) may result from the formation of each of the possible tetrameric combinations of the three δ-crystallin polypeptides differing in charge and size (Thomson et al 1978a; Patek and Clayton 1985). Detailed biochemical characterisation of crystallin proteins and their polypeptide subunits can be found in Clayton (1974), Bloemendal (1981), and Wistow and Piatigorsky (1988). The lenses of certain phylogenetic groups have been found to contain high levels of proteins unrelated to α-, ß/γ- or δ-crystallins. These taxon-specific crystallins are considered separately in relation to crystallin evolution and extralenticular crystallins.

Although the lens crystallin composition of the cow, rat, mouse and chicken, results from the expression of perhaps as few as 12-14 genes considerable additional diversity is generated by post-transcriptional mechanisms (reviewed by de Jong and Hendriks 1986; Piatigorsky 1989).

For example, in certain rodents and lower vertebrates a third α-crystallin polypeptide αA^{INS}-crystallin, is found which results from alternative RNA splicing of the αA-crystallin transcript (de Jong et al 1980; King and Piatigorsky 1983). Both the αA- and ß-crystallin polypeptides are also post-translationally modified by deamidation, phosphorylation and cleavage (for example see Voorter et al 1986,1989). αA-crystallin RNA from some species have an exceedingly long 3' untranslated sequence and a variable poly(A)$^+$ tail (Dodemont et al 1985). The ßA3- and ßA1-crystallin polypeptides, of chick and other species, are probably encoded by a single ßA3/A1-crystallin gene but generated by the alternative use of two different translation initiation sites (Peterson and Piatigorsky 1986). In the case of the two δ-crystallin genes of the chick the δ1-crystallin gene is capable of producing two δ-crystallin polypeptides by an, as yet, unknown co-translational mechanism (Wawrousek and Piatigorsky 1987) and the δ2-crystallin gene is also transcriptionally active in the chick lens (Parker et al 1988). Microheterogeneity in the polyadenylation site is evident in chicken ßB1-crystallin expression (Hejmancik et al 1986) and the rat γ-crystallins have multiple but closely clustered transcription initiation sites (van Leen et al 1986). The αB-

crystallin gene also produces two differently sized transcripts (Dodemont et al 1985; Iwaki et al 1990b).

Certain of these phenomena, particularly those involving 5' and 3' heterogeneity in mRNAs, are likely to be involved in the regulation of gene expression and probably do not figure in polypeptide diversity. Others, such as the differential splicing of αA-crystallin RNA and the two translation initiation codons of the βA3/A1-crystallin RNA clearly do contribute to polypeptide diversity.

Whether the diversity observed in lens proteins best conforms to a neutral or selective interpretation is debatable (see de Jong and Hendriks 1986; Wistow et al 1990). At the most gross level adaptation as an explanation for differences in crystallin expression between species is tenable. For example a comparison may be made between the hard, dehydrated, lenses of mice with high levels of nuclear δ-crystallins and the soft watery lens of the chick with high levels of nuclear δ-crystallin. It may be significant that the mouse is nocturnal requiring little accommodative capacity whereas the chick, prior to domestication, would presumably benefit from an ability to focus near for ground feeding and far to avoid predators.

iii. Developmental regulation of crystallin expression

The protein composition of lenses overall is known to differ between species but there is also a change in composition in individual species during development and protein localisation techniques suggest that this is due to the changing composition of successively formed lens fibre cells (see Clayton 1970,1974; Piatigorsky 1981). This developmental regulation is largely at the level of transcription, or at least relative-steady state RNA levels but may be modified by post-transcriptional events. No single obligate cascade of crystallin expression exists within or between species since the order in which the crystallins become detectable during lens development differ between rodents (α-crystallin first), newts (β-crystallin first) and chicks (δ-crystallin first) (see Clayton 1970; Piatigorsky 1981).

iv. Ontogenic changes in the chick

In the chick δ-crystallin is both the first crystallin to appear and the most abundant soluble lens protein throughout the embryonic period (Rabaey 1962; Zwaan and Ikeda 1968). During embryonic and post-hatch development the contribution of δ-crystallin diminishes and that of ß-crystallin increases correspondingly, while that of α-crystallin remains constant after an initial increase (Genis-Galvez et al 1968; Truman et al 1972; Pal and Modak 1984). These changes recorded at the level of the whole lens are probably driven by transcription or at least steady-state RNA levels since parallel changes are seen in the pattern of RNAs translated in vitro (Thomson et al 1978b; Ostrer et al 1981) and in the level of individual mRNAs assayed by hybridisation analysis (Bower et al 1983a; Errington et al 1985; Hejmancik et al 1985; Clayton et al 1986b).

δ-crystallin is first detectable around 50 hours of development in a few lens placode cells when invagination is initiated (Zwaan 1974). δ-crystallin synthesis and accumulation both rise sharply above background levels and increase steeply during the process of lens placode invagination at around 54 hours of development (Katoh and Yoshida 1973; Shinohara 1975) and the protein localises to the most posterior cells in the lens pit which are in closest apposition to the optic cup (Zwaan and Ikeda 1968; Brahma and van Doorenmaalen 1971). At the lens vesicle stage (2.5 days), δ-crystallin fluorescence localises uniformly to the elongating presumptive primary fibre cells but one day later the fluorescence has spread to the lens epithelium as well as the primary fibres and a peak is reached at the 7 day stage (Zwaan and Ikeda 1968; Brahma and van Doorenmaalen 1971; McDevitt and Clayton 1979). However, the level of δ-crystallin in the lens epithelium falls after 8 days of embryonic development and δ-crystallin is no longer detectable in the lens epithelium of hatched birds (Genis-Galvez et al 1968; Brahma and van Doorenmaalen 1971; McDevitt and Clayton 1979). This reduction results from a reduction in δ-crystallin synthesis followed by a loss of δ-crystallin RNA (Yoshida and Katoh 1971; Beebe and Piatigorsky 1981). Although still the most abundant water soluble lens protein throughout the embryonic period, comprising more than 60% at the 19 day embryo stage, its overall contribution continues to fall after hatch to around 14% in

the adult (Truman et al 1972; Pal and Modak 1984) due to a reduction in synthesis (Bagchi et al 1981) and due to mRNA degradation (Treton et al 1982). However, δ-crystallin protein is not lost from the cells in which it accumulates but its contribution to successively formed lens fibres does diminish (Zwaan 1968; Genis-Galvez et al 1968; Pal and Modak 1984).

Although ß-crystallin appears after δ-crystallin during chick lens morphogenesis (Zwaan and Ikeda 1968) the reported timing of appearance varies between reports. These apparent discrepancies are probably due, in part, to differences between antisera and the techniques employed in conjunction with the the complex antigenic nature and changing subunit composition of ß-crystallin (Truman et al 1972; Clayton and Truman 1974; Truman and Clayton 1974). All ß-crystallin antigenic determinants are present by 7.5 days and subsequent changes affect both the abundance and the relative levels of antigenic determinants (Truman et al 1972). Whether ß-crystallins are examined <u>in toto</u>, or as anodal and cathodal isoelectric sub-groupings they accumulate in the primary and secondary lens fibres and then spread to the overlying lens epithelium (Zwaan and Ikeda 1968; Waggoner et al 1976; McDevitt and Clayton 1979). Quantitative studies show that during post-hatch development the ß-crystallins become the predominant crystallin class (Truman et al 1972; Pal and Modak 1984) and this results from their increasing contribution to the most recently formed lens fibre cells (Zwaan 1968; Pal and Modak 1984). This process results from the relative increase in the abundance of total ß-crystallin RNA (Thomson et al 1978b; Ostrer et al 1981) and individual ß-crystallin RNAs (Hejmancik et al 1985; Clayton et al 1986b). The accumulation of ß-crystallin RNAs during ontogeny differs between lens epithelium and fibres (Ostrer et al 1981; Hejmancik et al 1985). Specifically, the ßA3/A1-crystallin RNA (formerly ß19/26) accumulation rises sharply during embryogenesis to a level over four times higher than any other ß-crystallin RNA assayed post-hatch and, before the end of the embryonic period, reaches absolute levels higher than that of δ-crystallin (Hejmancik et al 1985). The ßB1-crystallin (formerly ß35) RNA levels are comparatively low but unlike other ß-crystallin RNAs are largely restricted to the fibre cells at all stages examined (Ostrer et al 1981; Hejmancik et al 1985). This relative fibre cell specificity can also be seen in the relative abundance and

localisation of the ßB1-crystallin polypeptide (Patek and Clayton 1985; Brahma 1988).

α-crystallin is first detectable after ß- and δ-crystallin at 3.5 days of embryonic development (Zwaan and Ikeda 1968; Truman et al 1972; McDevitt and Clayton 1979). The protein localises to the most central primary fibres and spreads to encompass the entire fibre mass, annular pad and epithelium by 8 days of development and this pattern remains unchanged apart from a subsequent reduction in fluorescence from the nuclear fibres (Ikeda and Zwaan 1967; Brahma and van Doorenmaalen 1971; McDevitt and Clayton 1979). The relative proportions of the two α-crystallin subunits do not appear to change during development (Patek, Head and Clayton, in preparation) but differences in their inter- and intra-cellular localisation suggest they are independently regulated (Ueda 1989).

v. Ontogenic changes in rodents

A similar programme of morphological changes occur during lens differentiation in rodents and here too the crystallin classes and their polypeptide subunits are differentially regulated during development. However the rodent lens is different from that of the chicken in its relative proportions, size in relation to the rest of the eye, its anatomy and in the crystallins expressed. The δ-crystallin found at high levels in chicken lens fibres laid down during the embryonic period is not found in mammalian lenses. Although δ-crystallin is apparently replaced by γ-crystallins in rodents, it is α-crystallin which appears before ß- and γ-crystallins in the cells of the posterior lens pit in mice and rats (McAvoy 1978b; Zwaan 1983; Zwaan and Silver 1983).

ß- and γ-crystallin expression closely follows that of α-crystallin in the primary fibres prior to the completion of the elongation process. α-crystallin is found in the rodent lens epithelium and fibres, as it is in the chick, but unlike the chick, rodent ß- and γ-crystallins are expressed in the fibres, and not the epithelial cells (McAvoy 1978b; van Leen et al 1987a; Treton et al 1991).

The near synchrony of initiation of crystallin expression in rats and mice is followed by a quantitatively distinct pattern of accumulation and subsequent loss of crystallin mRNAs (van Leen et al 1987b; Murer-Orlando et al 1987; Treton et al 1988; Aarts et al 1989a; Treton et al 1991) which in most cases correlates well with the ontogenic changes in crystallin synthesis seen in rats (Voorter et al 1990). Two kinds of exceptions occur. Firstly, the αB-crystallin and γ_S (formerly β_S)-crystallin genes are activated later during embryonic development than other crystallin genes (Aarts et al 1989a). Secondly, there are disproportionately high levels of αB- and βB2-crystallin RNA present compared to their respective levels of synthesis, suggesting that their expression may be regulated or restricted by a relatively low translational efficiency (Aarts et al 1989a; Voorter et al 1990). Similarly, polyadenylation state may be a significant factor in the ontogenic down regulation of γ-crystallin RNAs (Treton et al 1988). The mature rodent lens nucleus is likely to be rich in βB1-, βB3- and γ-crystallins and the cortex rich in α-, βB2 and γ_S-crystallins (van Leen et al 1987a,b; Aarts et al 1989a; Voorter et al 1990).

vi. Species comparisons and evolution

The comparison of lens crystallin expression in chick and rodents shows a similar series of molecular mechanisms in operation but that these establish quite different patterns of protein composition. In some cases, by the use of different genes, δ-crystallin in chicks, γ-crystallins in rats. In other cases orthologous genes have clearly acquired species specific patterns of relative abundance as is seen in the case of βB2-crystallin RNA, the most abundant rodent β-crystallin transcript, and βA3/A1-crystallin RNA, the most abundant chick β-crystallin transcript (Hejmancik et al 1985; Aarts et al 1989a).

These data indicate that during vertebrate radiation different lineages have acquired developmental mechanisms regulating crystallin expression which are qualitatively and quantitatively distinct (see Clayton 1974). Such diversity is not restricted to comparisons made between relatively distant lineages. When other birds are compared with the chick qualitative and quantitative differences are also

apparent including differences in ontogeny (Brahma and van der Starre 1982), the expression of crystallins apparently not found in the chick such as ϵ-crystallin (Stapel et al 1985), differences in the expression of the two δ-crystallin genes (Piatigorsky et al 1988; Wistow and Piatigorsky 1990) and, in the case of one avian species at least, the absence of δ-crystallin from the lens altogether (Wistow et al 1990).

2. Extralenticular Crystallin Expression: Functional and Evolutionary Considerations

i. Introduction

There is a semantic problem involved in any discussion of extralenticular crystallins in that much of the relevant literature up until recently contained an implicit assumption of their lens specificity and specialisation as such. Not infrequently the absolute lens specificity of the crystallins was used as one of their definitive characteristics. However, the problem is more than one of nomenclature alone. One important aspect of embryonic development, namely cell differentiation, is effected, directly or indirectly by differential tissue-specific gene expression. Genes and their polypeptide products have conventionally been categorised into those which are present in some, if not all, cell types performing "housekeeping" functions or those present only in cells of a particular specialised type, the so called "luxury" gene products. Individually tissues or cell types may be characterised by the abundant expression of particular "luxury" proteins but it is also true that the functions and properties of these proteins have been inferred from, and become conceptually limited to the characteristics of the cell or tissue type in which they were initially found. Given that gene expression in a quantitative phenomenon the status of any given protein in the proposed luxury/housekeeping dichotomy is at least partially dependent on the sensitivity of available detection techniques. It is therefore not surprising that the relative abundance of the crystallins in the lens resulted in their initial categorisation as the structural luxury proteins of the lens, but with

the continuing refinement of existing methods and the introduction of techniques of increased sensitivity it has become increasingly clear that the crystallins along with many other luxury proteins can be found in ectopic (inappropriate) locations.

The molecular interactions of the crystallins at high concentration, in conjunction with other cellular features, may account for the transparency and refractive index gradient of the lens (Delaye and Tardieu 1983; Veretout and Tardieu 1989) but these characteristics of the organ as a whole cannot properly be attributed to any given polypeptide component per se.

Numerous gene products once thought to characterise single tissues or cell types such as actin, myosin, collagen, ovalbumin, insulin, S-100 and glial fibrillary acidic protein (GFAP) can all be found at lower levels in other tissues and cell types (see Clayton 1982, 1990). In some cases these "secondary" functions at lower concentration are clearly related to the "primary" function at high concentration as is thought to be the case in the proposed actin/myosin contractile system of lens epithelial cells (Rafferty et al 1990) and the expression of the intermediate filament proteins vimentin and GFAP in this same tissue (Boyer et al 1990). In other examples the low level functions in ectopic locations are less obvious as in the cases of neurotransmitter expression in immune system and germ cell types (see Persson et al 1990) or the expression of the renin-angiotensin system in cells of the central nervous system (see Milsted et al 1990).

In the cases of some crystallins it is now known that the functions performed in non-lens tissues are different from their structural role at high concentration in the lens and that these genes encode multifunctional proteins. Other crystallins or their RNAs may be detectable in non-lens tissues but their possible functions are not clear. The extralenticular expression of still other crystallins remains to be investigated. Viewed from an evolutionary perspective these studies may provide indications as to the structural features which have predisposed the ancestral proteins for selection as major lens proteins and whether this involved the classical route of gene duplication and divergence or the acquisition of independent tissue specific regulatory mechanisms for the expression of bi- or multi-

functional proteins.

ii. Extralenticular crystallins

In principle, immunological cross-reactivity between abundant lens antigens and those of non-lens tissues may be due to a minor component of the lens being a major component in non-lens tissues, the possession of similar or identical epitopes on otherwise unrelated molecules or the expression of abundant lens proteins at lower levels in non-lens tissues.

Clayton et al (1968) systematically tested this proposition and reviewed earlier studies, using hyperimmune sera to total lens protein and identifying cross-reacting antigens in non-lens tissues of both chick and Xenopus by electrophoretic and immunological means. These tests showed that lens crystallin antigens of the α-, ß- and δ-crystallin classes are present in a native molecular form partially or fully identical to the form in which they are found in the lens but at lower levels. Particularly prominent in this study was the identification of α-, ß- and δ-crystallin antigens with identical electrophoretic mobilities to lens antigens in the retina, but reactions of partial or complete identity to lens crystallins were also found to be present in iris, cornea, brain, liver, kidney and muscle. The alternative approach of using antisera raised against purified crystallin classes identified α-, anodal ß-, cathodal ß- and δ-crystallins in the 8 day embryo chick neural retina at between 0.2% and 1% of their respective levels in the day-old post-hatch lens (de Pomerai et al 1977).

The case for this immunological cross-reactivity resulting from the activation of the same genes as those expressed in the lens is strengthened by the finding that the most abundant lens mRNAs are also represented at low levels in 8 day embryo neural retina (NR) and retinal pigmented epithelium (PE) (Jackson et al 1978) corresponding to 0.025% and 0.043% of the cytoplasmic poly(A)$^+$ RNA of NR and PE respectively (Thomson et al 1979). During embryonic development the level of crystallin mRNA in the NR falls from the 3.5 days of egg incubation to the day-old post-hatch stage; however haemagglutination inhibition studies indicate that α- and ß-crystallin levels,

undetectable at the 3.5 day stage, increase approximately 3-fold between the 8 day and the day-old post-hatch stage (Clayton et al 1979).

The use of cDNA synthesised from abundant lens mRNA, though quantitatively accurate as a hybridisation probe, fails to discriminate between the transcripts of different crystallins. Crystallin classes and their polypeptide chains are distinguishable by immunological criteria (reviewed Clayton 1970, 1974) and crystallin polypeptides are encoded by apparently unrelated gene families (reviewed Wistow and Piatigorsky 1988). This suggests that their evolution and recruitment as lens proteins has been, to a large degree, independently achieved from genes or DNA sequences in existence prior to the evolution of the vertebrate lens (reviewed de Jong et al 1989; Piatigorsky and Wistow 1989). A corollary of this is that extralenticular crystallin expression may relate more closely to molecular properties of ancestral genes which predate the evolution of the vertebrate lens (Clayton et al 1986b). For these reasons the expression and putative functions of extralenticular crystallins are best dealt with individually for each crystallin gene family.

iii. δ-crystallin

δ-crystallin transcripts are detectable in Northern transfers of day-old post-hatch or embryonic chick neural retina (Agata et al 1983; Bower et al 1983a; Clayton et al 1986a,b), limb bud (Agata et al 1983), heart, lung, liver and kidney (Bower et al 1983b; Clayton et al 1986a) and brain (Agata et al 1983; Clayton et al 1986a) including the embryonic forebrain, optic lobes, hindbrain (Takagi 1986) and adenohypophysis (Ueda and Okada 1986). The levels detected are very much lower than those found in the lens. For example the poly(A)$^+$ δ-crystallin RNA found in the 3.5 and 8.5 day embryo neural retina is at 0.0028% of the level found in the day-old post-hatch lens (Agata 1985) and that found in the 3.5 day adenohypophysis is at less than one thousandth of the level found in the lens (Ueda and Okada 1986). δ-crystallin RNA accounts for 70-80% of the lens poly(A)$^+$ fraction (Piatigorsky 1984) whereas the δ-crystallin RNA found in the brain accounts for between 10^{-7} and 10^{-8} of the poly(A)$^+$ RNA and δ-crystallin for 10^{-5} of the soluble protein (Takagi 1986).

A characteristic feature of extralenticular δ-crystallin expression is the presence of high molecular weight δ-crystallin RNA in discrete size classes ranging from 2.5 kilobases (kb) to as large as 22kb in addition to, or instead of, the 2kb fully processed mature δ-crystallin transcripts found in the lens (Agata et al 1983; Bower et al 1983b; Agata 1985; Clayton et al 1986a,b). Similarly sized high molecular weight transcripts are also detectable in the lens total, cytoplasmic and poly(A)$^+$ RNA fractions (Bower et al 1982, 1983a; Agata et al 1983) and at later stages of development in the poly(A)$^-$ and nuclear RNA fractions (Bower et al 1983b). This suggests that the high molecular weight δ-crystallin RNA found in non-lens tissues represents processing intermediates (Agata et al 1983; Bower et al 1983b; Agata 1985). The proportions of the differently sized δ-crystallin RNA classes detected varies between tissues, stages of development and RNA fraction assayed. Any interpretation of comparisons made between differences in δ-crystallin RNA processing is problematic since a wide variety of RNA extraction procedures have been employed by different workers. This problem is compounded by a latent processing capacity evident in fresh neural retina, which may also occur in other tissues (Clayton et al 1986a,b).

Both the low levels of δ-crystallin RNA in non-lens tissues and the observation that a high proportion exist in a partially processed form may be used to suggest that extralenticular δ-crystallin expression represents "leakiness" in the mechanisms regulating this gene which must , de facto, be capable of high levels of expression in the lens. Several lines of evidence suggest that this is not the case.

Firstly, non-lens tissues which express δ-crystallin exhibit cellular heterogeneity such that a low overall level is a reflection of a relatively high level in a limited number of cells. When δ-crystallin RNA is localised by in situ hybridisation in squashes of neural retina, pigmented retina, adenohypophysis, epiphysis, otic vesicle and heart of chick embryos of between 3.5 and 6 days of development infrequent clusters of cells are seen to be heavily labelled (Jeanny et al 1985; Clayton et al 1986a,b). This RNA is has a predominantly nuclear location, presumably corresponding to the partially processed transcripts seen in Northern transfers, although some cytoplasmic label is also detectable in neural retina, adenohypophysis and

epiphysis (Jeanny et al 1985). In the case of the neural retina where δ-crystallin antigenicity had previously been reported (Clayton et al 1968; de Pomerai et al 1977) the presence of the 50 kilodalton (kD) δ-crystallin polypeptide is detectable in Western blots (Takagi 1986) and localises to a limited number of modified Muller glia which surround the optic nerve insertion (Linser and Irvin 1987). This immunoreactivity persists throughout the embryonic period. However the immunohistochemical localisation of δ-crystallin to the adenohypophysis (Barabanov 1977,1982) is apparently a transitory phenomenon detectable only between stages 17-30 but occurring in a larger proportion of cells, approximately 30% (Ueda and Okada 1986). In the adenohypophysis both fully processed 2kb RNA and 50kD δ-crystallin are detectable by Northern and Western blotting respectively (Ueda and Okada 1986). The optic cups (future retina) adenohypophysis (future pituitary) and epiphysis (future pineal) are all mesocephalic (midbrain) derivatives but the remaining brain regions, forebrain, optic lobes and hindbrain, also express mature δ-crystallin RNA and detectable protein but no restricted sub-populations have been identified (Takagi 1986).

This pattern of differential tissue-specific expression and localisation to cells defined by location or cell type is indicative of a functional cellular component. The chick genome contains two δ-crystallin genes (δ1- and δ2-crystallin) which are 91% identical at the amino acid level (Nickerson et al 1986). Both genes are expressed in the lens during embryonic development and early post-hatch stages although the level of δ2-crystallin mRNA is only 1-2% of that of the δ1-crystallin cytoplasmic poly(A)$^+$ RNA (Parker et al 1988). Both genes also exhibit a high degree of sequence homology with the enzyme argininosuccinate lyase (ASL) of yeast and humans, with the homology of the δ2-crystallin gene being greater than that of the δ1-crystallin gene (Wistow and Piatigorsky 1987). Other characteristics such as subunit molecular weight, isoelectric focusing point (pI) and secondary structure are also similar between δ-crystallin and ASL (Wistow and Piatigorsky 1987). Chick, duck and ostrich lens δ-crystallins have ASL activity (Piatigorsky et al 1988; Chiou et al 1991) and no chick or duck genomic sequences other than the two δ-crystallin genes cross-hybridise to a human ASL cDNA (Piatigorsky et al 1988). The ASL activity of the embryonic chick lens is around one

hundred times that of selected chick and duck non-lens tissues but the embryonic duck lens has an ASL activity of over one thousand times that of purified chick δ-crystallin which may be accounted for by the relatively high proportion of δ2-crystallin in duck lens as compared to the chick (Piatitgorsky et al 1988). This suggests that δ-crystallin is a bifunctional protein performing the role of a metabolic enzyme when expressed at low levels in extralenticular avian tissues but functioning as a structural protein when at high levels in avian lenses. However, whether this situation actually represents "gene sharing" or not depends on the functional properties and subunit composition of native δ-crystallin in lens and non-lens tissues. Since a direct approach to this question would present considerable technical difficulties, the question of the genetic source of extralenticular δ-crystallin, and by implication any differences in the functional properties of the two gene products, was addressed in this work at the level of the two δ-crystallin RNAs using oligonucleotide probes complementary to the most divergent regions of the δ1- and δ2-crystallin genes.

iv. Taxon-specific crystallins and their relationship to enzymes

The evolutionary recruitment of a metabolic enzyme and its expression at high levels in the vertebrate lens as a structural protein, as described for ASL and δ-crystallin, is not a unique isolated evolutionary event but represents a more general phenomenon particularly in the case of crystallins which display a restricted phylogenetic distribution as lens proteins (reviewed Wistow and Piatigorsky 1987; de Jong et al 1989; Piatigorsky and Wistow 1989). Moreover, an analogous situation appears to have occurred during the evolution of the cephalopod eye (Doolittle 1988) where the major squid lens protein exhibits a high degree of sequence homology to the enzyme glutathione S-transferase (Wistow and Piatigorsky 1987; Tomarev and Zenovieva 1988).

It is important to make a distinction between the evolution of crystallin genes by the more conventional route of duplication and divergence from a pre-existing gene (discussed in Clayton and Truman 1974) and that of gene sharing as described by Wistow and Piatigorsky

(1989). In the former case protein function, developmental regulation of expression and selection pressure act independently and apply separately to the duplicated genes. In the latter case the polypeptide is multifunctional, will evolve under multiple constraints and the developmental regulation of its expression will necessarily be both subtle and complex. The case for gene sharing has been made convincingly for ϵ- and τ-crystallin but it is not yet clear whether δ-, λ-, ρ-, μ-, or η-crystallins are coded for by genes which also encode functional enzymes or whether they are just closely related to specific enzymes.

ϵ-crystallin, which is present in variable amounts in some, but not all, avian and reptilian lenses (Stapel et al 1985), has lactate dehydrogenase activity (LDH) and is apparently identical to LDH-B4 expressed in the heart (Wistow et al 1987; Wistow and Piatigorsky 1987; Chiou et al 1988). In the duck and chick a single LDH-B4/ϵ-CRY gene encodes both the abundant lens protein ϵ-crystallin and the heart enzyme LDH-B4 (Hendriks et al 1988) but there is evidence for specific differences in post-translational modifications between lens and heart ϵ-CRY/LDH-B4 in ducks and swans (Hendriks et al 1988; Chiou et al 1989, 1990). The level of ϵ-CRY/LDH-B4 is very much higher in the lenses of ducks and swans, where it may act as a structural component, than in the lens of the chick (Stapel et al 1985), where a pattern of LDH isoenzyme activity is known to occur at levels compatible with other tissues (for example see Maisel et al 1965).

Similarly, τ-crystallin is present in variable amounts in the lenses of several species of birds, reptiles and fish (Stapel and de Jong 1983; Williams et al 1985). Comparison of τ-crystallin and human α-enolase suggest a close evolutionary relationship (Wistow and Piatigorsky 1987) and in the chicken and duck, at least, they are encoded by a single α-ENO/τ-CRY gene. Although there is approximately twenty-five times more α-ENO/ τ-CRY RNA in duck lens as compared to liver (Wistow et al 1988) the RNA and protein is more abundant in the lens epithelium than in the lens fibres and exists in an active enzymic form (Rudner et al 1990; Kim et al 1991). This contrasts with the situation found in the turtle where α-ENO/τ-CRY is more abundant in the lens fibres and has a markedly reduced enzyme activity (Wistow et al 1988; Rudner et al 1990).

It is not yet clear whether gene sharing can be inferred from the homologies between ρ-crystallin (expressed in frogs) and NADPH-dependent reductases (Watanabe et al 1988; Carper et al 1989), λ-crystallin (expressed in rabbits and hares) and hydroxyacyl-coenzyme A dehydrogenases (Mulders et al 1988), δ-crystallin (expressed in guinea pigs) and alcohol dehydrogenases (Borras et al 1989; Rodokanaki et al 1989) and η-crystallin (expressed in elephant shrews) and aldehyde dehydrogenases (Wistow and Kim 1991). These examples do however draw attention to a hitherto unrecognised diversity in mammalian lens proteins and suggest that this results from the recruitment of lens crystallins from pre-existing enzymes in mammalian as well as sauropsidans and amphibia.

v. The α-and ß/γ-crystallin gene families

It might be argued that the diversity of vertebrate lens crystallins of restricted phylogenetic distribution represents either adaptive or effectively neutral modulations in the protein composition of vertebrate lens fibre cells (de Jong et al 1989; Wistow et al 1990) but in either case it seems likely that these changes in certain lineages have been superimposed upon a pre-existing lens expressing α- and possibly ß/γ-crystallins (Wistow and Piatigorsky 1988; de Jong et al 1989). Members of both gene families are expressed in most, if not all, vertebrate lenses so far examined and their larger gene families and related sequences extend into the invertebrates and prokaryotes (reviewed Lubsen et al 1988; Wistow and Piatigorsky 1988).

The two α-crystallin genes (αA-crystallin and αB-crystallin) probably arose by duplication from a common ancestor, itself derived by exon shuffling (see Wistow and Piatigorsky 1988). The C-terminal region of α-crystallins displays homology with the small heat-shock proteins (SHSPs) of Drosophila (Ingolia and Craig 1982; de Jong et al 1988a), with the egg antigen p40 of the blood fluke Schistosoma mansoni (Nene et al 1986; de Jong et al 1988a) and with an antigen of the prokaryote Mycobacterium leprae (Lindquist and Craig 1988).

A detailed scheme for the phyletic derivation of the multigene gene ß/γ-crystallin super-gene family by gene duplication and intragenic

duplication and fusion is described in Lubsen et al (1988) and Wistow and Piatigorsky (1988) but the ß/ɣ-crystallin sequences also display structural homologies to the Ca^{2+}-binding spore coat protein S of the bacterium Myxococcus xanthus and to the encystment protein spherulin 3a of Physarum polycephalum (Wistow 1990) as well as to the human c-myc proto-oncogene (Crabbe 1985).

These structural homologies span vast phylogenetic distances: it is possible that the recruitment of α- and ß/ɣ-crystallins as lens proteins occurred by differential tissue-specific expression of ancestral α- and ß/ɣ-crystallins which performed functions outside of the lens in metazoan species. The reported current existence of extralenticular α-and ß-crystallins suggests that this is indeed the case. Since sequence comparisons fail to establish links between these crystallins with any polypeptides of known function in vertebrate non-lens tissues, characterisation of the pattern and regulation of α- and ß-crystallin expression may lead to a better understanding of their derivation and possible extralenticular functions.

vi. Extralenticular α- and ß-crystallins

α- and ß-crystallin antigens are known to be present in the chick iris and retina as well as in extra-ocular tissues of the chick and Xenopus (Clayton et al 1968; de Pomerai et al 1977). The levels detected in the chick retina, although always low compared to the lens, rise during embryonic development (Clayton et al 1979). In chick, mouse, rabbit and frog retina it is the Muller glia which are recognised by antisera raised against partially purified native α-crystallin (Moscona et al 1985).

Reports from several laboratories have subsequently shown that of the two α-crystallin genes, αB-crystallin RNA and protein are detectable in extra-ocular rodent tissues including heart, skeletal muscle, placenta, oesophogus, lung, kidney, skin and brain (Duguid et al 1988; Bhat and Nagineni 1989; Dubin et al 1989; Iwaki et al 1989). In the cases where the appropriate experiments have been performed extralenticular αB-crystallin expression is seen to increase during development (Bhat and Nagineni 1989; Iwaki et al, in press) but in

tissues other than lens and heart, only a subpopulation of cells in any given tissue are involved in αB-crystallin expression (Iwaki et al 1989).

In the rat central nervous system it is the oligodendrocytes and some astrocytes and in the peripheral nervous system it is the Schwaan cells which immunostain for αB-crystallin (Iwaki et al 1990a). αB-crystallin is detectable in human neural and pigmented retinas by Western blotting (Bhat and Nagineni 1989; Reddy et al 1991) but in the rat no immunostaining is seen in the neural retina other than in optic nerve astrocytes (Iwaki et al 1990a), whereas an anti-α-crystallin antiserum labels cat retina astrocytes and Muller glia (Lewis et al 1988) consistent with the earlier report of Moscona et al (1985) for chick, mouse, rabbit and frog.

Western blotting shows that both αA- and αB-crystallin polypeptides are present in the cat retina (Lewis et al 1988) and although αA-crystallin RNA was not detected in extralenticular tissues in mice (Dubin et al 1989), αA-crystallin RNA had previously been shown to be expressed in embryonic chick neural and pigmented retinas (Agata 1985; Errington et al 1985).

These apparent discrepancies between reports of the presence and localisation of the two α-crystallins in the retina may be due to differences in the experimental approach whether due to the comparison of different techniques (immunohistochemistry and Western blotting), the level of gene expression assayed (RNA or polypeptide) or the antibody specificity (relative titres and avidities for the two α-crystallin polypeptides). Alternatively they may reflect genuine ontogenic or species differences in the α-crystallin subunits expressed in the retina.

Although ß-crystallin antigenicity is detectable in the retina and other non-lens tissues of the chick (Clayton et al 1968; de Pomerai et al 1977; Clayton et al 1979) the molecular nature of this antigenic cross-reactivity has received little attention but is considered to be a matter of priority and is directly addressed in the work described here.

3. Regulatory Signals and Mechanisms Involved in Crystallin Expression

i. General introduction

There are several routes to the crystallin rich lens fibre cell phenotype. In vivo, nuclear lens fibres are formed by elongation of the posterior cells of the lens vesicle followed by successive layers of cortical fibres formed from equatorial lens epithelial cells. Lens regeneration from dorsal iris in urodeles follows a similar course of primary (nuclear) and secondary (cortical) fibre formation (Yamada 1967) as does the regeneration of this same tissue from outer cornea of urodele larvae and anuran tadpoles (Freeman 1963, Campbell 1963). Under suitable in vitro conditions lens fibre cells, identified by morphology and crystallin expression, can be derived from competent head ectoderm (Barabanov and Fedtsova 1982) lens epithelial explants (for example Phillpott and Coulombre 1965, 1968), dissociated chick lens epithelial cell cultures (Okada et al 1971) and newt iris (Yamada and McDevitt 1974). However, when dissociated and maintained in primary culture a potential for lens cell differentiation is seen in numerous tissues which are both developmentally unrelated to the lens cell lineage and which are not known to express the lens cell phenotype in vivo (reviewed Clayton 1982, Yamada 1982, Okada 1983). In vitro lens cell differentiation from lens epithelium and the transdifferentiation of other cell types allows both an investigation into the nature of competence, differentiation and cellular phenotypic stability and the directive cellular signals involved in lens cell differentiation. Similarly, crystallin gene expression must be responsive to these signals, the interdependence of lens cell differentiation and the expression of any individual or all crystallin genes is dependent on an understanding of the relevant signal transduction mechanisms. The changing crystallin composition of lens fibre cells formed during development and the expression of individual crystallin genes in extralenticular tissues both suggest that lens cell differentiation and crystallin expression are in principle independently regulable. It is therefore important to understand the mechanisms by which they are coordinately regulated in the lens.

ii. Growth, differentiation and maintenance factors

Lens determination in vivo is thought to involve a temporal sequence of inductive interactions between future head ectoderm and sequentially chordomesoderm, neuroectoderm, endoderm and mesoderm which establish a lens forming bias (competence) in head ectoderm. The exact timing and location of the lens rudiment resulting from an inductive signal from the evaginating optic vesicle (future retina) which initiates lens morphogenesis and differentiation (see Saha et al 1989 for a review and a comprehensive synthetic model). The existence of a single specific inducer of lens differentiation, from the optic vesicle, is in some doubt (discussed in McAvoy 1980a; Saha et al 1989) but there is evidence suggesting a role for a small heat-labile soluble factor in this process (see Piatigorsky 1981). Of the signals which induce lens formation little is known although retinal extracts, usually a rich source of fibroblast growth factor (FGF) can act as an inducer of lens (Mikhailov and Gorgolyuk 1979). FGF is known to function as an embryonic inducer in other tissue systems (Slack et al 1987, 1988, Grunz et al 1988) and the optic vesicle/head ectoderm and interfacial matrix all contain binding sites for bFGF (Fayein et al 1990).

Considerably more is known regarding the possible mechanisms which regulate the growth and differentiation of cortical or secondary lens fibres and their characteristic pattern of crystallin expression. The retina is directly implicated in the growth and differentiation of the lens. When the lens of a 5 day embryo chick is rotated through 180°, such that the lens epithelium faces the retina rather than the cornea, the existing fibres cease to elongate and the lens epithelial cells elongate. A partially complete anterior epithelium and a germinal zone both with the correct new polarity become established (Coulombre and Coulombre 1963). This phenomenon has also been demonstrated in the mouse and the identification of positive signals from the retina rather than an inhibitory effect via the anterior chamber clearly made (Yamamoto 1976). Surgical reorientation in amphibia similarly suggests that proximity to the retina determines the orientation of the regenerated lens (see Coulombre and Coulombre 1963). The exposure of cornea to growth factors contained in the vitreous after lentectomy has been suggested as the initial stimulus for lens regeneration from

cornea in Xenopus larvae (Bosco 1988).

Explanted central lens epithelia (LE) from 6 day chick embryos elongate in vitro in response to serum (Philpott and Coulombre 1965; Philpott 1970) or, after serum starvation, when implanted into a lentectomised eye or, to a more limited extent, when confronted with embryonic neural retina or mesenchyme in vitro (Philpott and Coulombre 1968). The elongated cells formed in response to serum closely resemble those embryonic lens fibre cells formed in vivo in terms of their morphology, ultrastructure and protein synthesis, including a 40-50% increase in δ-crystallin synthesis over a four week culture period (Piatigorsky et al 1972, Piatigorsky et al 1973). Elongation and a selective increase in δ-crystallin synthesis occurs during the first 24 hours of culture (Milstone and Piatigorsky 1975), for the first 5 hours in the absence of any increase of δ-crystallin mRNA followed by a period of increased synthesis and mRNA accumulation (Milstone et al 1976). Cell elongation and the pattern of δ-crystallin synthesis and RNA accumulation appears virtually identical when 15% foetal calf serum is replaced in this system by 1μg/ml insulin (Piatigorsky 1973; Milstone and Piatigorsky 1977) and these effects can also be seen using an extract of vitreous humour (Beebe et al 1980). The active vitreal factor (termed lentropin) differs from that found in foetal calf serum both in terms of its differing sensitivities to inactivating agents and in the relative synthesis of different crystallins affected in 6 day chick LE. The biochemical identity of lentropin is not known but it is immunologically related to the insulin-like growth factors (IGF-I and IGF-II) both of which can stimulate 6 day chick embryo LE cell elongation (Beebe et al 1987) and increase δ-crystallin transcription and mRNA levels in separately cultured epithelia and fibres (Alemany et al 1989). Circumstantial evidence suggests that these peptides may be the retina derived factors which regulate fibre cell differentiation and crystallin expression in vivo since insulin, IGF-I and IGF-II are expressed in the retina (Das et al 1987, Danias and Stylianopoulou 1990, Kukuchi et al 1991) and their receptors are present in the lens (Bassas et al 1987, Alemany et al 1989, Peralta Solar et al 1990, Bassnett and Beebe 1990) but it is not yet clear whether lentropin is a retina-derived form of any one of these factors nor is it clear whether any one of these peptides act individually or in concert with other candidate

growth and differentiation factors in vivo. A further complication arises from the finding that vitreous humour also contains an activity which inhibits protein synthesis in the lens (Singh and Bagchi 1989) which possibly originates from the iris-ciliary complex (Bagchi et al 1988).

The aqueous and vitreous humours probably contain several growth, differentiation and maintenance factors. Platelet derived growth factor (PDGF) is produced by the retinal pigmented epithelium (RPE) apparently for export (Campochiaro et al 1989) and may have effects on lens cells (Brewitt and Clark 1990). Nerve growth factor is also expressed by retinal cells (Ebendal and Persson 1988; Heuer et al 1990). The endothelial cells of the ciliary body produce transforming growth factor-ß (TGF-ß) apparently for secretion into the aqueous humour (Helbig et al 1991). These factors and other hormones and growth factors including eye and retina derived growth factors (EDGFs and RDGFs) and FGF are reported to fail to stimulate chick lens epithelial cell elongation (Beebe et al 1987) but negative results using a single concentration of any given factor should be interpreted with caution. FGF can, under appropriate conditions, stimulate fibre differentiation and crystallin expression (Mascarrelli et al 1986, Chamberlain and McAvoy 1987) and it may therefore be premature to discount a role for other such factors.

The retina is also a rich source of retinoids including retinoic acid (Chader 1982) and eye derived growth factors EDGFs (Barritault et al 1981) including the heparin binding growth factors aFGF and bFGF (Mascarelli et al 1987). The chick retina and vitreous contains both a and bFGF (Mascarelli et al 1987) and bFGF binding to the lens capsule has been observed in both chick and mouse (Cirillo et al 1990, Fayein et al 1990). Although the low affinity binding of FGF to heparan sulphate proteoglycans of the lens capsule is unlikely to elicit any biological response, in itself, it may act as a reservoir for high affinity cellular FGF receptors. Although no such evidence for retinoic acid in the vitreous humour or retinoic acid receptors on chick lens cells has been reported retinoic acid strongly promotes growth and lentoid body formation and crystallin expression in cell culture of day-old chick lens epithelium (LEC) (Patek and Clayton 1986,1990). Although all crystallin classes are increased there is a

strongly preferential effect upon δ-crystallin and although δ-crystallin expression is lost in second-passage cultures under standard culture conditions its expression is restored , possibly by de novo transcription, on treatment with retinoic acid (Patek and Clayton 1986,1990). Comparison of retinoic acid, insulin and a bovine retinal extract (which inter alia contains FGF) shows that each agency is mitogenic, fibrogenic and stimulates crystallin expression but their relative potency, the individual crystallins stimulated and the pattern of their loss of effect during serial sub-culture are distinct (Patek and Clayton 1986,1990 and in preparation).

Retinal extracts and EDGFs affect proliferation, alter morphology and increase protein synthesis in bovine lens epithelium in vitro (Arruti and Courtois 1978, Arruti et al 1985, Mascarelli et al 1986,1989). The establishment of an in vitro system using neonatal rat lens central epithelial explants by McAvoy and co-workers has provided evidence that a concentration gradient of retina derived FGF between vitreous and aqueous humours may be sufficient to account for the orderly recruitment and differentiation of equatorial lens epithelial cells into lens fibres cells (McAvoy and Chamberlain 1989). The correlation of the rate of lens cell mitosis with the appearance and distribution of the α-, β- and γ-crystallins during embryonic and neonatal development has been reported by McAvoy (1978a,b). By the onset of secondary fibre formation α-crystallin is represented in all lens fibres and epithelial cells but the β- and γ-crystallins are expressed in fibres only and β-crystallin accumulate prior to γ-crystallin during the elongation process. The pattern of mitotic activity, of elongation and of the synthesis of specific crystallins in neonatal rat lens cells correlates well with their relative position. Cells of the central epithelium are bathed by the aqueous humour and have a low mitotic index while that of the epithelial cells bounded by iris and ciliary process is higher and reaches a peak as the anterior border of the ciliary process is reached. Once the transition to vitreous humour is made the mitotic index falls to zero the rate of elongation increases dramatically and accumulation of first β- then γ-crystallins occur. These changes occur superimposed on the expression of α-crystallin in both lens epithelial and fibre cells (McAvoy 1978a,b).

The capacity of neonatal rat lens central epithelial explants to elongate in vitro may be induced by co-culture with rat neural retina or in rat neural retina conditioned medium (McAvoy 1980b), medium conditioned by bovine neural retina (McAvoy and Fernon 1984) or in response to purified a and bFGF, although the potency of bFGF is greater (Chamberlain and McAvoy 1987, 1989 Lovicu and McAvoy 1989). During this process the morphology and ultrastructural changes closely resemble those seen in lens fibre differentiation in vivo (Walton and McAvoy 1984, Lovicu and McAvoy 1989) and cells synthesise and accumulate ß- and δ-crystallin (McAvoy 1980b, Campbell and McAvoy 1984, Chamberlain and McAvoy 1987, 1989). Most importantly, the effects of bFGF on rat lens epithelia are concentration dependent, high levels differentially stimulate fibre differentiation, intermediate levels, cell migration and low levels a proliferative response (McAvoy and Chamberlain 1989). The model is proposed to work as follows:- The most posterior epithelial cells are stimulated by high concentration of FGF in the vitreous and differentiate into fibre cells. These cells are replaced by cell migration from the mitotically active germinative zone, which is bathed by aqueous of the posterior chamber (proposed to have a lower FGF concentration). The cells of the central epithelium neither differentiate nor proliferate being bathed in aqueous humour of the anterior chamber and isolated from the FGF of the vitreous (Chamberlain and McAvoy 1987, 1989 Lovicu and McAvoy 1989).

The implication of bFGF of retinal origin as the fibrogenic stimulus in vivo is supported by the findings that bFGF is both more potent than aFGF to lens cells (Chamberlain and McAvoy 1987, 1989, Lovicu and McAvoy 1989) and the more abundant form of FGF in the retina (Baird et al 1985a). The increased potency of bFGF applies to the stimulation of protein synthesis in germinative zone and superficial cortical fibres but the effects of a and bFGF are similar on the central epithelium (Mascarrelli et al 1989). The levels of bFGF in human vitreous are consistent with the concentration required to stimulate fibre differentiation in vitro (Baird et al 1985b, McAvoy and Chamberlain 1989). However, a and bFGFs are also expressed by lens epithelial cells themselves and possibly exported in association with heparan sulphate proteoglycans suggesting that FGF sensitivity in vivo is potentially an example of autocrine as well as paracrine stimulation

(Schweigerer et al 1988).

The capacity and response of chick and rat lens epithelial cells to serum, insulin, retinoic acid and FGF is determined at least in part by age. The capacity of chick lens epithelial cells to elongate in vitro in response to serum is lost during embryonic development (Piatigorsky and Rothschild 1972) and the response of the lens epithelium from older animals to IGFs and FGFs tend towards proliferation rather than fibre differentiation (Reddan and Dziendzic 1982, Arruti et al 1985). Lens epithelial cells from day-old post-hatch chicks readily differentiate to fibre cells in primary cultures but this capacity is progressively lost during ageing in vitro during serial subculture. The protein profiles of successively formed chick lens fibre cells exhibit a shift from the expression of δ-crystallin to α- and ß-crystallins (Pal and Modak 1984; Patek and Clayton 1985, 1986). Both the progressive reduction in the extent of fibre differentiation with age and the concomitant changes in crystallin composition closely resemble the pattern seen in vivo and argue for an intrinsic age related programme of response (Patek and Clayton 1986, 1988, 1990 and reviewed in Clayton et al, in press).

A similar situation is evident in ontogenic studies using retina conditioned medium or bFGF to stimulate rat lens epithelial explants of increasing donor age. The capacity for fibre differentiation is progressively lost, the time lag before crystallin appearance progressively increases and the levels of crystallin accumulation are reduced (Richardson and McAvoy 1988, 1990). The sequence of crystallin classes lost in this system (δ- followed by ß- followed by α-crystallin) is significant since this parallels the changing composition of lens fibres laid down in vivo (Richardson and McAvoy 1990).

In principle the reduced rate of fibre differentiation may be effected by an age related decrease in overall level of any putative growth factor or by a decrease in the capacity to respond, for example by a change in the number, availability or location of specific receptors. There is indirect evidence for an age related fall in the levels of retinal EDGFs (see Karim and de Pomerai 1990) but no information currently exists regarding ontogenic changes either in low affinity (

lens capsule heparan sulphate proteoglycan) or high affinity (cell surface) FGF receptors during the embryonic period in the chick lens. In the case of insulin the level of binding to the epithelial cells remains constant during embryonic development and binding to epithelial cells is significantly higher than to fibre cells. However, both lens epithelium and lens fibres show a sharp decrease in IGF-I binding from initially high levels at 6 to 19 days of embryonic development which may be partially responsible for the declining rate of proliferation (Bassas et al 1987). Although IGF-I receptors are present at comparable levels in 6 day embryo epithelium and fibres (Bassas et al 1987) fibre cell receptors fail to internalise IGF-I and binding may be effectively non-functional (Peralta Soler et al 1990).

Although the changes in IGF-I and insulin receptor levels and function in the LE and fibres is suggestive of a role in differentiation, IGF-I receptors appear evenly distributed over the central epithelial, annular pad and the basal tips of fibre cells at both 6 and 19 days of embryonic development (Bassnett and Beebe 1990).

Changes in the levels or availability of combinations of growth factors and changes in the levels, distribution and behaviour of their receptors may account for the orderly spatial pattern of fibre differentiation seen during development and quite possibly the concomitant elevation of crystallin polypeptides seen during fibre differentiation. The proposition that changes in extrinsic signals themselves directly regulate the ontogenic changes in successively formed lens fibres seems less likely since, firstly, the numbers of regulatory factors, if a one to one correspondence were assumed, would necessarily be large. Secondly, both chick and rat lens epithelial cells in standardised <u>in vitro</u> conditions exhibit an intrinsic age related programme of crystallin expression similar to that seen <u>in vivo</u> (Patek and Clayton 1986, 1990; Richardson and McAvoy 1988, 1990; Clayton et al , in press and presented here).

One such possible intrinsic cellular property is the cell cycle itself. The δ:ß-crystallin ratio in chick lens fibres ranges from close to 1:0 in primary and early secondary fibres when the mitotic index of germinative zone cells is high, to effectively 0:1 in those fibre cells formed in the adult when the mitotic index has fallen

considerably. Genotypes or culture conditions which increase the mitotic index favour δ-crystallin expression whereas those which decrease the mitotic index favours ß-crystallin expression (for example see de Pomerai et al 1978; Clayton 1982; Patek and Clayton 1985; 1986, 1988, 1990). The increased mitotic index has been proposed as a possible mechanism by which δ-crystallin expression is regulated (Randall et al 1979; Clayton et al 1980) and this applies to retinoic acid treatment of chick lens epithelial cell differentiation in vitro and to FGF treatment of transdifferentiating chick neural retina (Patek and Clayton 1990; Karim and de Pomerai 1990).

Lens fibre cells in vivo progressively accumulate single and double stranded DNA breakage and finally the nuclei degenerate (Modak and Perdue 1970; Appleby and Modak 1977; Muel et al 1989). It is therefore likely that the stabilisation of patterns of crystallin synthesis in lens fibres which are still synthetically active is post-transcriptional and is achieved by crystallin RNA stabilisation and a reduction in protein turn-over, both of which are noted characteristics of lens fibre cells (Reeder and Bell 1965; Stewart and Papaconstantinou 1967; Clayton et al 1972).

Autocrine and paracrine growth factors appear to be both semi-ubiquitous and multifunctional (Spor and Roberts 1988). Their specific effects on cells are dependent both on their concentration (for example McAvoy and Chamberlain 1989) and the prior state of target cell differentiation (for example Patek and Clayton 1990; Richardson and McAvoy 1990; Peralta Soler et al 1990). It is therefore likely that growth factor stimulation has a strongly permissive rather than fully directive nature in developing systems, effects being combinatorial and quantitative. These considerations are clearly demonstrated by the findings that as well as a suggested role in normal lens differentiation, FGF alone can stimulate such diverse cellular phenomena as primary embryonic induction (Slack et al 1988; Grunz et al 1988), lens regeneration from amphibian iris (Cuny et al 1986; Connelly and Green 1987) transdifferentiation of chick neural retina to lens (Karim and de Pomerai 1990) and neural retina regeneration from chick retinal pigmented epithelium (Park and Hollenberg 1989).

iii. DNA methylation as a regulatory mechanism

A small proportion of the cytosine residues in DNA of vertebrates are methylated, as 5-methylcytosine (5mC), usually in the form of the dinucleotide CpG. Since DNA methylation is a reversible event and an inverse correlation between methylation and transcription has been observed for a wide range of vertebrate genes, DNA methylation has been invoked as a regulatory mechanism involved in cell differentiation (see Adams and Burdon 1985).

Both δ-crystallin genes are hypomethylated in the embryonic chick lens but methylated in non-lens tissues (Jones et al 1991). During lens induction and morphogenesis there is a sequential and progressive hypomethylation of CpG sites but although one such site in one of the δ-crystallin genes becomes hypomethylated around the time of the initiation of high level δ-crystallin transcription in the lens other sites only become hypomethylated by 96 hours well after transcriptional activation (Sullivan and Grainger 1987). Other developmental studies have shown that in tissues such as chick liver and kidney, where very low levels of δ-crystallin RNA are detectable, several sites become hypomethylated during development but that the methylation status does not correlate with the differing levels of RNA detectable when tissues are compared (Bower et al 1983b; Sullivan et al 1989). The significance of the observation that kidney δ-crystallin genes become relatively hypomethylated during development as compared to other non-lens tissues and that their pattern of methylation comes to resemble closely that seen in the lens (Bower et al 1983b) has only recently become apparent (Head et al, in press). Although the levels of δ-crystallin RNA are high in 8 day embryo chick neural retina as compared to day-old post-hatch neural retina this is not reflected in their comparative methylation status (Bower et al 1983b; Cooper et al 1983). Neither is any change evident in the pattern of δ-crystallin gene methylation during transdifferentiation of 8 day chick embryo neural retina to lens where δ-crystallin RNA levels rise through two orders of magnitude (Errington et al 1983). The chromatin conformation of the δ-crystallin locus does change during transdifferentiation from DNase-resistant (nucleosomal chromatin) to largely DNase-sensitive (smooth-fibre chromatin) and the pattern of DNase-I-hypersensitivity in 5' flanking region of the δ1-

crystallin gene comes to resemble that seen in the lens (de Pomerai 1988).

These data suggest that hypomethylation is neither a regulatory mechanism for, nor a result of δ-crystallin gene activation in chick tissues. However, a number of theoretical and technical considerations should be noted. Firstly, of the possible methylation sites in the δ-crystallin genes, only those recognised by the individual restriction enzymes used will be assayed. Secondly, the sites identified are dependent on the region of the gene covered by the hybridisation probe employed. Thirdly, the methylation status of closely clustered sites cannot be identified since the size of the restriction fragments generated are both poorly resolved and bound during Southern blotting. Fourthly, where cellular heterogeneity is involved, as is known to be the case for δ-crystallin expression in non-lens tissues (Jeanny et al 1985), the methylation pattern observed is likely to reflect the status of the tissue as a whole rather than that of any given subpopulation of cells.

It may be that only the methylation status of certain sites are significant, such as those which lie in or close to regulatory DNA elements. Two such sites have been identified which become hypomethylated early during lens differentiation (Sullivan et al 1989) and their hypomethylation is restricted to the lens (Sullivan et al 1991). These two sites are adjacent to a viral core enhancer-like element in the second intron of the δ-crystallin gene (Sullivan et al 1989) but the kinetics of hypomethylation lags behind that of δ-crystallin expression (Sullivan et al 1991). The identification of methylation sites in the 5' promoter region (Nickerson et al 1985; Ohno et al 1985) or in the strongly lens-specific enhancer element which is located in the third intron of the δ1-crystallin gene (Hayashi et al 1987) would do much to clarify the role, if any, of methylation in δ-crystallin expression.

A strong case for hypomethylation as a regulatory mechanism involved in rat lens δ-crystallin expression has recently been made by Peek et al (1991). Hypomethylation of sites in the promoter and 5' but not 3' regions correlates with the lens abundant expression of these genes and with their activation during lens fibre differentiation from lens

epithelial cells in vitro. Furthermore, the expression of γ-crystallin promoter driven recombinant constructs in transitory assays in tranfected lens cells is dependent on chemically induced modification of the methylation status of cytosine residues (Peek et al 1991).

iv. Crystallin promoter and enhancer elements

Considerable advances have been made in recent years in the analysis of the DNA sequences which are involved in the regulation of crystallin genes at the level of transcription. The overall aim is the identification of cis-acting elements (promoters and enhancers) and isolation of trans-acting factors (DNA binding proteins) which effect the complex spatio-temporal pattern of crystallin gene activation and expression during induction, morphogenesis and differentiation of lens cells.

These studies have employed the introduction of mutated sequences , usually deletions, ligated to reporter genes such as CAT (bacterial chloramphenicol acetyl transferase) into homologous and heterologous cell types and species in transient expression assays by microinjection or transfection in vitro and by the production of transgenic animals. The differing experimental rationales and procedures are described by Piatigorsky (1987), Kondoh et al (1988) and Okada (1988).

Crystallin promoters exhibit a high degree of cross-species lens specificity. For example the 5' α-crystallin sequences of both chick and mouse function equally well in transfected lens epithelia (LE) of either species and selectively so as compared to other cell types (Okazaki et al 1985; Chepelinsky et al 1985) suggesting that promoter sequences and trans-acting factors are well conserved between these species. More surprisingly, since mammalian lenses lack δ-crystallin, the δ-crystallin gene exhibits a high degree of lens specificity when microinjected into mouse lens epithelial cells as compared to other cell types (Kondoh et al 1983) and when used to generate transgenic mice (Kondoh et al 1987; Takahashi et al 1988). Similarly, chick lens

epithelial cells selectively recognise promoters from several mouse and human γ-crystallin genes (Lok et al 1985; Meakin et al 1987) although the chick lens in vivo expresses a distantly related γ-crystallin, γs (Treton et al 1984; van Rens et al 1991). Furthermore rat γ-crystallin promoters are recognised by lens cells derived by transdifferentiation from the embryonic chick neural retina (Peek et al 1990).

There are, however, phylogenetic limits to promoter recognition and specificity in that a δ-crystallin transgenic fish expresses δ-crystallin in most cells of virtually all tissue types (Ozato 1986 et al; Inoue et al 1989), and, although the promoters of other human genes can direct transcription in transfected yeast cells, the αA-crystallin promoter does not (Toyama and Okayama 1990).

Thompson et al (1987) report the presence of a 15base-pair consensus sequence in the 5' region of α-, ß-, γ-, and δ-crystallin genes of rodents, chick and humans. The degree of matching, position relative to TATA box and orientation were highly variable and the proposition that this sequence itself has any regulatory function awaits experimental verification. Two of the four such consensus sequences found 5' to the chick αA-crystallin gene lie in or flank the region (-242 to -189) found to be required for high-level expression of a chick αA-crystallin promoter/ δ-crystallin coding sequence construct and microinjected into mouse lens cells or fibroblasts. This region exhibits many of the features of an enhancer since its proximity and position relative to the transcription initiation site and its orientation do not appreciably affect its activity. Replacement of this element with enhancers of viral origin maintain activity but abolishes the lens specificity (Okazaki et al 1985).

Considerably more is known about the 5' sequence elements which regulate the expression of the murine αA-crystallin gene. The sequences necessary to confer lens specificity on a murine αA-crystallin promoter/CAT construct are known to lie between -366 and +46 both for transient expression in the chick lens epithelium and in lenses of transgenic mice (Chepelinsky et al 1985; Overbeek et al 1985). This region contains two interactive but distinct functional elements, one distal at -111 to -88 and one proximal at -88 to -60 in

transient expression assays in chick lens epithelium (Chepelinsky et al 1987). The -88 to +46 region is sufficient for lens specificity and the developmental timing of activation in transgenic mice (Wawrousek et al 1990). Gel retardation assays indicate that these two regions bind different nuclear proteins but these proteins are unlikely, in themselves, to confer lens specificity since they, or similar proteins, are present in Hela cells and chick erythrocytes as well as in lens cells (Sommer et al 1988). The -66 to -57 αA-crystallin binding protein, designated CRYBPI has been cloned and found to be closely related to known transacting factors for non-crystallin genes (Nakamura et al 1990). This, in conjunction with its expression in many cell types other than lens, makes its involvement in tissue specific expression unlikely (Nakamura et al 1990).

Similarly, the octamer binding-like and polyomavirus enhancer-like promoter elements found 5' to the chicken βB1-crystallin gene are unlikely to account for the abundant expression of this gene in lens fibre cells since the transcription factors that they bind, or closely related nuclear binding proteins, can also be found in many tissues (Roth et al 1991).

Initial studies of the promoter region of the δ-crystallin genes took advantage of the absence of δ-crystallin in rodent lenses and assayed directly for δ-crystallin expression by immunological means in mouse cells into which the δ-crystallin promoter and coding sequence had been transferred (Kondoh et al 1983; Kondoh et al 1984; Hayashi et al 1985). The region lying between -93 and +58 was implicated in conferring the elevated levels of expression seen in lens cells as compared to fibroblasts whereas expression in the latter cell type requires an additional 12 base pairs 3' to this (Hayashi et al 1985). This larger region contains several regulatory elements including two putative consensus binding sites for proteins (Hayashi and Kondoh 1986; Das and Piatigorsky 1986; Kondoh et al 1986). The specificity of this promoter region for lens expression and its involvement in developmental regulation were apparently confirmed by the expression and developmental timing of activation of δ-crystallin in the lenses of transgenic mice (Kondoh et al 1987; Takahashi et al 1988). However when the δ-crystallin coding sequence was replaced by the CAT reporter gene for transfection studies in chick lens epithelium the lens

specificity was lost, associated with loss of a 1kb long lens specific enhancer spanning the third intron of the structural gene (Hayashi et al 1987). The function of the enhancer element was unaffected by position and orientation and activates transcription from the δ-crystallin promoter 20-40 fold in lens cells (Hayashi et al 1987). Although it contains a 120bp core with high lens specificity this element functions co-operatively with other adjoining elements which display a broader cell type specificity (Goto et al 1990). Evidence for a 5' negative regulatory element (between -803 and -120) as well as the positive promoter region (between -120 and -43) has been reported by Borras et al (1988) who suggest a role in developmental down regulation of the δ-crystallin gene seen in the post-hatch chick lens (Treton et al 1982). In the absence of the δ1-crystallin enhancer in transfection experiments the -120 to -43 δ1-crystallin promoter region mediates the transcriptional stimulatory activity of insulin and insulin-like growth factor-I (Alemany et al 1990). How directly IGF-I is involved in δ-crystallin transcriptional regulation remains to be shown but, once internalised in lens cells, a proportion is translocated to the nucleus (Peralta Soler et al 1990).

The δ1-crystallin enhancer is unlikely to account for the higher steady-state levels of δ1- over δ2-crystallin RNA found in the lens (Parker et al 1988; Head et al 1991) since the δ1-crystallin enhancer region is highly conserved in the δ2-crystallin gene (see Hayashi et al 1987). Difference in the relative strengths of their promoters have been reported by Borras et al (1985) and Das and Piatigorsky (1986) but more recent studies using the promoter and enhancer regions of the δ1- and δ2-crystallin genes show them to be functionally equivalent when pSVOCAT recombinants are assayed in chick lens epithelia (Thomas et al 1990). It may be that, as yet unidentified elements regulation the δ1/δ2-crystallin ratio (Thomas et al 1990) or that post-translational events play an important role (see Head et al 1991)

It is important to note that although the levels of expression detected in δ-crystallin microinjection experiments are high in lens cells, other cell types also express the exogenous gene but with a lower efficiency (Kondoh et al 1983; Kondoh et al 1986) and that transgenic mice, irrespective of the techniques used in their production, express δ-crystallin in the CNS (pyramidal neurones of the

piriform cortex) as well as the lens (Kondoh et al 1987; Takahashi et al 1988).

αB-crystallin is also expressed in extralenticular tissues whereas αA-crystallin expression is reportedly lens-specific in mice but the regions which determine its lenticular and extralenticular pattern of expression are, as yet, poorly defined (Dubin et al 1989) but in humans must be outside the immediate vicinity of the transcription initiation site, possibly lying in the 3' flanking region (Dubin et al 1990). The -661 to +44 region of the αB-crystallin gene is necessary for expression in both the lens and skeletal muscle in transgenic mice. Transfection experiments identify two protein binding elements one necessary for expression in myotubes (-426 to -257), the other (3' to -115) required for expression in lens cells (Dubin et al 1991) Unlike the αA-crystallin gene, a near perfect heat-shock consensus sequence is located 5' to the hamster αB-crystallin gene (Quax-Jeuken et al 1985; Thompson et al 1987). This sequence is involved in the increased synthesis of αB-crystallin in fibroblasts heat-shocked or exposed to Cd^{++} (Klemenz et al 1991a,b). Neither the αA- nor αB-crystallin genes are responsive to heat-shock in the lens (de Jong et al 1986) perhaps because both genes are already expressed in lens cells at their maximum possible levels.

4. Aims and Objectives

The regulation of crystallin gene expression can be examined in terms of molecular biology, and may therefore, be considered in terms of the factors and sequences involved in differential gene expression. Alternatively crystallin expression, and therefore, necessarily, its regulation, may be viewed in the larger context of the evolution and embryonic development of the eye lens.

The existence of extralenticular crystallins suggests that the crystallins may have retained functions which predate the evolution of the vertebrate lens and that the evolution of the lens involved the utilisation of pre-existing genetic resources by a mechanism of differential tissue-specific expression or gene duplication and divergence. However, such an hypothesis is dependent on the genetic identity of lens and non-lens crystallins and on the identification of putative functions for individual crystallins in non-lens structures.

The work described here has sought to address the genetic basis and possible functions of the α-, ß- and δ-crystallins which were previously detected in extralenticular tissues, including embryonic retina in the chick, and in the case of the ß-crystallins extend these findings to the mammalian retina. These studies also address the relationship between the expression of crystallins and the lens fibre cell phenotype. The findings underscore the independent regulation of the crystallin genes but they also contribute to a better understanding of the possible functions of extralenticular crystallins and provide evidence as to the path leading to the evolution of the lens as an organ defined, at least in part, by the super-abundant expression of a limited number of gene products.

Specifically, the following issues are addressed:-

1) The relative contribution and localisation of the two δ-crystallin genes to chick lens tissues and during lens fibre cell induction and subsequent differentiation.

2) The genetic basis, tissue distribution, developmental regulation and localisation of extralenticular ß-crystallin expression.

3) The nature of the requirement for individual crystallins by an examination of the consequences of interfering with their expression

in lens and retina by targeting crystallin transcripts using antisense oligonucleotides in vitro.

The following research papers are ordered according to the specific topics addressed and are not, therefore, in strict chronological order. Papers 1, 2 and 3 directly address the expression of the δ-crystallin genes in lens and non-lens tissues but addition information concerning extralenticular δ-crystallin expression may be found in papers 5 and 7. Papers 4, 5 and 6 are largely concerned with extralenticular ß-crystallin while paper 7 addresses the issue of αA-crystallin in the retina.

EXPERIMENTAL CELL RESEARCH 193, 370-374 (1991)

Independent Regulation of Two Coexpressed δ-Crystallin Genes in Chick Lens and Nonlens Tissues

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It is known that δ-crystallin is superabundant in the early chick lens, but it is found at lower levels in certain other tissues. Ninety-nine percent of the lens δ-crystallin poly(A)⁺ RNA is from the δ1-crystallin gene. We report here that the δ1- and δ2-crystallin genes are both transcribed in the chick lens and retina throughout embryonic development and that both RNAs are found in embryo adenohypophysis and epiphysis and in day-old posthatch chick tibiofemoral chondrocytes and striated muscle. δ1-crystallin RNA is more abundant in lens tissues, while δ2-crystallin RNA is more abundant in all nonlens tissues. However, δ1-crystallin RNA is processed more efficiently than δ2-crystallin RNA in all early embryonic tissues examined. A comparison of lens epithelium and fibers established that levels of δ2-crystallin RNA are the same but those of δ1-crystallin RNA are over 100-fold higher in fibers compared to epithelial cells. The evidence implies independent regulation both of transcription and of post-transcriptional events for these two genes. © 1991 Academic Press, Inc.

INTRODUCTION

δ-Crystallin is the major water-soluble protein in the embryonic chick lens [1] and the first to be synthesized following induction [2, 3]: its synthesis declines gradually during development and ceases in the young adult [4]. During the developmental period, the differentiation of fiber cells is accompanied by an increase in the level of δ-crystallin [5].

There are two δ-crystallin genes in the chick [6, 7], the δ1-crystallin gene and δ2-crystallin gene. They exhibit a high degree of sequence homology [8] and have homologies to arginosuccinate lyase (ASL) [9]. They may be distinguished from each other by probes directed to the most divergent regions of the two genes [10]. Both δ1-crystallin RNA and δ2-crystallin RNA are transcribed in the lens: δ2-crystallin, which has ASL activity, is a minor component in chick lens comprising some 1% of the lens δ-crystallin poly(A)⁺ RNA, while the major δ-crystallin component, δ1-crystallin, appears to have little or no ASL activity [10, 11].

δ-crystallin RNA is also transcribed at low levels in several nonlens tissues [12–15]. Low levels of the RNA are found in kidney, liver, and heart which appear to be δ2-crystallin transcripts [16]: examination of embryo heart shows that this RNA is transcribed in a small subpopulation of the cells [15]. Tissues such as neural retina, which have the potential for transdifferentiation to lens [17–21], express higher levels of δ-crystallin RNA than tissues which do not transdifferentiate [12–14]. As in all other nonlens tissues examined, this RNA is expressed in a subpopulation of cells [15]. The levels of δ-crystallin RNA rise steeply during transdifferentiation of retina to lens [22–24].

Other crystallins are also expressed at low levels in retina. α-Crystallin (possibly both α-crystallins) and two of the β-crystallins, (one acidic and one basic) are detected in chick retina ([25, 26], Head, Peter, and Clayton, unpublished) as are their RNAs [27–30]. Like δ-crystallin RNA, they appear to have a restricted distribution in the retina cell population ([29, 30]; Head, Peter and Clayton, unpublished; Head, Triplett, and Clayton, unpublished).

Examination of the expression of different crystallin RNAs and their proteins in cells of the neural retina during development and transdifferentiation may contribute to an understanding of the putative roles for these crystallins in extralenticular sites; permit an assessment of possible relationships between the expression of a particular crystallin and transdifferentiation potential; and, finally, determine the characteristics of crystallin regulation in different cell types.

We report here on the differential expression of δ1- and δ2-crystallin RNA, both of which we find expressed in lens, retina, and several other embryonic or day-old chick tissues, although δ1 is the majority component in lens tissues and the minority component in the non-lens tissues. Our data imply that δ1-crystallin may have a noncrystallin, nonenzyme function in some cells and indicate that the relative level of these two RNAs may be regulated by at least two independent mechanisms.

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0014-4827/91 $3.00

In this paper we use the following abbreviations: δ1- or δ2-RNA for δ1- or δ2-crystallin RNA, δ1- or δ2-gene for δ1- or δ2-crystallin gene, and δ1- or δ2-probe for δ1- or δ2-crystallin antisense oligonucleotide probe.

MATERIALS AND METHODS

cDNA and oligonucleotide probes. The *Pst*1 insert of the δ-crystallin cDNA clone pM56 [31] was labeled with [α-^{32}P]dCTP (>400 Ci/mmol, Amersham) by random primed synthesis [32]. The two oligonucleotide probes [10], specific for sequences in the second exon of the δ1- and δ2-genes, respectively, were synthesized by the Oswel DNA Service (Department of Chemistry, University of Edinburgh). Quantities (1 μg) of oligonucleotide DNA were labeled with 50 μCi [α-^{32}P]-dCTP (>400 Ci/mmol, Amersham) using 25 U of calf thymus terminal transferase (BCL) [33]. Oligonucleotide probes were purified by chromatography and collected by ethanol precipitation [33]. Cerenkov counting of aliquots from probe preparations gave specific activities of 5.8×10^7 and 5.2×10^7 dpm/μg for δ1- and δ2-probes, respectively. The specific activity of the probes used in Fig. 4 were 3.0×10^7 for the δ1-probe and 3.5×10^7 for the δ2-probe.

RNA preparation and blotting. Tissues from several embryonic stages of incubation and day-old posthatched birds were dissected rapidly and frozen in liquid nitrogen. Removal of one or two tissues only from each embryo ensured that each sample was frozen within 2 min of opening the egg.

Total cellular RNA was prepared by homogenization of tissues as they thawed in the denaturing extraction buffer [34]. Northern transfers were prepared by electrophoresis of glyoxal/DMSO-denatured RNA through 1.2% agarose gels [35]. Gels were preequilibrated and RNA was transferred overnight to Zeta Probe nylon membranes (BRL) by capiliary blotting in 10 m*M* NaOH. Dot blots were prepared by glyoxal denaturation [35], serial dilution, and vacuum filtration using a Hybri-Dot Manifold (BRL) onto Zeta Probe nylon membranes. RNA was bound to membranes by baking at 80°C for 2 h at reduced pressure. Filter-bound RNA was deglyoxalated prior to hybridization by immersion in boiling 20 m*M* Tris–HCl, pH 8.0.

Northern transfers screened with the δ1- and δ2-probes were prepared and processed together from the same RNA preparations to avoid artifacts due to differential processing or degradation of transcripts [23, 24].

Hybridization and wash conditions. Blots were incubated for 2 h at 65°C in 0.1× SSC, 0.5% SDS followed by prehybridization and hybridization and with oligonucleotide probes present at 10 ng/ml [36]. Washing included two 30-min washes in 3× SSC, 0.1% SDS at 37°C. The cDNA clone was hybridized and washed under highly stringent conditions [37].

RESULTS

Northern transfers of embryonic lens were hybridized to the cDNA probe to show the position of the fully processed 1.6-kb δ-crystallin RNA, and duplicate transfers were then screened with the δ1- or the δ2-probes, respectively. Both oligonucleotide probes detected the 1.6-kb transcript, together with a second 5.2-kb component, corresponding to the partially processed transcript previously detected [23, 24]. However, while the δ1-probe hybridized mainly to the 1.6-kb species, with only a trace of the 5.2-kb RNA, the δ2-probe detected approximately equal amounts of both RNA species (Fig. 1). This considerable difference in relative abundance indicates that the δ1- and δ2-probes distinguish the two δ-crystallin RNAs, and this specificity is confirmed by differential hybridization to total cellular RNA from lens and nonlens tissues (Figs. 2 and 3). Differences are also clearly visible in the size classes of the RNA detected by the two probes in nonlens tissues (Fig. 1). The δ1-probe detects roughly equal amounts of the 1.6-kb and the 5.2-kb RNA in neural retina, retinal pigmented epithelium, adenohypophysis, and epiphysis but the δ2-probe detects only the 5.2-kb form in these same tissues.

Figure 2 shows δ1-RNA at very high steady-state lev-

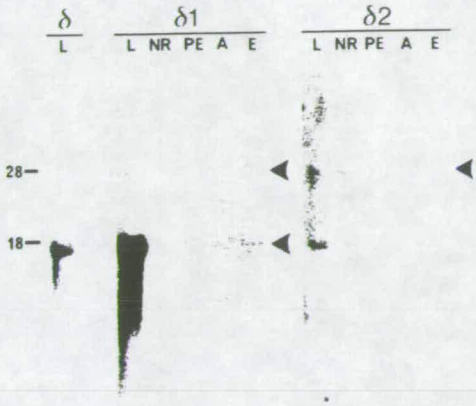

FIG. 1. Hybridization of δ1- and δ2-crystallin RNA-specific oligonucleotide probes and a δ-crystallin cDNA probe to duplicate Northern transfers of 20 μg total cellular RNA extracted from 8-day embryo lens (L), neural retina (NR), retinal pigmented epithelium (PE), adenohypophysis (A), and epiphysis (E). Arrows indicate the major size classes of transcripts. The transfers probed with the oligonucleotides were exposed for 18 days and the transfer probed with the δDNA clone was exposed for 24 h. The positions of the 28 s and 18 s ribosomal subunits are shown for reference.

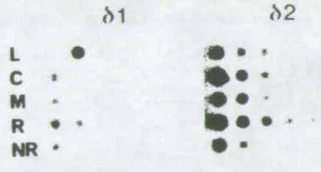

FIG. 2. Dot blot hybridization of δ1- and δ2-crystallin RNA-specific oligonucleotide probes to duplicate blots of total cellular RNA extracted from day-old posthatched lens (L), tibiofemoral chondrocytes (C), striated muscle (M), retina (R), and neural retina (NR). Initial dots, extreme left, represent 20 μg RNA and 10-fold serial dilutions except for the initial dot of lens RNA hybridized to the δ1-probe which contained 2 μg only, but was hybridized, washed, and exposed on the same filter as the other dots shown. Duplicate blots probed with each oligonucleotide were processed simultaneously and exposed for 18 days.

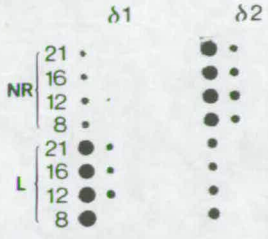

FIG. 3. Dot-blot hybridization of δ1- and δ2-crystallin RNA-specific oligonucleotide probes to total cellular RNA extracted from fresh lens (L) and neural retina (NR) after 8, 12, 16, and 21 days of embryonic development. Initial dots, left, represent 20 μg NR RNA; 2 μg lens RNA and 10-fold serial dilutions exposed for 18 days.

els in lens and at trace levels in day-old chick retina, chondrocyte, and muscle. δ2-RNA is detectable as the minor δ-crystallin RNA component of lens but is the more abundant δ-crystallin RNA component in all non-lens tissues tested.

The rapid and complete removal and freezing of day-old posthatched chick retina precluded separation of tissues and it therefore comprised both neural and pigmented layers. It expresses higher steady-state levels of both δ1-RNA and δ2-RNA than chondrocyte, muscle, or neural retina alone (Fig. 2).

The relative steady-state abundance of both the δ1- and δ2-RNA remains fairly constant in both lens and retina from the 8-day embryo to posthatch stage (Fig. 3), but between the 8- and 12-day stages of lens development there is a relative decrease in δ2-RNA and an increase in δ1-RNA (Fig. 3).

A comparison of the level of δ1- and δ2-RNA in day-old posthatched lens shows that the contribution of δ2-RNA is similar in lens epithelium and in the lens fiber mass but that δ1-RNA is over 100-fold more abundant in fiber cells compared to the epithelium (Fig. 4).

DISCUSSION

Trace levels of δ-crystallin have been detected in several non-lens tissues, including retina [25, 26]; low levels of δ-crystallin RNA were found in heart, lung, liver, kidney, brain [13, 23]; and at higher levels were found in retina [12, 14]. However, these levels of δ-crystallin RNA and protein in non-lens tissues represent appreciable levels on a per cell basis, since *in situ* hybridization [15, 23, 24] and immunofluorescence [38, 39] show a subpopulation of positive cells in an otherwise negative tissue. This nonrandom distribution in the retina implies possible function ([12, 15, 39]; Head, Triplett, and Clayton, unpublished). These reports do not distinguish between the two δ-crystallins, which have very similar RNA and protein sequences [8]. Using discriminant probes, δ2- but not δ1-RNA was found in heart, liver, and kidney [16], and the results reported here, using probes with the same sequences as those used in that laboratory [10], show that the steady-state level of δ2-RNA is higher than that of δ1-RNA in three other extralenticular tissues, retina, chondrocyte, and muscle, but we report here that δ1-RNA is also expressed at very low levels in all these tissues. Posthatch muscle and chondrocyte were not examined further. The levels of the two RNAs, the genes for which are located in tandem separated by 4 kb, appear to be independently regulable, since the RNAs show a reciprocal relationship in lens and nonlens tissues, and the levels of δ1-crystallin RNA increase independently of δ2-RNA during fiber formation, both in lens and during lentoid formation in transdifferentiating neural retina (data not shown).

We analyzed the expression of the two δ-crystallin genes in lens and in neural retina, retinal pigmented epithelium, adenohypophysis, and epiphysis at an earlier stage of development by Northern transfer. These nonlens tissues are all neuroectodermal in origin, are topologically homologous, have previously been shown to contain δ-crystallin RNA, and can transdifferentiate to the lens phenotype [17]. The reciprocal pattern of δ1- and δ2-RNA levels in lens and non-lens tissues is again apparent. We also found differences in the relative proportions of the fully processed and the partially processed RNAs. The vast excess of δ1-RNA found in the lens Northern transfer is in the fully processed form, unlike that found in other tissues where the partially and fully processed forms are represented in roughly equal amounts. However, in the case of δ2-RNA, while somewhat more than half appears to be fully processed in the lens, virtually all the δ2-RNA seen in the Northern transfers of non-lens tissues is in the partially processed form.

While the levels detected may be affected by a number of post-transcriptional events, one possible explanation is that δ2-RNA is processed less efficiently than δ1-RNA in the tissues in which both RNAs are expressed. For example, we find that the levels of δ2-RNA in total cellular RNA are approximately 10% of those of δ1-RNA, yet a previous report [10] found that the level of δ2-RNA was only 1% of the δ1-RNA in the lens

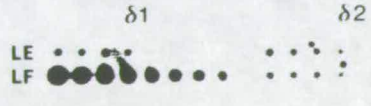

FIG. 4. Dot blot hybridization of δ1- and δ2-crystallin-specific oligonucleotide probes to duplicate blots of total cellular RNA extracted from day-old posthatched lens epithelium (LE) and lens fibers (LF). Initial dots on the left represent 20 μg RNA, doubling dilutions from left to right. Blots probed with each oligonucleotide probe were processed simultaneously and exposed for 14 days.

poly(A)$^+$ RNA fraction. We do not yet know whether this actually implies less efficient processing or a more rapid turnover rate, but in either case, the amount of δ2-RNA available for translation is relatively lower than that of the δ1-RNA available for translation.

Independent regulation of δ-crystallin RNA transcription and processing is shown during lens development and during neural retina transdifferentiation: transcription outruns processing capacity in the very young chick embryo lens [40] and in the earlier stages of transdifferentiation of neural retina to lens when most of the δ-crystallin RNA shows a predominantly nuclear location [12]. This independence is also demonstrated by the possession of latent processing capacity of 8-day embryo neural retina [23, 24].

The enhancer of the δ1-gene, and presumably of the δ2-gene, is in the third intron [41]. The δ1-gene enhancer contains both lens-specific and non-tissue-specific elements [42]. These lens-specific elements are presumably missing in the δ2-gene but it is not yet known whether the δ2-gene may have enhancer elements which permit some relative nonlens tissue specificity.

The relative amounts of the fully processed δ1- and δ2-RNAs raise the question of the relative levels of the respective proteins which may be translated. Thirty percent of adenohypophysis cells express δ-crystallin RNA [38] but its electrophoretic mobility differs from that of the lens protein [43] and its function in this tissue is unknown. The homology of δ-crystallin to arginosuccinate lyase, ASL [9], implies a possible function for extralenticular δ-crystallin. ASL activity is high in duck lens δ-crystallin which expresses both δ1- and δ2-proteins, but ASL activity and δ2 levels are both low in chick lens [11], suggesting that δ1-crystallin has lost most or all ASL activity. Other characteristics of δ-crystallin may be relevant to possible extralenticular function: it binds to the lens plasma membrane *in vivo* and *in vitro* [44] and binds calcium *in vitro* by virtue of calmodulin-like motifs [45]. The relationship between the concentration of a crystallin, the properties expressed, and its role in the differentiation of that cell merits investigation as does the relationship of extralenticular crystallin expression to transdifferentiation potential.

We thank A. F. R. C. Roslin for the provision of fertile J-line eggs, Frank Johnstone for his photographic skills, and Mary Ramsay for preparing the manuscript.

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Received July 31, 1990
Revised version received October 23, 1990

LOCALIZATION OF δ-CRYSTALLIN RNA DURING LENS MORPHOGENESIS AND DIFFERENTIATION IN THE CHICK EMBRYO I: δ-CRYSTALLIN EXPRESSION DURING NORMAL EMBRYOGENESIS

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Short title: Localization of δ-crystallin RNA

Key words: δ-crystallin; RNA localization; lens induction; lens fiber differentiation; *in situ* hybridisation

With 5 figures and 49 references.

Abstract

δ-crystallin is both the first crystallin to appear during lens morphogenesis and the most abundant lens protein throughout the embryonic period in the chick. Ontogenic changes of δ-crystallin RNA levels, protein synthesis and accumulation have been the subject of considerable study, but the cellular localization of δ-crystallin RNA during lens morphogenesis has not previously been reported. *In situ* hybridization to tissue squashes shows that δ-crystallin RNA is present at low levels in the nuclei of lens placode cells and that subsequent development is characterised both by an increase in the levels detected and the proportion of the RNA found in the cytoplasm. *In situ* hybridization to sectioned early embryos and later eyes show that the formation of both primary and secondary fibers is associated with an increase in the steady state levels of δ-crystallin RNA but that this occurs prior to cell elongation. The ontogeny and distribution of αA- and δ-crystallin trancripts are distinct from each other but both closely resemble their known pattern of protein accumulation.

Introduction

Inductive relationships between embryonic tissues provide important cues to the subsequent morphogenesis and differentiation of the tissues involved (reviewed by Gurdon 1987). The transparent vertebrate lens is derived from competent head ectoderm after an inductive interaction with the evaginating neuroectoderm (optic vesicle). 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 constitute the annular pad and they have columnar morphology in contrast to the contiguous cuboidal anterior lens epithelium. 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 (for example see Barabanov and Fedtsova 1982) and its competence is thought to result from earlier inductive interactions during gastrulation (reviewed by Saha et al 1989).

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 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 hybridisation using embryo head RNA shows that δ-crystallin RNA accumulates steadily prior to the point at which protein synthesis rises, and the authors extrapolate these results backwards in time to identify 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 every 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). These data may account for the report of Perlmann and de Vincentiis (1961) of crystallin antigens distributed throughout the chick embryo before lens formation. 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 dissected lens fiber mass than in the central or equatorial epithelium (Pal and Modak 1984; Hejtmancik et al 1985).

Transcripts of both δ-crystallin genes are found in the lens but the level of δ1-crystallin RNA is both higher than that of δ2-crystallin RNA in the lens as a whole (Parker et al 1988) and higher in the lens fiber mass than in the epithelium (Thomas et al 1990; Head et al 1991). The identification of significant levels of putative δ-crystallin precursor mRNA in Northern transfers of whole 6 day embryo lens RNA suggests that processing can be a rate limiting step in the early embryonic lens (Bower et al 1983) and dissproportionately affects the the δ2-crystallin transcripts (Head et al 1991).

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 mammals lack both δ-crystallin as a major lens protein and an annular pad region as a distinct morphological structure.

We have used *in situ* hybridization with a cDNA probe to sectioned embryos and lens squashes to study the ontogeny and inter- and intracellular localization of δ-crystallin RNA during lens morphogenesis in the chick in order to examine the relationship between lens induction, fiber formation and the transcription and processing of δ-crystallin RNA. We have also used this same technique at a later stage of development in order to compare the distribution of δ- with that of αA-crystallin in the cortical and nuclear fibers and at the annular pad/cortical fiber boundary in order to examine the relationship between secondary fiber cell differentiation and crystallin RNA expression.

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 st.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 st.12 cells of the invaginating lens placode are moderately heavily labelled (Fig. 1C,D) and by st.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 st.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 labelled intensely (Fig. 2C,D). Hybridisation with the αA- 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 (st. 21) (Fig. 3C). Hybridisation 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 labelled 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.

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 δ-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 and not lens placode formation 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 lens placode cells. No such nuclear δ-crystallin RNA was detected 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). We do not yet 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 st. 10 head ectoderm represents <10 transcripts/cell 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, Sullivan et al (1991) report only fully processed δ-crystallin RNA whereas the transcripts found in lens placode squashes here are nuclear in location and therefore probably unprocessed or only partially processed.

These data suggest that although Shinohara and Piatigorsky (1976) are correct in identifying the lens placode stage in the transcription of δ-crystallin, this RNA is predominantly nuclear in location and would not be expected to contribute significantly to the embryo head cytoplasmic poly(A)$^+$ RNA which these authors used. However the low levels of mature δ-crystallin RNA detected in head ectoderm, head mesoderm and neural tube by Sullivan et al (1991) may have obscured the finding we report here that the accumulation of abundant cytoplasmic δ-crystallin 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.

In situ hybridization to sectioned eyes 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 hybridizable δ-crystallin RNA which appears evenly distributed throughout the cytoplasm. 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 cells. The localization of cells with even higher levels of δ-crystallin RNA in the annular pad/cortical fiber region suggests that the initiation of high level δ-crystallin expression is

associated with fiber differentiation. 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. The spatial distribution of αA-crystallin RNA, as shown here by *in situ* hybridization closely parallels, and presumably accounts for, the pattern of αA-crystallin accumulation as identified by immunofluorescence at this same stage of development (Ueda 1989). The increase in the steady state level of δ-crystallin RNA as annular pad cells approach the annular pad/cortical fiber boundary is not gradual but is relatively abrupt and occurs just prior to cell elongation. Although a gradient of δ-crystallin RNA levels can be seen decreasing from the lens cortex to the lens nucleus considerable caution should be employed in the interpretation of this observation, since there is a corresponding increase in cell length and cell volume (Beebe et al 1982).

δ-crystallin expression appears therefore to be regulated during lens development by two successive mechanisms. Firstly, in presumptive primary (nuclear) fibers during lens induction, by intimate contact with the optic cup, and secondly during secondary (cortical) fiber formation, when any retinal influence could only be indirect, via the vitreous humor. 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 synthesised in the retina, present in the vitreous humor and have binding sites in the lens (Beebe et al 1980; Mascarelli et al 1986; Bassas et al 1987; Beebe et al 1987; Mascarelli et al 1987; Alemany et al 1989; Bassnett and Beebe 1990; Patek and Clayton 1990). bFGF binds to the heparan sulfate proteoglycan components of both the optic vesicle/lens placode interfacial matrix and the lens capsule (Fayein et al 1990) and elicits differing age-dependent fiberogenic responses from lens epithelial cells (Richardson and McAvoy 1990). bFGF is therefore a good candidate inducer of lens fiber differentiation, in the case of primary fibers, during intimate contact between neuroectoderm and head ectoderm, and in the case of secondary fibers, in a soluble form in the viteous humor.

Whether the changing pattern of crystallin composition of successively formed lens fiber cells results from changes in the extrinsic regulatory signals to which their progenitor lens epithelial cells are exposed or due to an intrinsic age-related program remains

unclear. However both the capacity of lens epithelial cells to respond *in vitro* to external fibrogenic stimuli and the specific nature of the response, as judged by resultant fiber cell crystallin composition is age-related and resembles that seen in the *in vivo* situation both in the chick (Patek and Clayton 1990) and in the rat (Richardson and McAvoy 1990).

Irrespective of the exact nature of the stimulus, or stimuli, the resultant terminal differentiation of lens fibers from epithelial cells involves coordinated changes in cell morphology and biochemistry which may, in part, be mediated by changes in growth factor receptors (Bassas et al 1987; Bassnett and Beebe 1990; Perlata Soler et al 1990), proto-oncogene expression (Nath et al 1987; Zelenka et al 1988; Zelenka et al 1989) and ribosomal protein phosphorylation (Vu and Zelenka 1987) as well as changes in the levels of crystallin and non-crystallin structural components and metabolic enzymes. The use of cultured lens epithelial cells, whether dissociated or grown as explants, has inherent limitations in the study of fibrogenic stimuli and cellular responses, since the epithelial cells which respond *in vivo* are strictly defined by location and presumably position relative to other lens cells, the lens capsule and the ocular compartment occupied. The use of an *in vitro* whole lens culture system using a defined medium and purified growth factors (such as that described by Brewitt and Clark 1990), and subsequent localization of specific growth factor receptors (Basnett and Beebe 1990; Cirillo et al 1990) and analysis of reponsive genes by *in situ* hybridization, as described here, would provide valuable information concerning the causal links and the mechanism involved in the ontogenic changes in crystallin expression seen during lens development *in vivo*.

Materials and Methods

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) the were labelled with ^{3}H-dCTP (>1.85TBq/mmol, Amersham, UK) by random primed synthesis (Feinberg and Vogelstain 1983). Fertile eggs of the N-J genotype (described in Patek and Clayton 1988) were incubated for defined periods and the stage (st.) 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 hybridisation buffer consisting of 50% formamide, 0.6M NaCl, 10mM Tris-HCl. pH7.0, 1mM EDTA, 1x Denhardt's solution, 1% dextran sulfate, 250 µg/ml heat sheared herring sperm DNA, 500µg/ml yeast tRNA and ^{3}H-labelled probe present at 1µg/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 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.

Acknowledgements 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.

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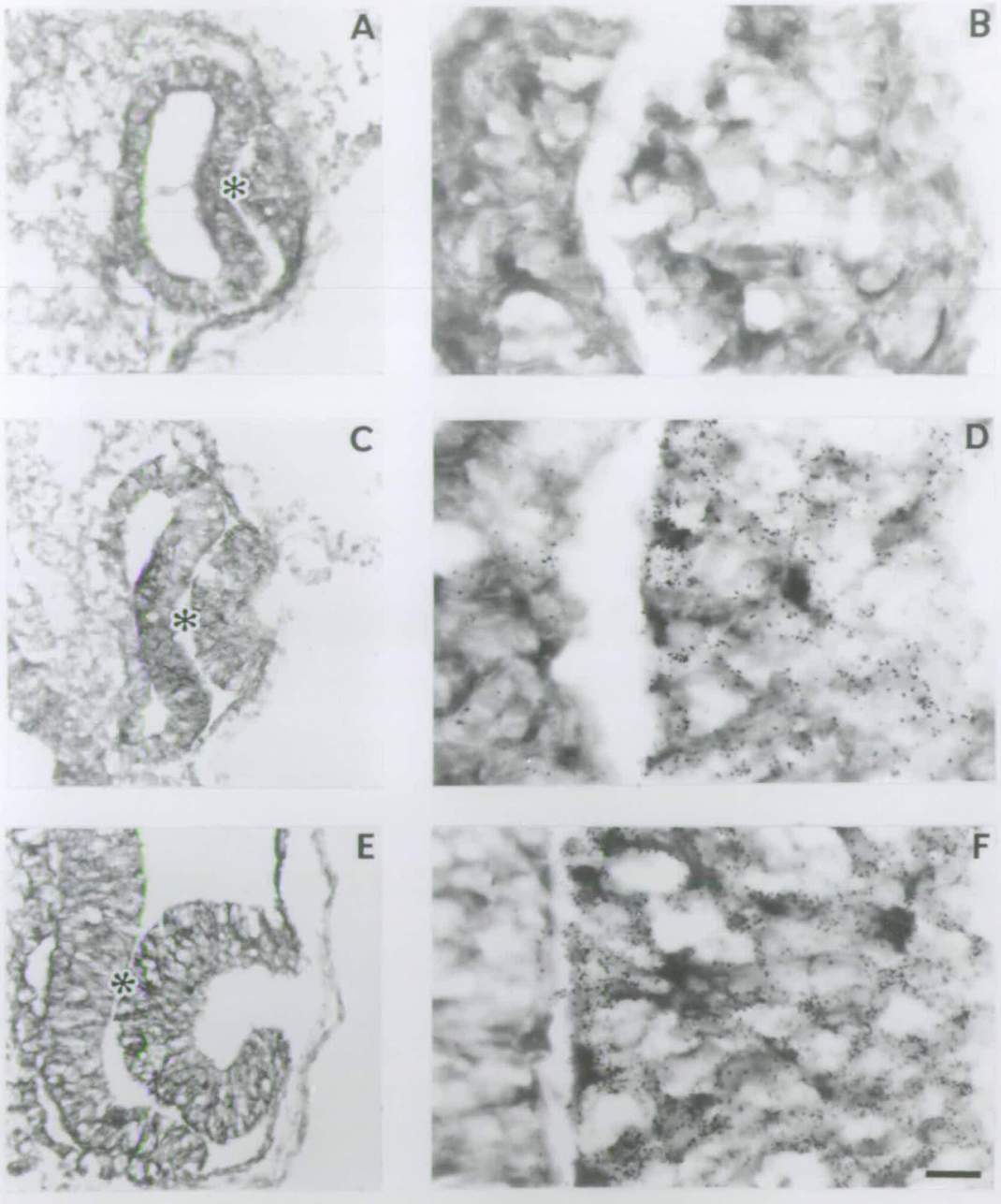
Fig.1 Localization of δ-crystallin RNA by *in situ* hybridization in transverse sections of chick embryos at st.11.5 (A,B), st.12 (C,D) and st.15 (E,F). The regions of A,C and E shown at a higher magnification in B,D and F are indicated. Scale bar for B,D and F represents 10μm.


A
B
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E
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Fig. 2 Localization of δ-crystallin RNA by *in situ* hybridization in transverse sections of a st.15 chick embryos. 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 30μm.

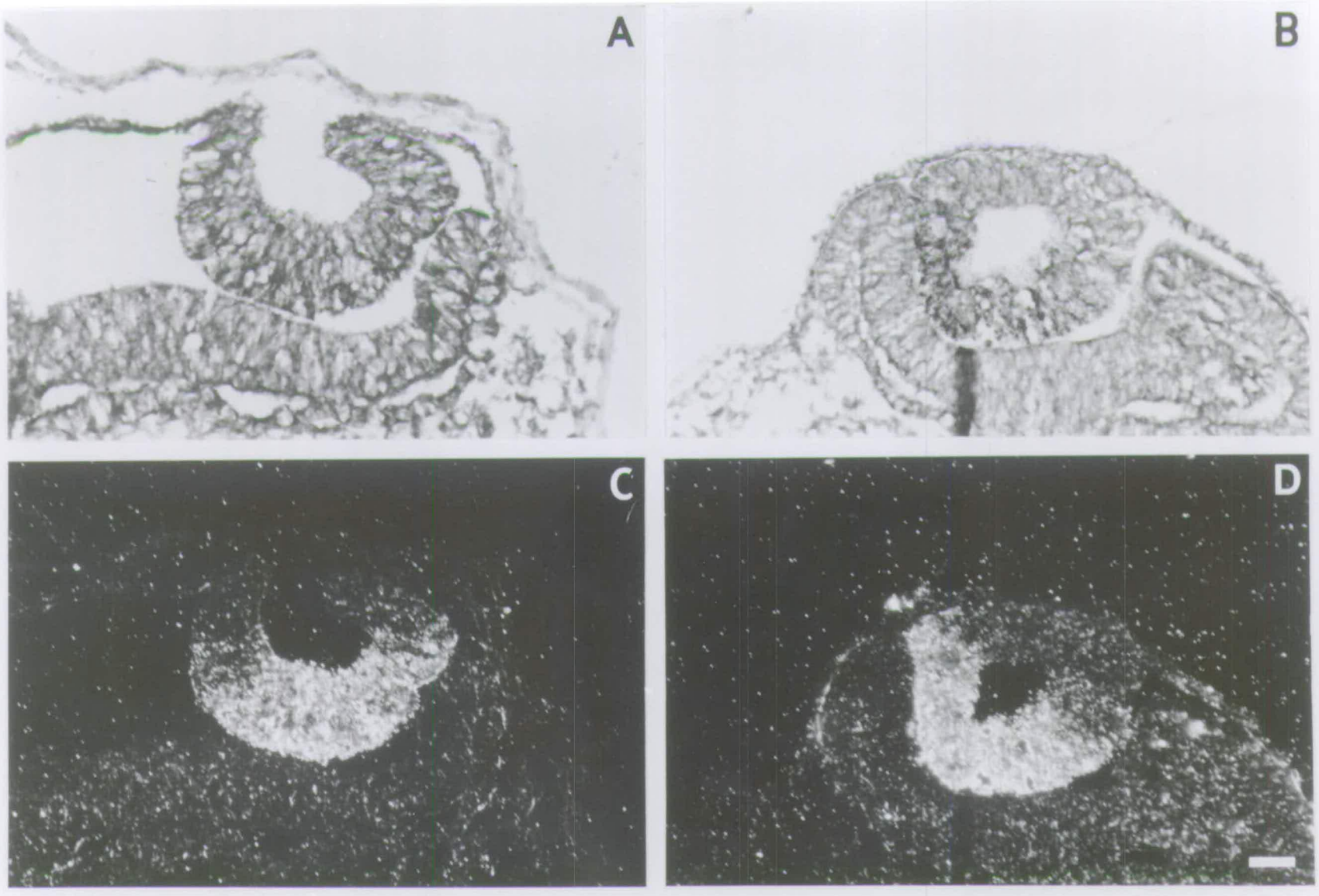
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Fig. 3 Localization of δ-crystallin RNA in tissue squashes of dissected lens placode (A), lens vesicle (B) and a 3.5 day embryo lens (C). Hybridization using a ß-crystallin probe and the 3.5 day embryo lens is shown for comparison (D). Scale bar represents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

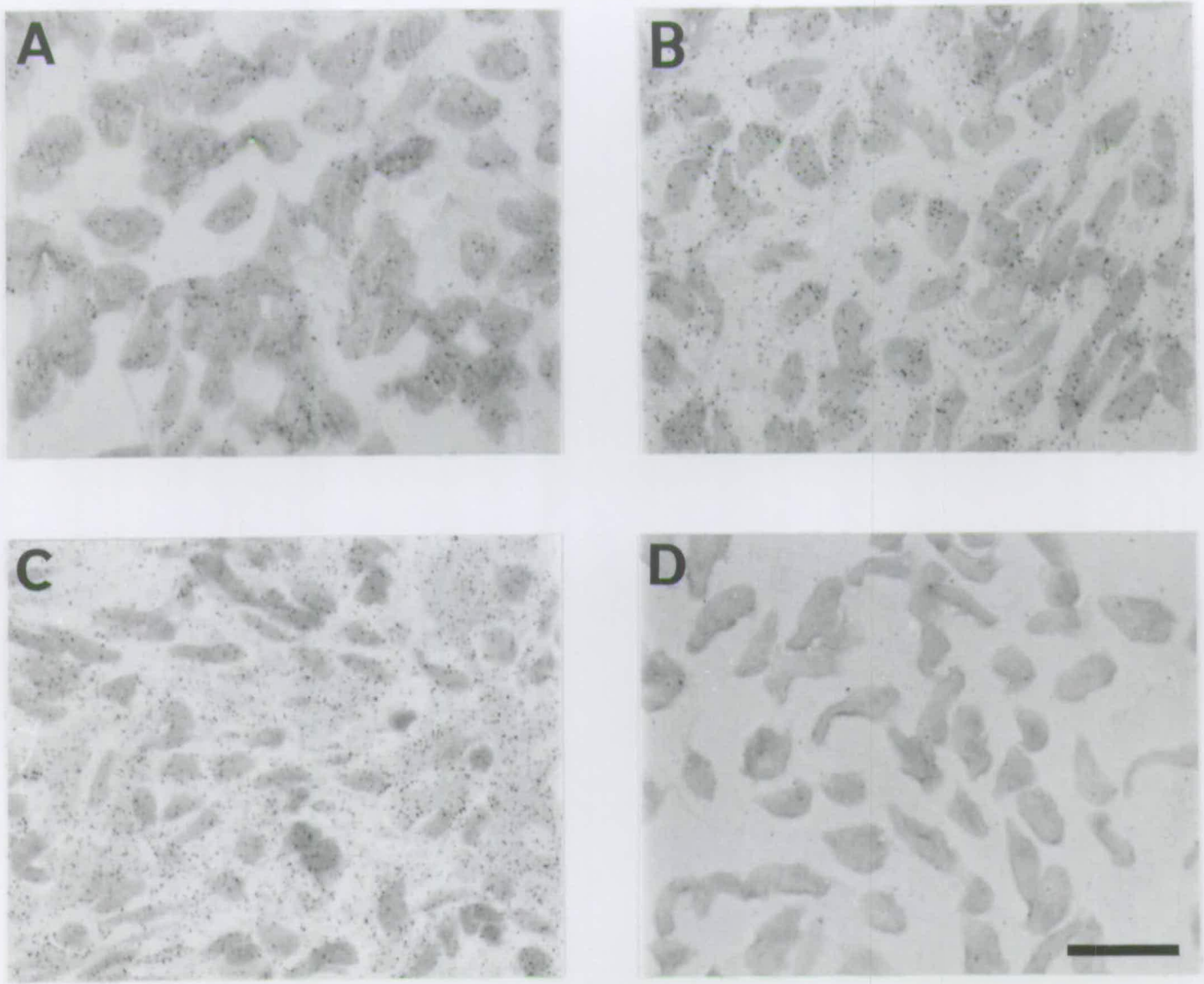
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Fig. 4 Localization of δ-crystallin RNA by *in situ* hybridization to median sagittal sections of 14 day chick embryo eye showing ; lens bow region (A) including the cortical fibers (CF) and the annular pad (AP), annular pad/cortical lens fibre boundary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

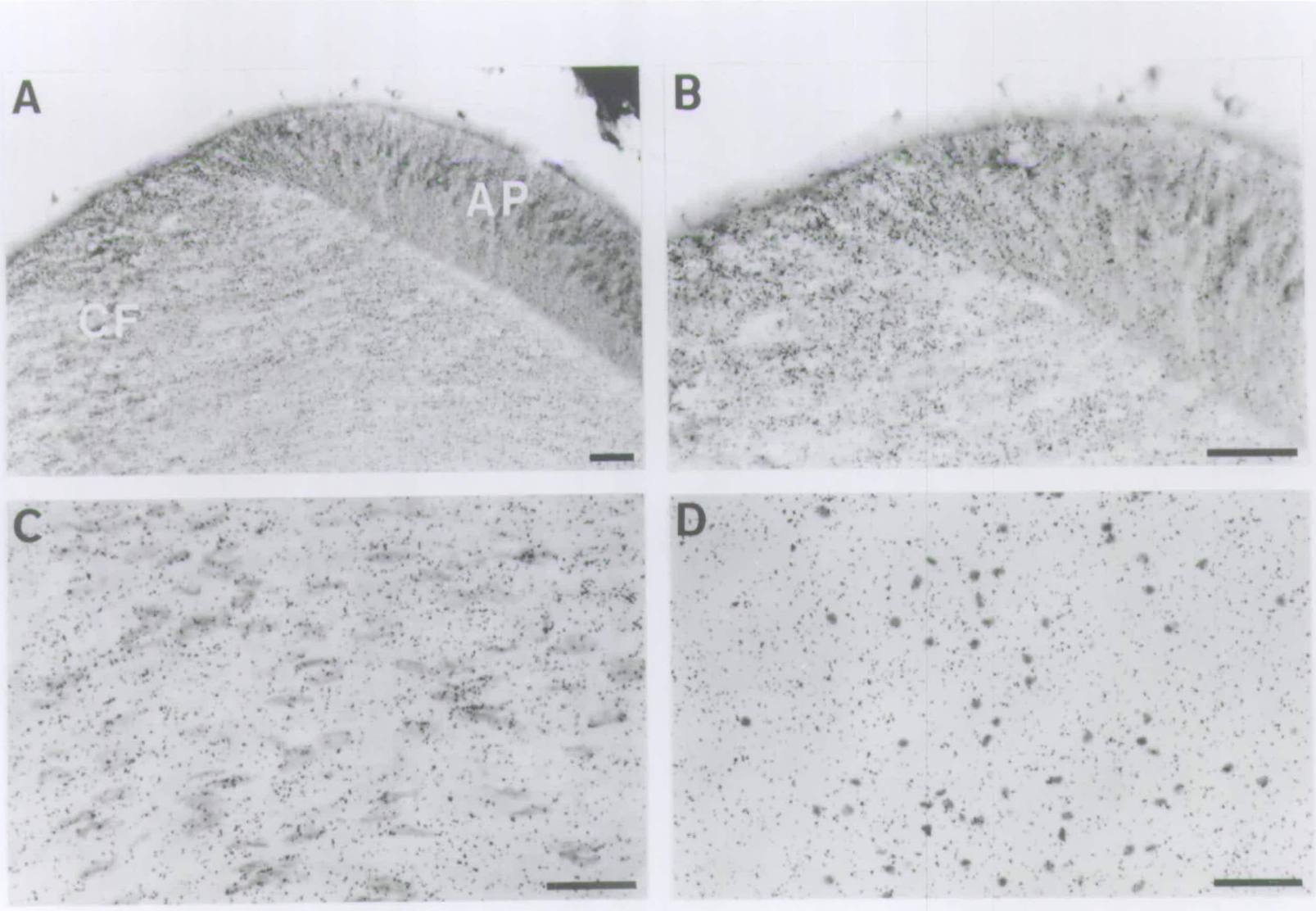
A
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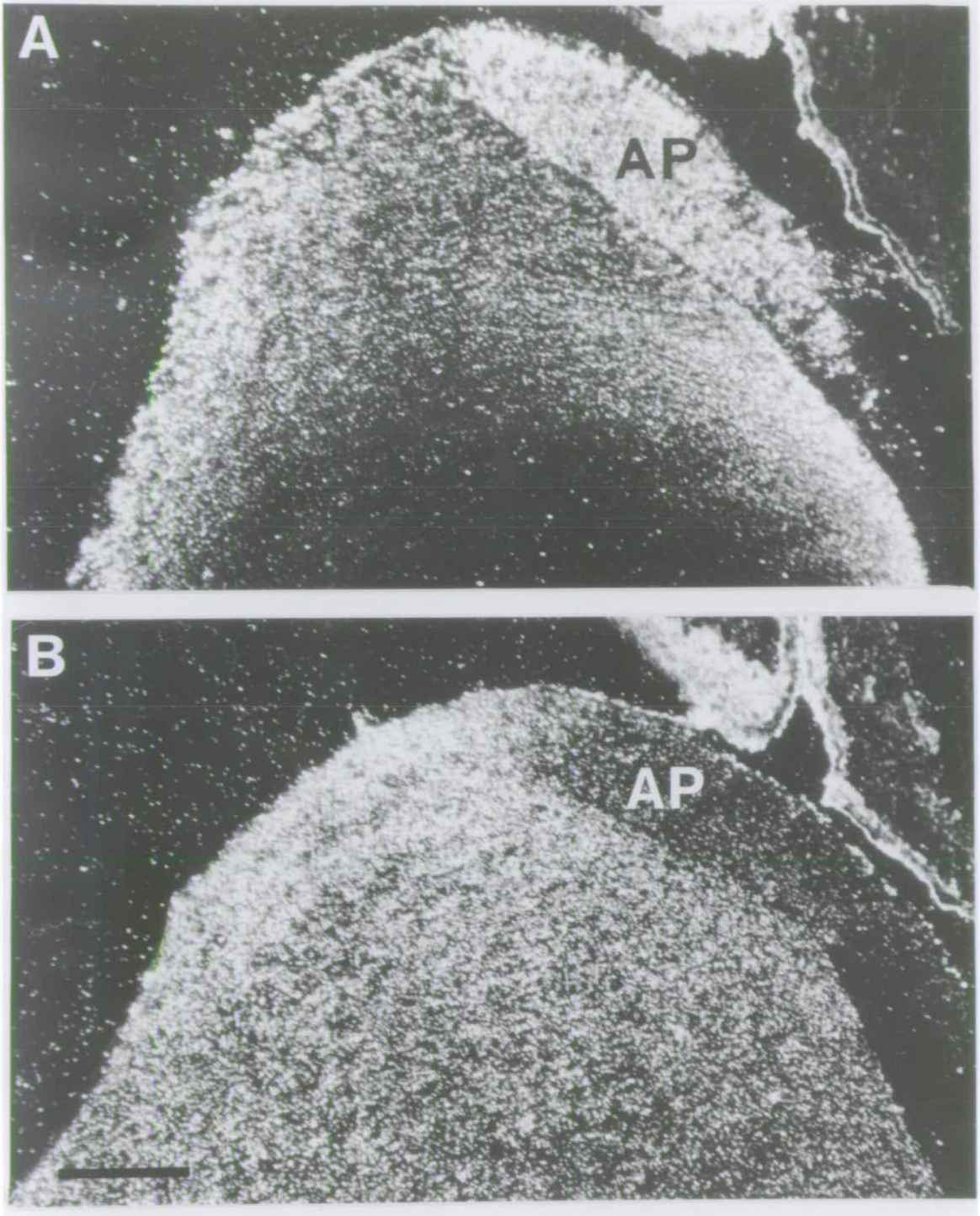
Fig. 5 Localization of αA-crystallin RNA (A) and δ-crystallin RNA (B) by *in situ* hybridization to median sagittal sections of 14 day chick embryo eye photographed under dark-field illumination. Scale bar represents 100μm.


A
AP
B
AP

LOCALIZATION OF δ-CRYSTALLIN RNA DURING LENS MORPHOGENESIS AND DIFFERENTIATION IN THE CHICK EMBRYO II: δ1- AND δ2-CRYSTALLIN EXPRESSION DURING NORMAL LENS DEVELOPMENT AND ECTOPIC LENS DIFFERENTIATION IN THE *TALPID*^{3} MUTANT.

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Short title: Ectopic expression of δ1-crystallin

Key words: δ1- and δ2-crystallin; RNA localization; lens induction; antisense oligonucleotides probes; *in situ* hybridisation.

With 2 figures and 28 references.

Abstract

During early embryonic development in the *talpid*^{3} chick mutant ectopic lens differentiation occurs in the form of an ectodermal lens bridge connecting the eye lenses and in the production of ectopic lenses in the ventral head mesenchyme. *In situ* hybridisation with antisense oligonucleotide probes detects high levels of δ1-crystallin RNA in the eye lenses and in the lens bridge. Low levels of δ2-crystallin/argininosuccinate lyase RNA are detected in all lens and non-lens tissues examined. The results point to the inductive effect of the optic vesicle and optic cup on competent head ectoderm in the production of the lens bridge and the accumulation of δ1-crystallin RNA.

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. Surgical manipulations of early embryos demonstrate that the competence for lens differentiation extends over a larger area of head ectoderm than is normally induced to form lens by the evaginating neuroectodermal optic vesicle (reviewed by Waddington 1952; Saha et al 1989). Although a latent competence for lens differentiation is maintained in a number of embryonic chick tissues, as evidenced by their capacity to transdifferentiate to the lens phenotype *in vitro* (reviewed Clayton 1990), reports of ectopic lens formation *in vivo* are restricted to three independently observed autosomal recessive mutations in the domestic fowl termed *talpid* (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 corresponds to the shortest distance between the eye lens induction and the variable production of small and large ectopic lenses in 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 of cellular response in terms of the activation of crystallin gene expression and its relationship to proximity to the optic cup (future retina).

δ-crystallin is both the first crystallin to appear after lens induction and the most abundant soluble protein in the lens throughout the embryonic period in normal chick embryos (Rabaey 1962; Zwaan and Ikeda 1968) and is, therefore, a convenient marker for lens differentiation. We have recently used *in situ* hybridization to localize and assess the ontogenic changes in δ-crystallin RNA accumulation during lens morphogenesis and have found that high levels of δ-crystallin RNA accumulate during the formation of the lens pit, specifically in those cells in closest proximity to the optic cup (Head, et al, submitted for publication). We have, therefore, used *in situ* hybridization to determine whether crystallin expression during ectopic lens differentiation in *talpid*^{3} is regulated similarly to that seen in ventral eye lens formation in *talpid*^{3} embryos and in lateral eye lens formation in normal embryos.

The situation is complicated by the existence of two δ-crystallin genes in the chick genome which exhibit a very high degree of sequence homology (Nickerson et al 1986). Both genes are transcribed in late embryonic and post-hatch chick lens, and at lower levels in some non-lens tissues but their relative steady state abundance displays a reciprocal pattern with δ1-crystallin RNA the more abundant transcript in the lens (Parker et al 1988; Head et al 1991) and δ2-crystallin RNA the more abundant in non-lens tissues (Thomas et al 1990; Head et al 1991). It seems increasingly likely that the function of δ2-crystallin gene expression in non-lens tissues is that of the urea cycle enzyme argininosuccinate lyase (ASL) (Piatigorsky et al 1988; Kondoh et al 1991; de Pomerai et al 1991). In order to discriminate between the transcripts of the δ1- and δ2-crystallin genes, we have employed the gene specific oligonucleotide probes, described previously (Parker et al 1988; Head et al 1991), and adapted our *in situ* hybridization technique accordingly.

Results

At stage 15 (st.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. 1A) as compared to the labeling pattern seen when the δ2-crystallin probe is used (Fig. 1B).

The primary lens fibers seen at st.18 and the primary and secondary lens fibers seen at st.21 are uniformly and heavily labeled by the δ1-crystallin probe whereas the corresponding lens epithelium is only lightly labeled (Fig. 1C,E). Only low or background levels of silver grains are seen over tissues other than lens with the δ1-crystallin probe (Fig. 1A,C,E) but above background levels of label are seen over all tissues, including lens, at each stage examined using the δ2-crystallin probe (Fig. 1B,D,F).

Transverse sections through the head region of a *talpid*^{3} homozygote at 3.5 days of embryonic development show the eyes displaced ventrally (Fig, 2A, B) and the eye lenses (L) connected by a lens bridge (LB) composed of palisaded cells (Fig. 2A). *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. 2C, 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. 2F). 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. 2B). Hybridization with the δ1-crystallin probe labels the eye lenses (Fig. 2D), whereas the δ2-crystallin probe labels all cellular structures (Fig. 2G).

Discussion

Embryonic chick secondary (cortical) lens fiber cell differentiation is characterized by the accumulation of high levels of δ-crystallin protein and RNA (Pal and Modak 1984; Hejtmancik et al 1985) and this is due to a selective increase in the level of δ1-crystallin transcripts as compared to those of the δ2-crystallin gene (Thomas et al 1990; Head et al 1991). We have recently used *in situ* hybridization to localize the appearance and accumulation of δ-crystallin RNA during lens morphogenesis and provided evidence in support of the proposition that the eye cup continues to play an important role in the regional differences in δ-crystallin expression seen during primary lens fiber differentiation (Head et al, submitted for publication). The results presented here clearly demonstrate that the increase in δ-crystallin RNA abundance during lens placode invagination and primary lens fiber formation result from 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 largely those of δ2-crystallin.

Here we show that the cells of the lens bridge seen in the *talpid*^{3} mutant accumulate high levels of δ1-crystallin RNA, as do the eye lens fiber cells of both *talpid*^{3} and normal chick embryos in contrast to extralenticular tissues which express low levels of δ2-crystallin RNA. We cannot be certain whether the the vesicle-like structure seen in Fig. 2B is a small ectopic lens or whether it represents the abnormal Rathke's pouch found in *talpid*^{3} (see Ede and Kelly 1964) but in either case it expresses low levels of δ2-crystallin RNA rather than high levels of δ1-crystallin RNA. 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 developmentally analogous to the invaginating lens placode seen during normal development and the larger scale morphology of the lens bridge is suggestive of an ongoing invagination into the mesenchyme in the ventral region between the eyes.

Several growth factors have been found in ocular tissues which have been shown to be fibrogenic for lens epithelial cells (see Head et al, submitted for publication). Lens induction may involve a soluble inducer (reviewed in Piatigorsky 1981; Saha et al 1989) and FGF is known to function as an embryonic inducer in other tissue systems (Slack et al 1987,1988; Grunz et al 1988). Extracts of retina, a rich source of FGF (Mascarelli et al 1987), can act as an inducer of lens (Mikhailov and Gorgolyuk 1979), while bFGF is also a potent fibrogenic stimulus in its soluble form (Chamberlain and McAvoy 1987). 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 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 which is 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.

Materials and Methods

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 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 labelled with ^{3}H-dCTP (>1.85TBq/m mol, Amersham, UK) and purified as according to Head et al (1991). Hybridization was carried out with ^{3}H-labelled probe present at 0.1μg/ml in 0.9M NaCl, 90mM Tris-HCl pH7.5, 9mM EDTA, 5X Denhardt's solution, 1% dextran sulfate, 250μg/ml heat sheared herring sperm DNA and 500μg/ml yeast tRNA for 18 hr 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). Hybridisation slides were photographed under transmitted light and dark-field illumination using an Olympus Vanox microscope.

Acknowledgments: We thank C. Nicolson for assistance in the preparation of frozen sections, F. Johnstone and G. Brown for photography and P. Robinson for preparing the manuscript.

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Fig. 1 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 st. 15 (A,B), st. 18 (C,D) and st. 21, 3.5 days of incubation, (E,F). Scale bar represents 100μm.

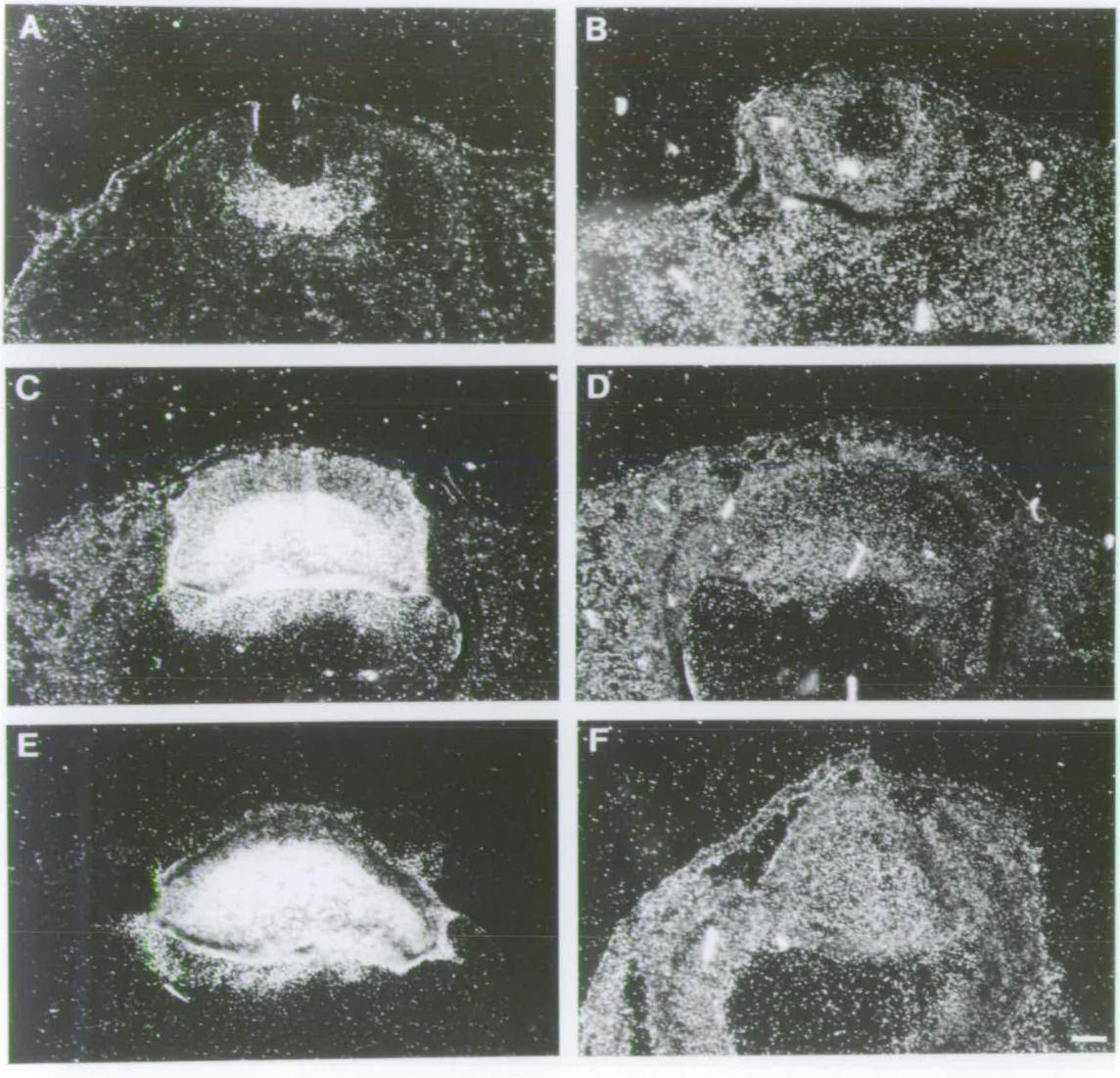
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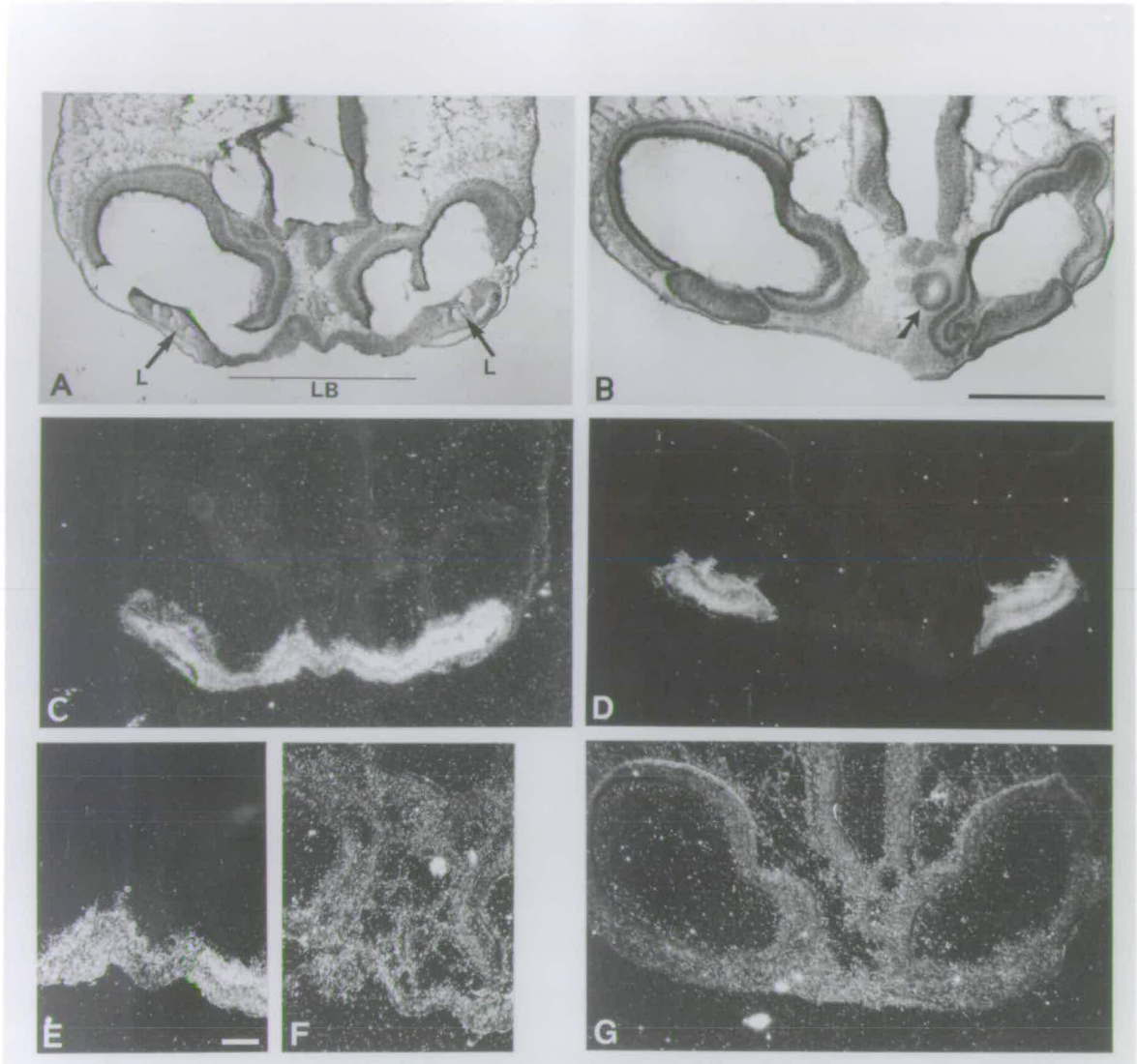
Fig. 2 Localization of δ1-crystallin RNA (A-E) and δ2-crystallin RNA (F,G) by *in situ* hybridization in transverse sections through the head region of a *talpid*^{3} homozygote at 3.5 days of embryonic development photographed under transmitted light (A,B) or dark field illumination (C-G). The eye lens, 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 500μm scale bar. The scale of E and F is shown in E by a 100μm scale bar.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Molecular Biology of the Eye: Genes, Vision, and Ocular Disease, pages 239-247

β-CRYSTALLIN TRANSCRIPTION IN EMBRYONIC CHICK RETINA CELLS

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ABSTRACT β-crystallin RNAs were found in a proportion of cells of 6.5 and 8-day embryonic chick neural retina. The number of cells which transcribe δ-crystallin and β-crystallin RNAs imply that some cells transcribe more than one crystallin RNA species. There is some evidence that there is an ontogenic change in distribution of such cells during development and that some at least of the transcription may not be random with respect to cell type.

INTRODUCTION

Although crystallins had long been regarded as quintessential organ-specific proteins, several authors, using anticrystallin antibodies, reported finding crystallins in certain non-lens tissues. The earlier data have been reviewed and discussed elsewhere (1).

More recently, immunohistology with mono-specific antibodies to δ-crystallin, the major component of the embryonic chick lens, have detected δ-crystallin antigenicity in 30% of the cells of the embryonic adenohypophysis (2,3), in specific neuronal tracts of the embryonic avian midbrain (4) and in a specifically located sub-population of retinal glial cells (5). cDNA probes to coding sequences of δ-crystallin RNA detect its presence in several non-lens tissues (6,7).

The potential for transdifferentiation of retina to lens was found to be associated with the expression of crystallin RNA at moderate or intermediate levels (8) but in situ hybridisation showed that tissues which transcribe δ-crystallin RNA are heterogeneous, with groups of cells transcribing at relatively high levels amongst

histologically similar cells without such transcripts (9).

α-crystallin protein was reported in retina and iris by several investigators (1) and an antiserum to a fraction enriched with α-crystallin localised in retinal Muller-glia cells (10) while αA-crystallin RNA was detected in freshly isolated 8-day embryo neural retina (11).

β-crystallin antigenicity has been detected in retina. The simplest interpretation of the early data is that only some of the β-crystallin polypeptides are detectable (1). The 25kD β-crystallin RNA was not detected in freshly isolated 8-day embryo neural retina (12); however other β-crystallins remain to be investigated.

We report here on some data from a preliminary exploration of β-crystallin RNAs in the embryonic neural retina, and on a preliminary attempt to obtain a partial separation of cell types in order to examine the distribution among them of different crystallin RNAs.

MATERIALS AND METHODS

We are currently using cDNA probes to the following crystallin RNAs: δ-crystallin RNA (pM56) (13), 25kD β-crystallin (026) (12) and three β-crystallin probes which were a generous gift from Dr. J. Piatigorsky (pCβ19/26 Cr42, pCβ23 Cr52, pCβ25 Cr61 (14). All probes were constructed by dG dC homopolymer tailing and were inserted into the Pst 1 site of the plasmid vector, pBR322. In situ hybridisation to sectioned whole eyes at 6.5 days of incubation, to squashes of neural retina at 8 days of incubation, and to cultured cells from 8-day neural retina was carried out as in Bower et al (15) and Jeanny et al (9) but under conditions of higher stringency as in Pardue (16). Cells or tissue fragments were cultured for 24 hours in medium containing sodium valproate or phenytoin, and neurotransmitter uptake and glial enzyme activities were measured according to Sedowofia and Clayton and Sedowofia et al (17,18). Cells were also grown in control medium, in valproate or phenytoin from days 17 to 24, and harvested on day 24. Total cell RNA was prepared according to Chirgwin et al (19) and preparation of dot blots, hybridisation, highly stringent washing and hybrid detection were carried out according to Anderson and Young (20): SDS PAGE was as in Patek and Clayton (21).

RESULTS

We are currently examining sectioned eyes and tissue squashes of eyes from embryos of 3.5 to 16 days of incubation, but report here only on 6.5 and 8-day embryonic material, for which we have examined several eyes from two different chick strains.

6.5-day embryo eyes. δ-crystallin RNA was found located in both nuclei and cytoplasm of lens cells (Fig. 1A) while 19/26kD β, 23kD β and 25kD β RNAs were mainly nuclear in these cells Fig. 1 BCD). Fig. 1 EFGH represent sections through the central posterior neural retina and pigment epithelium of the corresponding eyes. All four RNA species were found in numerous cells scattered throughout the neural retina. Occasional pigment epithelium cells were also labelled by hybridisation to each of the four probes. The number of positive cells for each probe implies that at least some cells in neural retina transcribe more than one crystallin RNA.

8-day embryo neural retina. Some degree of cellular heterogeneity, both quantitative and qualitative is shown in squashes of all four RNAs, (Fig. 2 ABCD). This is especially marked for 25kD β-RNA, where small groups of heavily labelled cells are surrounded by cells of indistinguishable morphology which exhibit little or no hybridisation to the probe.

Cultured 8-day embryo neural retina cells. The 21-day cultured cells shown in Fig. 2 (FGHI) show that all four RNAs are partially processed to the cytoplasm.

Partial selection of retina cells. Phenytoin treatment for 24 hrs led to a 36% increase in the uptake of dopamine, a 177% increase in the uptake of noradrenaline and to a relative increase in 19/26kD RNA (Fig. 3A). Sodium valproate treatment for 7 days resulted in a 48% diminution in GABA uptake and a 170% increase in noradrenaline uptake in the neurons, and an increase of 27% and 29% respectively in the activity of glial carbonic anhydrase and cyclic nucleotide phosphorylase. There was a virtual absence of αA and αB crystallins, 22kD β-crystallin and 50kD δ-crystallin in 24 day old cultured cells. Further information will be presented elsewhere (Head and Clayton in prepn.).

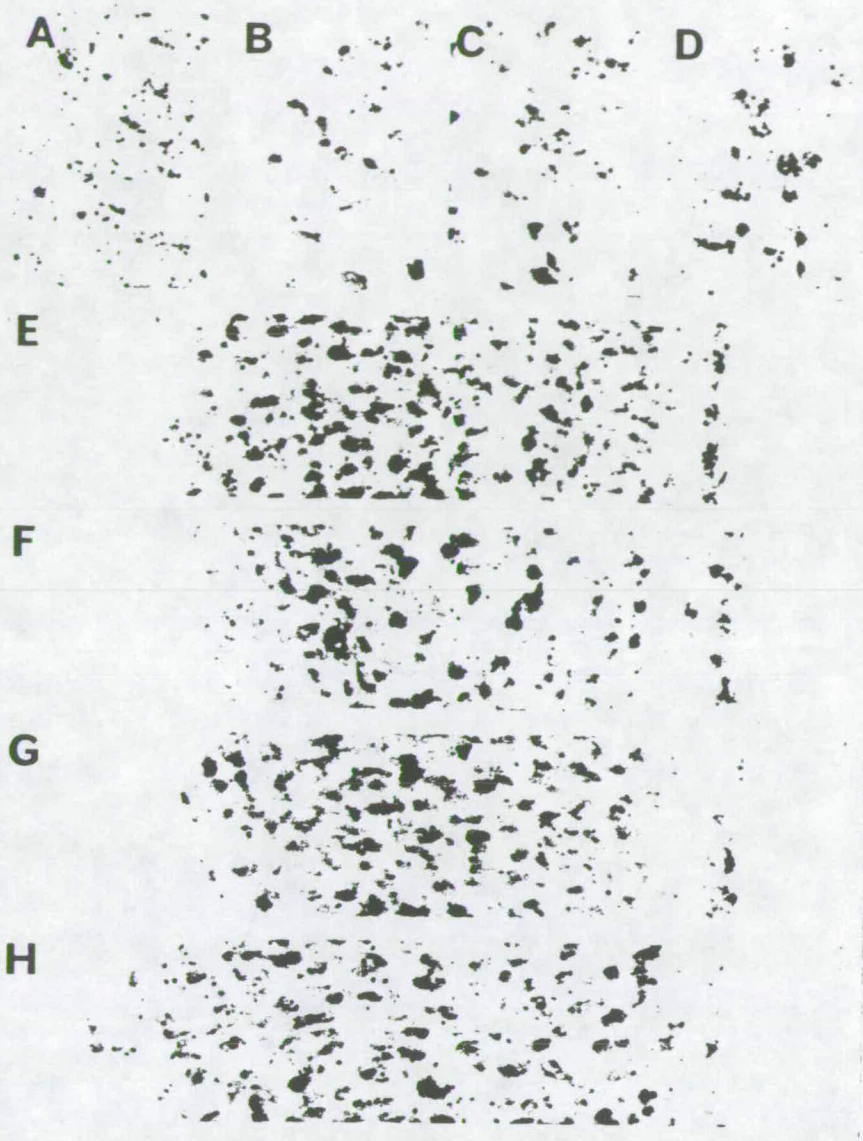

FIGURE 1. In situ hybridisation of δ- and β-crystallin cDNA probes to sectioned 6.5 day embryo chick retina and lens. A,B,C, and D, show lens fibre nuclei. E, F, G, and H, posterior retina: vitreal border on the left; pigmented epithelium on the right. Paired lens and retina fields come from the same section. All fields x400. Specificity of probes: (AE) δ-crystallin: (B,F) 19/26 β-crystallin (C,G) 23 β-crystallin: (D,H) 25 β-crystallin.

FIGURE 2. In situ hybridisation of δ- and β-crystallin cDNA probes to squashes. Fresh 8 day embryonic neural retina (A-E). 8 day neural retina cultured for 21 days (F-J): specificity of probes: (A,F) δ-crystallin (B,G) 19/26 β-crystallin (C,H) 23 β-crystallin (D,I) 25 β-crystallin (E,J) pBR322, plasmid vector control.

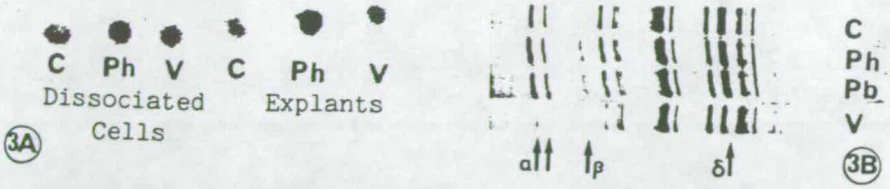

FIGURE 3 A. Dot blot hybridisation of the 19/26 β-crystallin cDNA probe to 10μg of total cellular RNA from 8-day embryo neural retina, cultured for 24 hours.
B. SDS PAGE of water soluble protein from 8-day embryo neural retina cultures treated on days 17 to 24 inclusive and harvested on day 24. A and B: C control medium, Ph phenytoin, Pb phenobarbitone, V valproate.

DISCUSSION

Our data suggest that at least a proportion of retina cells transcribe more than one crystallin RNA for at least some period during development. Total crystallin RNA declines during development (8). A comparison of 3.5 and 6.5 day sections shows that the distribution of transcribing cells may also change (15 and this report).

The levels of crystallin proteins detected in whole retina are relatively low (8) but may not be insignificant at the cellular level if expression is restricted to particular cells, as has been found for α-crystallin and δ-crystallin (10,5).

Crystallin synthesis does not imply a lens-like morphology. δ-crystallin is the major component of the early chick lens, yet cells expressing δ-crystallin alone are not lens-like (22): the 35kD β-crystallin characterises lens cell elongation (23) but it is also found in ageing lens cell cultures with no lens fibres (21). Lens cell morphology and function may require the coprescence of several crystallins, at sufficient cellular concentrations, and within the range of relative proportions evidenced by ontogenic progressions and species comparison.

Tissues which synthesise crystallins at moderate levels during their development are not randomly distributed (24). It is therefore possible that extra-lenticular crystallin expression reflects a still active ancestral function of these proteins, antedating the evolution of the vertebrate lens, but not as ancient as is implied by the sequence

homologies found between crystallin and non-crystallin proteins: for example between the enzyme arginosuccinate lyase and δ-crystallin, which has no enzyme activity (25).

The role of crystallin RNA in retina cells is more puzzling. In situ hybridisation with crystallin RNAs show a mainly supranuclear location (9,26 and this report) and Northern blots show that most of the extra-lenticular δ-crystallin RNA is not fully processed (6,7): the 19/26kD β-crystallin RNA is also incompletely processed (Head and Clayton, in prepn.). Are these RNAs transcribed randomly with respect to cell type in the retina? 24 hours exposure to phenytoin enriches the retina cell population for adrenergic and dopaminergic neurotransmitters and also for the 19/26kD β-crystallin (Fig. 3A). The effects of valproate are more complex, possibly because cells were assessed later, during transdifferentiation. Late valproate treatment led to selective diminution or loss of four crystallin polypeptides only, and an increase in CNP and CA activities. This suggests heterogeneity among glial cells, perhaps between cells synthesising glial enzymes and some crystallins and cells synthesising different crystallins. Even although we are not yet able to interpret these results in detail, the data suggest that these effects are not due to adaptive changes within a cell, but to partial cell selection, and that cellular subpopulations of neurones and glia might also be characterised by the expression of different crystallin transcripts.

ACKNOWLEDGEMENTS

We are deeply grateful to Drs. J. Cuthbert, J-C. Jeanny and C.E. Patek for help and discussion and to M. Ramsay for preparing the manuscript.

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Differentiation (1991) ■■:■■-■■

Differentiation
Ontogeny, Neoplasia and Differentiation Therapy

Evidence for the extralenticular expression of members of the β-crystallin gene family in the chick and a comparison with δ-crystallin during differentiation and transdifferentiation

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Accepted in revised form September 27, 1991

Abstract. The β-crystallins are major water soluble proteins of vertebrate lens fibre cells and have previously been regarded as lens-specific proteins: however βB2- and βA3/A1-crystallin RNAs are transcribed and β-crystallin polypeptides are detectable in the developing chick retina. The β-crystallin RNA is transcribed in a subpopulation of retina cells and the number of transcribing cells and the level of β-crystallin polypeptides increase during the differentiation of the retina. Several tissues express β-crystallin polypeptides but individual tissues are characterised by qualitative and quantitative differences in the β- and δ-crystallin polypeptides expressed. The expression of β-crystallins appears to be non-random as defined by tissue distribution, cellular localisation and ontogeny, implying a function for extralenticular β-crystallins and a complex mechanism for the regulation of their expression.

Introduction

Chick lens β-crystallins are heteropolymers composed of members of a family of at least six major related polypeptides [43, 54, 63]. The overall contribution of β-crystallins to successively formed lens fibres increases during development and the β-crystallin polypeptides and their RNAs display differential accumulation and localisation during this process (for example [30, 40, 44, 46, 66]).

In both chick and *Xenopus* many non-lens tissues, including brain and retina, express low levels of some of the major lens proteins. In chick, these are the α-, and δ-crystallins and some of the β-crystallins [8, 13, 15].

Embryonic chick neural retina has been shown to express αA-crystallin RNA [2, 24] and δ-crystallin RNA [3, 10] including both δ1- and δ2-transcripts [28]. Localisation studies indicate heterogeneity of the retina cell population in the expression of crystallin RNA or crystallin protein of both δ- and α-crystallins in chickens [33, 36, 42] and α-crystallin in the cat [35]. Several extraocular tissues in the chick express δ-crystallin RNA [3, 11, 28, 57, 64] and αB-crystallin RNA and protein has been found in extraocular rodent tissues [6, 21, 22, 31, 32].

Several embryonic chick non-lens tissues, including the neural retina, can transdifferentiate in vitro producing lentoid bodies, lens fibre cell masses, which contain high levels of crystallin RNAs and proteins (recent reviews see [13, 23, 51]). It has been suggested that the expression of crystallin RNAs or protein at sufficient levels and in a sufficient number of cells is a precondition for the potential for transdifferentiation to lens cells [13, 16, 61].

The β-crystallin antigenicity detected in the retina was partial and it was suggested that this reflected the expression of some but not all of the β-crystallin antigenic determinants [15]. Both anodal and cathodal components were subsequently detected in fresh 8 day embryonic neural retina and it was shown that the level of each of these components falls below detectable levels after cell dissociation and then rises steeply and differentially during transdifferentiation [52].

Mammalian β-crystallin genes, mRNAs and their corresponding primary gene products are well characterised and have a rational nomenclature based on polypeptide isoelectric point (pI), designated βB1, βB2, βB3, βA1, βA2, βA3, and βA4 in order of decreasing pI [5]. The extension of this nomenclature to cover the homologous chicken β-crystallin genes and polypeptides is problematic since where direct homology has been demonstrated at the nucleotide or amino acid level the chicken genes have diverged sufficiently to encode polypeptides with radically different pIs from their mammalian homologues [65]. Even where hybrid-selected in vitro translation data exists for the chick β-crystallin cDNAs [25, 29, 47] the pattern of β-crystallin polypeptides evident in two dimensional (2D) gels makes a single unambiguous assignment difficult. For this reason we

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Springer-Verlag, Heidelberg / H. Stürtz AG, Würzburg
Provisorische Seitenzahlen / Provisional page numbers 1. Korr. Date 21.10.91 F

present our data using the rational nomenclature for the cDNA/mRNA hybridisation experiments and our own molecular weight estimations to identify polypeptides in SDS-PAGE and Western blots.

We previously reported the presence of β-crystallin RNAs in embryonic retina: in situ hybridisation showed that the βB2-crystallin RNA occurred at relatively high levels in small clusters of neural retina cells. The βA3/A1-crystallin RNA detected in the same tissue was shown to be enriched by transitory phenytoin treatment of neural retina cells in vitro which also enriches the assessed levels of dopamine and noradrenalin. This polypeptide was therefore provisionally ascribed to neurones rather than to glia [19]. Although, in an earlier study, we failed to detect the 26 kD β-crystallin in the neural retina of hatched birds [13] we are now using techniques of greater sensitivity and antisera of higher titres that as reported here, demonstrate its presence.

Here we report on the tissue distribution of β-crystallin polypeptides and RNAs in embryonic chick tissues, on ontogenic changes during retina and lens differentiation, and on changes during transdifferentiation of neural retina to lens in vitro.

Methods

Cell culture. Neural retinas from 8 day chick embryos were dissected, dissociated and maintained in primary spreading culture as described previously [52].

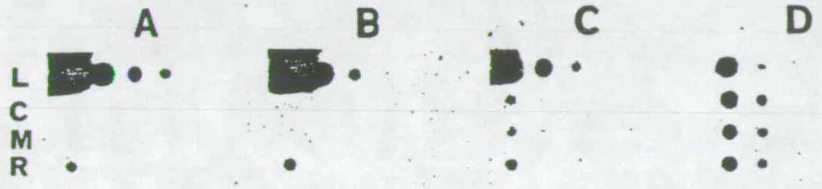

Fig. 1. Dot-blot hybridisation of βA3/A1-crystallin (Rsa 1 insert of pCβ19/26Cr42) (**A**), βB2-crystallin (Rsa 1 insert of pCβ25Cr61) (**B**), δ1-crystallin (**C**) and δ2-crystallin (**D**) probes to: Lens (*L*), tibio-femoral chondrocyte (*C*), striated muscle (*M*) and retina (*R*) RNA, extracted from day-old post-hatch chicks. The blots show the presence of βA3/A1- and βB2-crystallin RNA at high levels in lens and at lower levels in retina. Initial dot, extreme left, 20 μg of total cellular RNA; tenfold dilutions extending from left to right. Filters A, C and D exposed for 18 days, filter B exposed for 14 days

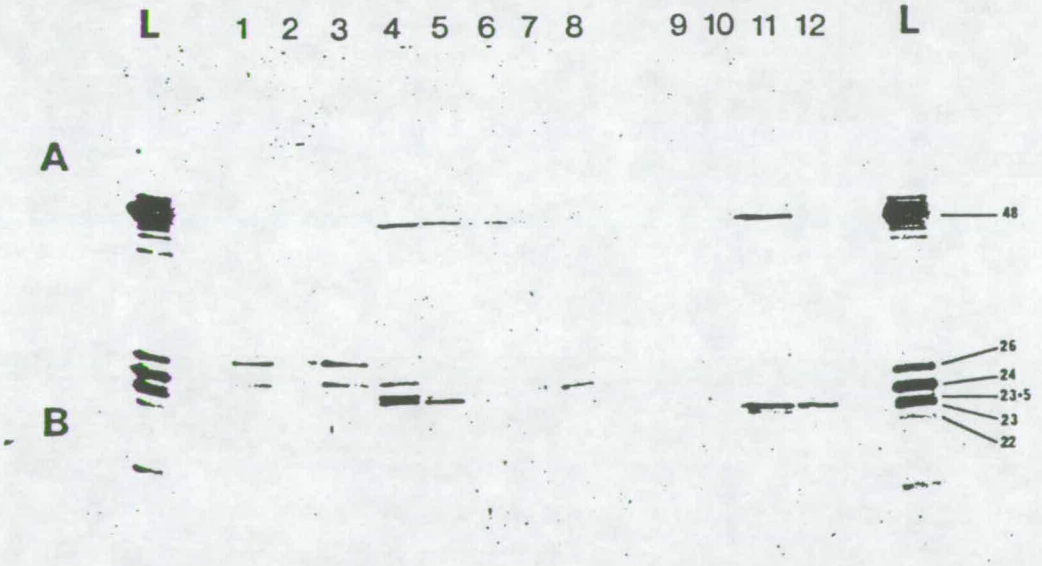

Fig. 2. SDS-PAGE separation of accumulated water soluble proteins from day-old post-hatch chick lens (*L*), total retina (*1*), optic nerve and retinal area 2 mm in diameter containing the optic nerve insertion (*2*), total retina with the area in 2 above removed (*3*), iris (*4*), cornea (*5*), cerebrum (*6*), mid-brain (*7*), optic lobe (*8*), striated muscle (*9*), heart (*10*), kidney (*11*) and liver (*12*) ~~and~~ analysed by Western blotting with a monospecific anti-δ-crystallin antiserum (**A**) and an anti-β-crystallin antiserum (**B**). Lens protein: 10 μg/lane, all other lanes 100 μg. *Molecular weights* are given for lens crystallins in kilodaltons

of these were l.c.

Protein electrophoresis and Western blotting. SDS-PAGE was carried out as previously described [46]. Two-dimensional electrophoresis, 2D(IEF/SDS)-PAGE, was by electrofocusing in the first dimension [20], followed by SDS-PAGE of sliced isoelectric focusing (IEF) rod gels pre-equilibrated in SDS-PAGE sample buffer. Gels were electroblotted for >16 h at 4° C and low voltage (30 V) using a Trans-Blot Cell (Bio-Rad Laboratories, Hemel Hempstead, UK) and a 20% Methanol, 25 m*M* TRIS, 192 m*M* Glycine buffer [62]. Immunological identification employed rabbit antibodies to total water soluble chick lens proteins or to chick β- or δ-crystallins purified by gel filtration [60] as primary antibody, a secondary horseradish peroxidase-labelled donkey anti-rabbit antiserum (kindly provided by the Scottish Antibody Production Unit, Carluke Hospital, Carluke, UK) and the 4-Chloro-1-naphthol/H_2O_2 colour reaction [27]. Western blots analysed for both δ- and β-crystallin polypeptides were made from a single SDS-PAGE separation by cutting the transfer prior to incubation in the primary antisera. Confirmation of protein estimations and gel loadings were assessed by Coomassie blue staining of duplicate gels. The anti-δ-crystallin antiserum was made monospecific by removing contaminant traces of anti-α- and anti-β-crystallin antibodies by repeated chromatography over Sepharose 4B (Pharmacia, Milton Keynes, UK) with calf lens protein as the immunoabsorbant ligand. The anti-β-crystallin antibody was found to give a strong reaction with four β-crystallin polypeptides (23 kD, 23.5 kD, and 24 kD and 26 kD) which are the four major components of the β_L-crystallin (β-light or low molecular weight oligomeric β-crystallin) immunising fraction. Faint reactions with some other crystallins are also seen when lens samples are analysed by Western blotting. These contaminating reactions are not seen after short incubations (2 h) but only following extended incubations (overnight to 24 h), with primary antibody at high concentrations (1:250 dilution): conditions we find necessary to detect the relatively low levels of crystallins in non-lens tissues.

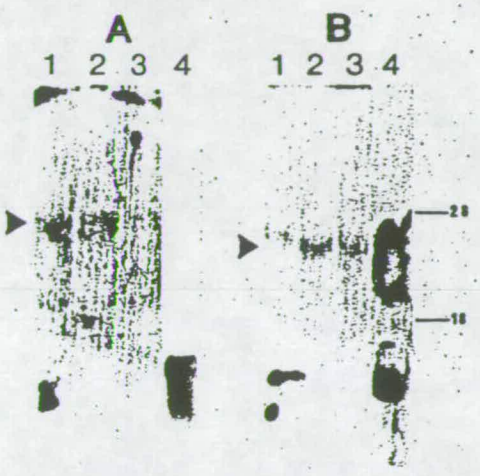

Fig. 3. Hybridisation of the βA3/A1-crystallin (pCβ19/26Cr41) (**A**) and βB2-crystallin (O26) (**B**) probes to Northern transfers of total cellular RNA from 8 day embryo neural retina (*1*), 16 day embryo neural retina (*2*), day-old post-hatch neural retina (*3*) and day-old post-hatch lens (*4*). Transfers in A show 20 μg RNA/lane and were exposed for 12 days while those in B show 15 μg RNA/lane and were exposed for 4 days. The positions of the 28S and 18S ribosomal subunits are indicated.

RNA extraction and dot-blot hybridisation. Total cellular RNA was prepared using the Guanidinium thiocyanate/Caesium chloride method [12], denatured with Glyoxal and spotted directly onto Schleicher and Schuell nitrocellulose filters (Anderman, Kingston-Upon-Thames, UK) [67] or applied to Zeta probe nylon filters (Bio-Rad Laboratories, Hemel Hempstead, UK) by vacuum filtration using a Hybri-Dot manifold (Gibco BRL, Paisley, UK). Northern transfers were prepared as described previously [9, 28]. Inserts or whole plasmids of the βB2- and βA3/A1-crystallin cDNA clones, pCβ25Cr61 and pCβ19/26Cr42 [29], the βB2-crystallin, βO26 [25] and the δ-crystallin cDNA clone M56 [9], were labelled with 1.85 mBq/μg α^{32}P-dCTP (>15 TBq/mmol, Amersham, Aylesbury, UK) by nick translation [39], or random primed synthesis [26] and purified by chromatography over Sephadex G50 [39]. Hybridisation and hybrid detection employed 10% Dextran sulphate, heterologous competitor DNA and RNA and competitor homopolymeric DNAs [4]. Highly stringent washing conditions were used [4] including two washes of 30 min each in 0.1 × standard

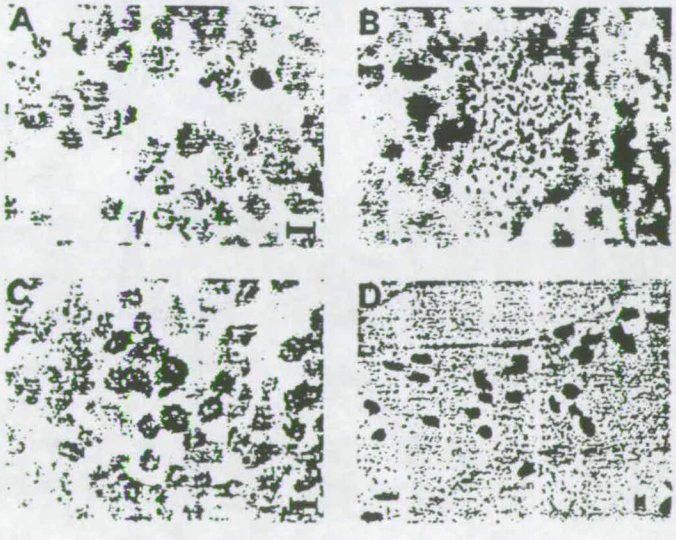

Fig. 4. In situ hybridisation of βB2-crystallin cDNA probes to squashes of freshly dissected 8 day embryo neural retina (pCβ25Cr61) **A**, 14 day embryo neural retina (Pst 1 insert of O26) **B**, 8 day embryo neural retina (pBR322, the plasmid vector with no cDNA insert) **C**, and 14 day embryo lens (Pst 1 insert of O26) **D**. The change in intracellular location of βB2-crystallin RNA in positive neural retina cell clusters can be seen. *Scale bar* represents 10 μm

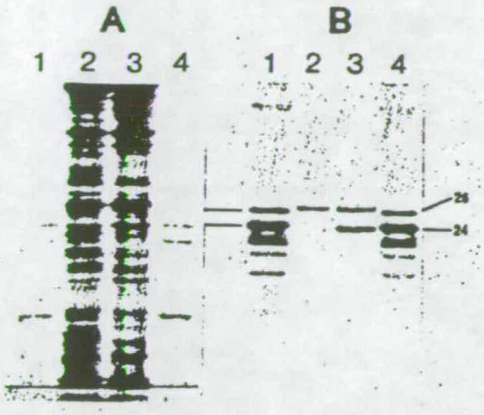

Fig. 5. SDS-PAGE separations of water-soluble proteins from day-old post-hatch chick lens (*1 and 4*), neural retina from 8 day embryos (*2*), and day-old post-hatch chicks (*3*), visualised by Coomassie Blue staining, (**A**), or Western blot analysis with an anti-β-crystallin antiserum (**B**). The presence of low levels of the 26 kD β-crystallin polypeptide is seen in the retina at both stages and the appearance of the 24 kD β-crystallin polypeptide is seen at the day-old post-hatch stage. Lens protein 10 μg/lane, neural retina protein 100 μg/lane

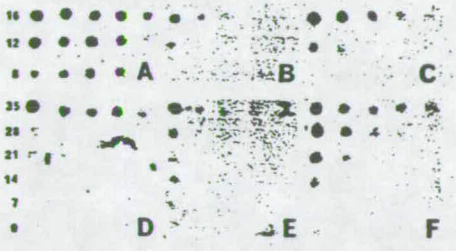

Fig. 6. Dot-blot hybridisation of crystallin cDNA probes to total cellular RNA, extracted from fresh embryonic lens (**A**, **B** and **C**), and cultured 8 day embryo neural retina (**D**, **E** and **F**), showing the early appearance and rapid accumulation of relatively high steady state levels of βB2-crystallin RNA during transdifferentiation. *Initial dots*: 0.25 μg lens RNA and 2.5 μg neural retina RNA, with doubling dilutions. Days of embryonic development for lens, and days in culture for neural retina are shown. *O* indicates freshly dissected 8 day neural retina. Probe specificities were as follows: **A** and **D**, δ-crystallin clone M56, **B** and **E** βA3/A1-crystallin clone pCβ19/26Cr42, **C** and **F** βB2-crystallin clone pCβ25Cr61. Blot A exposed for 3 days, all other blots exposed for 18 days

δ1 e/

saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) at 65° C to avoid β-crystallin cross-hybridisation [29]. Dot-blot hybridisation using δ1 and δ2-crystallin RNA specific oligonucleotide probes (Oswel DNA Service, University of Edinburgh, UK) was exactly as described previously [28].

S DNA/DNA hybridization analysis of the β-crystallin cDNA O26 [25] to four β-crystallin cDNAs described elsewhere [29] indicates that it corresponds to the β-crystallin cDNA clone pCβ25Cr61 (data not shown). Both cDNAs select mRNA encoding a β-crystallin polypeptide of approximately 25 kD [25, 29] identified as βB2-crystallin [48]. The β-crystallin clone pCβ19/26Cr41 has been

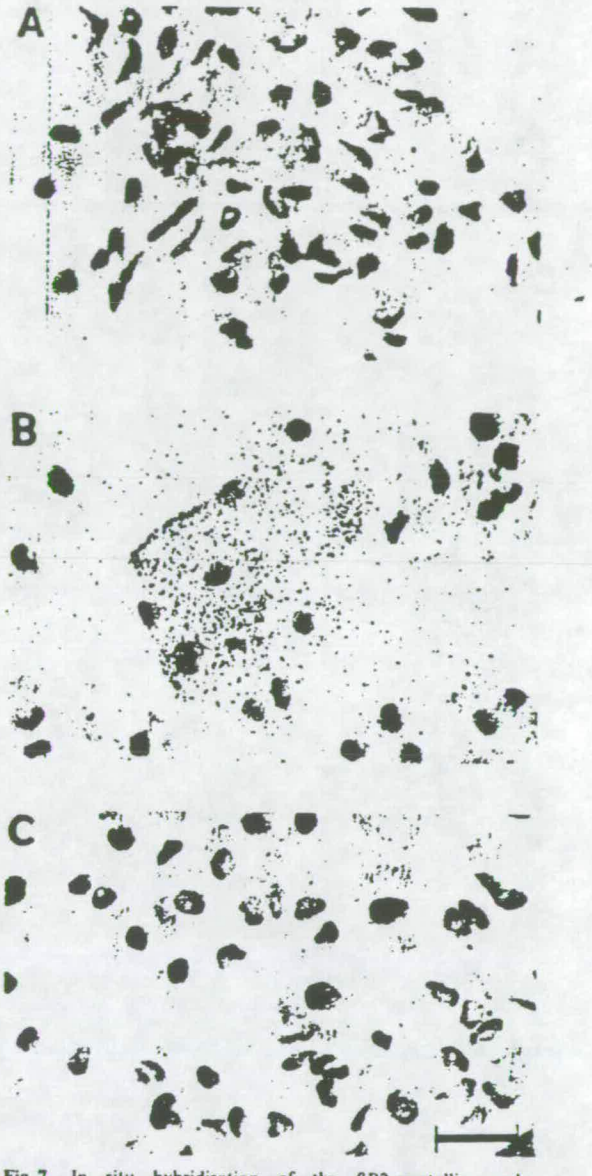

Fig. 7. In situ hybridisation of the βB2-crystallin probe (pCβ25Cr61) to 8 day embryo neural retina cell cultures grown for 21 days (**A**) and 28 days (**B**). A control hybridisation at the 28 day stage using the plasmid vector pBR322 with no cDNA insert (**C**) is shown to indicate the low experimental background level of silver grains. *Scale bar* represents 50 μm

shown to encode the chicken homologue of the mammalian βA3/A1-crystallin that, by virtue of two alternative translation initiation codons, encodes both a 25 kD (βA3) and a 23 kD (βA1) polypeptide [47] (estimates ~~derived~~ from 26 kD and 19 kD respectively [29]). For these reasons we suggest provisionally that the β-crystallin cDNAs O26 and pCβ25Cr61 both encode the basic major 24 kD β-crystallin (βB2) and that the β-crystallin cDNA pCβ19/ δ1 revised/

26Cr41 encodes both the more basic of the two 26 kD β-crystallins (βA3) and another lower molecular weight β-crystallin (βA1).

In situ hybridisation. Neural retinas of 14 day chick embryos were divided into eight regions comprising the inner and outer parts of quadrants obtained by cutting along and at right angles to the axis of the choroid fissure. Hybridisation to squashes of the freshly excised tissues of chick embryos and tissue culture monolayers was as described previously [33] but with a modified hybridisation buffer consisting of 50% Formamide, 0.6 *M* NaCl, 10 m*M* TRIS-HCl pH 7.0, 1 m*M* EDTA, 1 × Denhardt's solution, 1% Dextran sulphate, 250 μg/ml heat sheared Herring sperm DNA, 500 μg/ml Yeast tRNA and ^{3}H-labelled probe present at 1 μg/ml. In situ hybridisation probes were prepared and purified as for dot-blot hybridisation but using 1.85 MBq/μg ^{3}H-dCTP (> 1.85 TBq/mmol, Amersham, Aylesbury, UK).

Results

Dot blot hybridisation to total cellular RNA from day-old post-hatch tissues shows that the βA3/A1-crystallin RNA is represented in the total retina at levels between 1/100 and 1/1000 of those in the lens (Fig. 1A). The βB2-crystallin RNA was found at a higher relative level: around 1/100 of that in the lens (Fig. 1B). Under these experimental conditions neither transcript was detectable in chondrocyte nor in striated muscle tissue (Fig. 1A, B). Hybridisation of a δ2-crystallin oligonucleotide probe to the same RNA samples demonstrates the presence of δ2-crystallin RNA at relatively high levels in each of these non-lens tissues and lower levels of δ1-crystallin RNA in these same tissues (Fig. 1C, D).

Western blot analysis of day-old post-hatch tissues shows that δ- and β-crystallins are present in retina, iris, cornea, brain, heart, kidney and liver (Fig. 2). The 26 kD and 24 kD β-crystallins polypeptides are the major crystallins detected in total retina (lane 1) but are reduced in abundance in the region of the optic nerve insertion, whereas low levels of δ-crystallin are just detectable in the original blots in this region (lane 2). Cornea contains the 23.5 kD and 23 kD β-crystallins and δ-crystallin at higher levels than in retina (lane 4) and iris contains the 23 kD, 23.5 kD, 24 kD and 26 kD β-crystallins and δ-crystallin at even higher levels than in cornea (lane 5). The three brain regions assayed (cerebrum, mid-brain and optic lobes) express δ- and the 23.5 kD β-crystallin polypeptides but at different relative levels in each (lanes 6,7 & 8). Kidney and liver (lanes 11 & 12) express the 23.5 kD β-crystallin at levels similar to those in iris but δ-crystallin is found at higher levels in kidney (lane 11) than in any other non-lens tissue assayed. β- and δ-crystallins are barely detectable in heart (lane 10), and are undetectable in striated muscle (lane 9) under the conditions employed here.

Hybridisation to Northern transfers of total cellular RNA from embryonic and day-old post-hatch neural retina shows that βB2- and βA3/A1-crystallin transcripts present are in the form of high molecular weight partially processed RNA. The abundance of the βA3/A1-crystallin RNA between the 8 day and the day-old post-hatch stages falls whereas that of βB2-crystallin rises (Fig. 3). Preliminary experiments with poly(A)+ RNA of total retina from the day-old post-hatch stage indicates that at least some βB2-crystallin RNA is processed to the same size as the mature transcripts seen in the lens (data not shown).

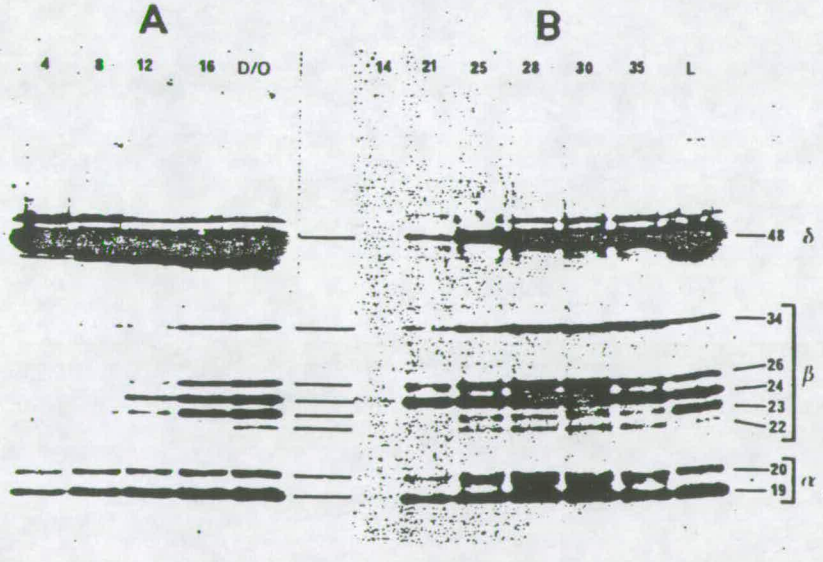

Fig. 8. Pattern of crystallin accumulation during embryonic lens development (A) and during transdifferentiation of neural retina (B) by Western blotting of an SDS-PAGE separation, using 50 μg protein per lane, and an anti-chick lens protein antiserum. Days of development are shown for lens and days in culture for neural retinal cells. *D/O* and *L* indicate day-old post-hatch chick lens protein. Molecular weights are given for lens crystallins in kilodaltons

In situ hybridisation of tissue squashes of 8 day embryo neural retina to the βB2-crystallin probe shows a small intensely labelled subpopulation of cells (Fig. 4A), suggesting high levels of βB2-crystallin RNA per cell, most of which is located in the nucleus. At 14 days of development, these clusters are more frequent and the label is clearly cytoplasmic (Fig. 4B). Control hybridisations of the plasmid vector alone to 8 day neural retina were virtually negative (Fig. 4C) with positive control hybridisation of the βB2-crystallin probe to 14 day lens tissue resulting in both nuclear and cytoplasmic labelling (Fig. 4D). The location of the positive clusters in 14 day neural retina is largely in the anterior dorsal region. No such positive cell clusters, nor indeed any labelling above background, was detectable in the 3.5 day embryo neural retina (data not shown). Western blot analysis with an antiserum to β-crystallin detects only trace levels of the 24 kD β-crystallin polypeptide in 8 day neural retina, but appreciable amounts are found in the day-old post-hatch neural retina (Fig. 5B). A polypeptide with β-crystallin antigenicity and the electrophoretic mobility on SDS-PAGE of the 26 kD β-crystallin polypeptide was detectable in both the 8 day embryo and the day-old post-hatch chick (Fig. 5B).

The βB2-crystallin RNA appears early during transdifferentiation, increasing steadily from day 14 to day 35 (Fig. 6F), whereas the δ- and βA3/A1-crystallin RNAs, although detectable during this period, increase in abundance only after day 28 (Fig. 6D, E). Cell culture squashes examined by in situ hybridisation using both the βB2- and the βA3/A1-crystallin probes show these RNAs to be cytoplasmic and evenly distributed throughout the glial cell sheet after 21 days in culture (Fig. 7A) however raised local levels of these RNAs became detectable after 28 days in culture (Fig. 7B) in putative lentoid bodies or pre-lentoid body cell condensates (data shown for βB2-crystallin only). The relative abundance of βB2-crystallin RNA is reflected in the early appearance of the 24 kD β-crystallin polypeptide at 14 days of culture (Figs. 8B, 9A), before the detection of any other crystallin polypeptide, and its predominance at the 21-day stage (Figs. 8B, 9B).

The sequence of events during transdifferentiation differs markedly from the pattern of crystallin expression during embryonic lens development both at the levels of RNAs and polypeptides. Dot-blot hybridisation to embryonic lens RNA (Fig. 6A, B, C) confirms the presence of δ-crystallin RNA from 8 days, the earliest stage examined, to 16 days of development and the appearance of β-crystallin RNAs during this period. This contrasts with the increased abundance of βB2-crystallin RNA before that of δ-crystallin RNA during transdifferentiation, as described above. Differences in the pattern of crystallin polypeptide accumulation was assessed by Western blotting. The use of a single anti-total chick lens protein antiserum to screen Western blots of accumulated soluble proteins during lens development and transdifferentiation of neural retina to lens allows direct comparison of individual crystallin polypeptides during these two processes. Although a direct comparison between the relative abundance levels of different crystallin

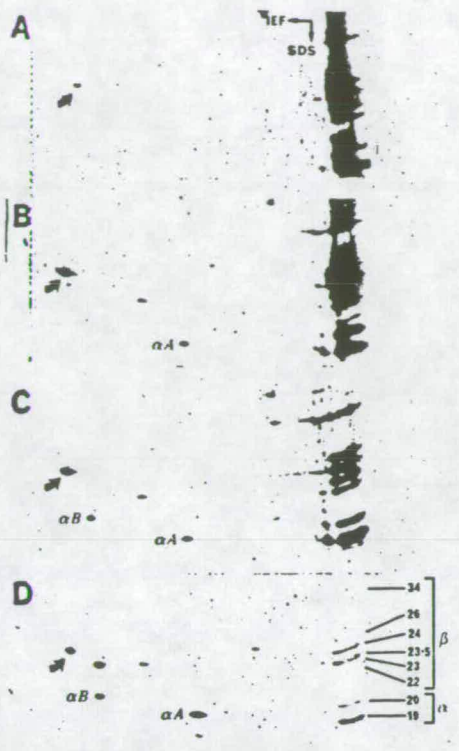

Fig. 9. Pattern of crystallin accumulation during transdifferentiation of neural retina by Western blotting of 2D (IEF/SDS)-PAGE separation of 200 μg water-soluble proteins after 14 (A), 21 (B) and 28 (C) days in culture using an anti-chick lens protein antiserum. D shows the total protein of the day-old post-hatch lens stained with Coomassie Blue. Each blot and gel has a reference lane of 50 μg chick lens protein separated by SDS-PAGE only. The *horizontal arrow* indicates the direction of the acidic to basic pH gradient of the IEF separation. Molecular weights are given for lens crystallins. The position of the early appearing 24 kilodalton β-crystallin is marked with an *arrow*.

polypeptides would depend on the relative titres and avidities of the antiserum for each polypeptide, the sequence of changes in relative amounts is clear (Figs. 8, 9). During normal lens development, the anti-total crystallin antiserum detects a high level of δ-crystallin, and both αA- and αB-crystallin polypeptides but only trace levels of β-crystallins at the 4 and 8 day stages. During transdifferentiation from 8 day embryo neural retina, however, the 24 kD β-crystallin polypeptide is detected before either the δ- or α-crystallins (Figs. 8, 9). The temporal sequence of changes in the β-crystallin polypeptides in developing lens and in transdifferentiating neural retina also differs. These observations are in general agreement with data on the pattern of accumulation and loss of crystallin polypeptides in long-term neural retina cell cultures (Patek, Jeanny and Clayton, in preparation).

Discussion

Lens cell differentiation, transdifferentiation and crystallin expression

Lens cells expressing high levels of crystallin proteins are derived in vivo by successive rounds of terminal differentiation of the epithelial cells at the lens equator. Lentoid bodies (lens fibre cell masses) may also be derived by differentiation of the lens epithelial cells in vitro or by transdifferentiation of certain extralenticular tissues in vitro [69].

Chick tissues which can transdifferentiate to the lens phenotype express low levels of α-, β-, and δ-crystallins [15, 16, 52] and their RNAs [2, 3, 10, 24, 28]. All the available evidence suggests that although low levels of α-, β- and δ-crystallin RNAs and antigens are present in 8 day embryo retina, these fall below detectable levels after cell dissociation and then reappear and accumulate differentially prior to lentoid body formation ([10, 24, 52] and Figs. 6, 8). This loss may be a general response to cell dissociation since a similar transitory loss of crystallin expression occurs following dissociation of chick lens epithelial cells [52].

Previous studies of the differentiation of lens fibre cells from lens epithelium and from neural retina showed that crystallin expression is regulated by both transcriptional and post-transcriptional mechanisms, and also that the different crystallins are independently regulated [11, 17, 18, 19, 24, 52]. At some stage during the programme of changes in crystallin expression seen during transdifferentiation any concentration- or interaction-dependent functions of the crystallins must also change. We have continued our comparison of events in normal differentiation and transdifferentiation in order to clarify the relevant conditions and events, as this is a prerequisite for assessment of possible functions. The temporal sequence of crystallin gene expression during transdifferentiation of embryonic neural retina to lens is different from that seen during lens development, both at the level of RNAs and of polypeptides ([18] and Figs. 6, 8).

Extra-lenticular tissues which express δ- or α- crystallin are characterised by cellular heterogeneity and have groups of positive cells in an otherwise negative cell population. Such cells may be grouped by location or correspond to particular cell types [10, 32, 33, 35, 36, 57, 64]. We do not yet know whether more than one crystallin RNA may be co-expressed in a single non-lens cell. Transdifferentiation potential is high in the early embryo retina but falls during development as does the overall level of lens-abundant RNA sequences [16] and the numbers of cells involved in their transcription (Head and Clayton, unpublished observation). This suggests that a future investigation of the relationship of crystallin levels per cell, the number of crystallin expressing cells and the individual contribution of crystallin RNAs expressed in a cell may be relevant to transdifferentiation potential.

β- and δ-crystallin expression in the developing retina

Although the levels of lens ~~abundant~~ sequences in fresh neural retina fall during the embryonic period, β-crystallin antigenicity, undetectable at 3.5 days, becomes detectable and increases by more than threefold from the 8 day to the day-old post-hatch stage [16]. The in situ hybridisation data presented here demonstrates that βB2-crystallin RNA, though undetectable at the 3.5 day stage undergoes a predominantly nuclear to predominantly cytoplasmic transition in increasing numbers of clusters of neural retina cells between 8 and 14 days of embryonic development (Fig. 4) and that a 24 kD β-crystallin polypeptide becomes detectable in Western blots at the day-old post-hatch stage (Fig. 5). Both βB2- and βA3/A1-crystallin RNAs contribute to fresh day-old post-hatch retina at between 1×10^{-2} and 1×10^{-3} of their steady state level in the lens at this same stage (Fig. 1) but Northern transfers of neural retina total cellular RNA show that a large proportion of these RNAs are only partially processed and that the abundance of βB2-crystallin RNA increases during embryonic development whereas that of βA3/A1-crystallin RNA falls (Fig. 3).

The apparent discrepancy between the abundance of δ2-crystallin RNA (Fig. 1) and the low level of δ-crystallin protein in in day-old retina (just detectable in the original blots, Fig. 2) probably results from the high proportion of incompletely processed δ-crystallin RNA evident from Northern transfers [17, 18, 28] and appears to reflect the limited number of cells which accumulate δ-crystallin [36]. There is a sharp drop in δ-crystallin protein levels detected in fresh neural retina in Western blots between 7 and 11 days of embryonic development with no corresponding drop in the δ-crystallin mRNA abundance seen in Northern transfers [53].

Tissue distribution of β- and δ-crystallin expression

When other ocular and extra-ocular tissues from post-hatch chicks are analysed, a complex pattern of δ- and β-crystallin expression is evident (Fig. 2). δ-crystallin RNA had previously been detected at very low levels in some of these tissues [11, 17, 57]. δ-crystallin protein can be seen to accumulate to higher levels in iris, cornea, brain and kidney than that seen in the retina (Fig. 2). This protein probably results from expression of the δ2-crystallin gene since all non-lens tissues so far examined contain higher levels of δ2- than δ1-crystallin RNA [28, 59]. Nevertheless, the δ-crystallin polypeptide detected here in kidney and other non-lens tissues is the same size as the major δ-crystallin polypeptide found in the lens.

Taking together all the extralenticular tissues shown in Fig. 2 it would appear that each of four of the β-crystallins, (26 kD, 24 kD, 23.5 kD and 23 kD) is found to contribute, whether singly or together with one or more of the other three, to cross-reactive tissues. The relative levels of each of these, both in relation to the other constituents of the tissue concerned, and, where

more than one is present, with respect to each other, is tissue-specific. Are these components true β-crystallins? Clearly it is important to determine whether the β-crystallin antigens detected in the retina reflect the presence of antigenic β-crystallin polypeptides or the presence of β-crystallin type epitopes on non-crystallin molecular species. Although sequence data may be required to firmly establish their nature, we believe that the weight of evidence to date points to the presence of β-crystallin polypeptides which are the same as lens β-crystallins or, at most, only slightly modified.

First the anti-β_L-crystallin antiserum reacts strongly with four of the six β-crystallin polypeptides in SDS-PAGE and 2D Western blots of lens (Figs. 2, 5), while reactions with non-β-crystallin contaminants of the β_L fraction are extremely weak and hard to detect. It might be argued that this antibody recognises one or more β-crystallin epitopes on a non-crystallin polypeptide found in non-lens tissues, which coincidentally has a similar mobility in SDS-PAGE to a lens β-crystallin. However, this argument becomes increasingly implausible with each additional such coincidence, and it does not provide a straight-forward explanation for the four distinct polypeptides, each of them electrophoretically indistinguishable from one of the four known lens β-crystallins, and each found in different combinations and at different relative levels in several non-lens tissues. A third datum is the presence of extralenticular β-crystallin transcripts, as judged by in situ hybridisation (Fig. 4), dot-blots (Fig. 1) and Northern transfers (Fig. 3) in the embryonic and post-hatch retina.

These data support an earlier observation that several β-crystallin antigenicities were found distributed in several non-lens tissues and the suggestion that "much (tissue and cell) differentiation is combinatorial and quantitative" [15]. We have not yet co-localised individual β-crystallin polypeptides or RNAs in non-lens tissues and do not know whether extralenticular β-crystallins can form associations similar to those in the lens or whether they mark separate cells, function independently, and may therefore perform functions different from those they exhibit in the lens.

Extralenticular β- and δ-crystallins: evolution and function

The presence of extra-lenticular crystallins may reflect molecular properties which predate the evolution of the vertebrate lens [13, 18]. whether they have evolved by a mechanism of duplication and divergence [14] or by the mechanism of gene sharing [49].

Crystallins may be bi- or multi-functional proteins [49]. Current theories regarding the extralenticular function of δ-crystallin are well borne out by an examination of the novel finding reported here of δ-crystallin protein in chick kidney. High molecular weight δ-crystallin RNA was reported in brain, heart, lung, liver and kidney of day-old post-hatch chicks but kidney had both the highest levels of poly(A)$^+$ δ-crystallin RNA and the highest proportion of fully processed transcripts [11, 17]. These data correlate well with the finding of higher levels of δ-crystallin protein in the kidney than any other non-lens tissue tested at this stage (Fig. 2). The identity of the δ-crystallin in kidney is not known but all non-lens tissues so far examined express higher levels of δ2- than δ1-crystallin RNA [28, 59] although the reverse is true in the lens [28, 45]. δ2-crystallin has argininosuccinate lyase (ASL) activity [34, 50]. These findings taken together with the reports that chicken kidney but not liver contains mRNA hybridising to a human ASL cDNA [40] and that chicken kidney exhibits a high ASL activity [58] strongly suggest that the δ-crystallin detected in kidney is involved in arginine metabolism.

β-crystallins form a super-gene family with the γ-crystallins sequences (reviewed [37]) but structural similarities between β/γ-crystallins and microorganism dormancy proteins suggest a common ancestor prior to the prokaryotic/eukaryotic split [68]. A distantly related member of this gene family which has recently been isolated and partially characterised exhibits both lenticular and extralenticular expression in *Xenopus* [56]. Crystallins which exhibit both lenticular and extra-lenticular expression will presumably have evolved under multiple constraints. It is noteworthy that βA3/A1-crystallin is even more highly conserved than the αA-crystallin gene [1]. Any statements regarding possible extralenticular functions for β-crystallins are at present speculative, but βB2-crystallin has been shown to be heat stable like α-crystallin, but unlike the other β-crystallin subunits [38]. The capacity for Ca^{++} binding by β-crystallins [55] may be of relevance in non-lens tissues, as may be their ability to form associations with cytoskeletal and cell membrane components [7].

In earlier experiments, we detected β-crystallin antigens in both chick and *Xenopus* tissues [15] and in a recent pilot experiment we have detected the βB2-crystallin polypeptide in both murine and feline non-lens ocular tissues. This implies that β-crystallins are expressed in avian, amphibian, and mammalian retinas, and that the βB2-crystallin polypeptide is expressed at appreciable levels in extralenticular sites in both mammalian and avian species. This cross-species conservation of low level β-crystallin expression in extralenticular sites is indicative of a functional cellular component. We are currently testing this hypothesis using antisense oligonucleotides for selective blocking of β-crystallin expression in vitro, a procedure we have recently found useful in the case of retinal α-crystallin.

Acknowledgements. We would like to thank Drs. E.L. Triplett, S.K.A. Sedowofia, J. Cuthbert, C.E. Patek and J.-C. Jeanny for valuable help and discussion, F. Johnstone for his photographic skills, C. Nicolson and A. Edgar for technical assistance in some experiments, M. Ramsay for typing the manuscript and AFRC (Roslin) and Ross Breeders (Newbridge) for the generous provision of fertile eggs and day-old post hatch chicks. The chick β-crystallin clones pCβ19/26Cr42 and pCβ25Cr61 were a generous gift from Dr. J. Piatigorsky.

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Fig.1 Dot-blot hybridisation of ßA3/A1-crystallin (Rsa 1 insert of pCß19/26Cr42) (A), ßB2-crystallin (Rsa 1 insert of pCß25Cr61) (B), δ1-crystallin (C) and δ2-crystallin (D) probes to: Lens (L), tibio-femoral chondrocyte (C), striated muscle (M) and retina (R) RNA, extracted from day-old post-hatch chicks. The blots show the presence of ßA3/A1- and ßB2-crystallin RNA at high levels in lens and at lower levels in retina. Initial dot, extreme left, 20µg of total cellular RNA; 10-fold dilutions extending from left to right. Filters A, C and D exposed for 18 days, filter B exposed for 14 days.

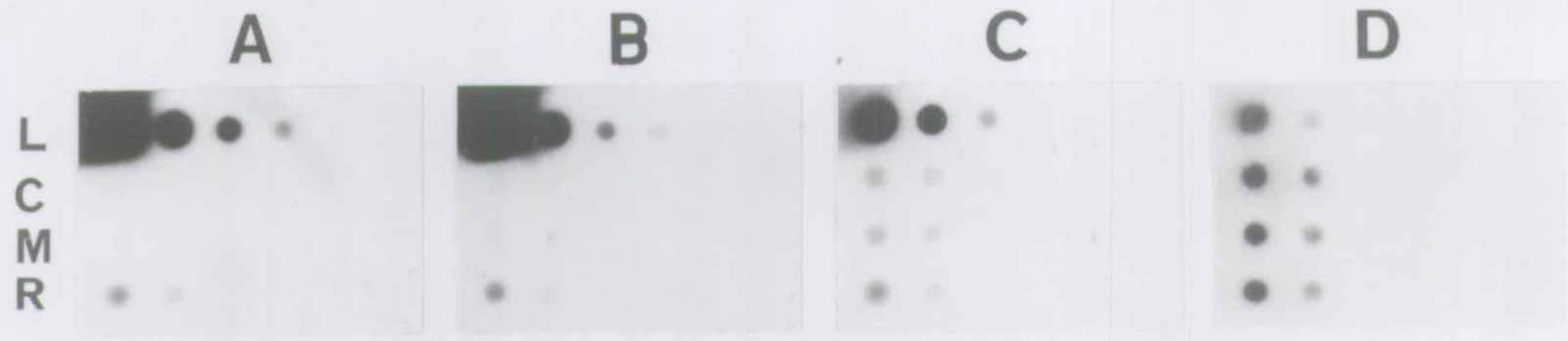
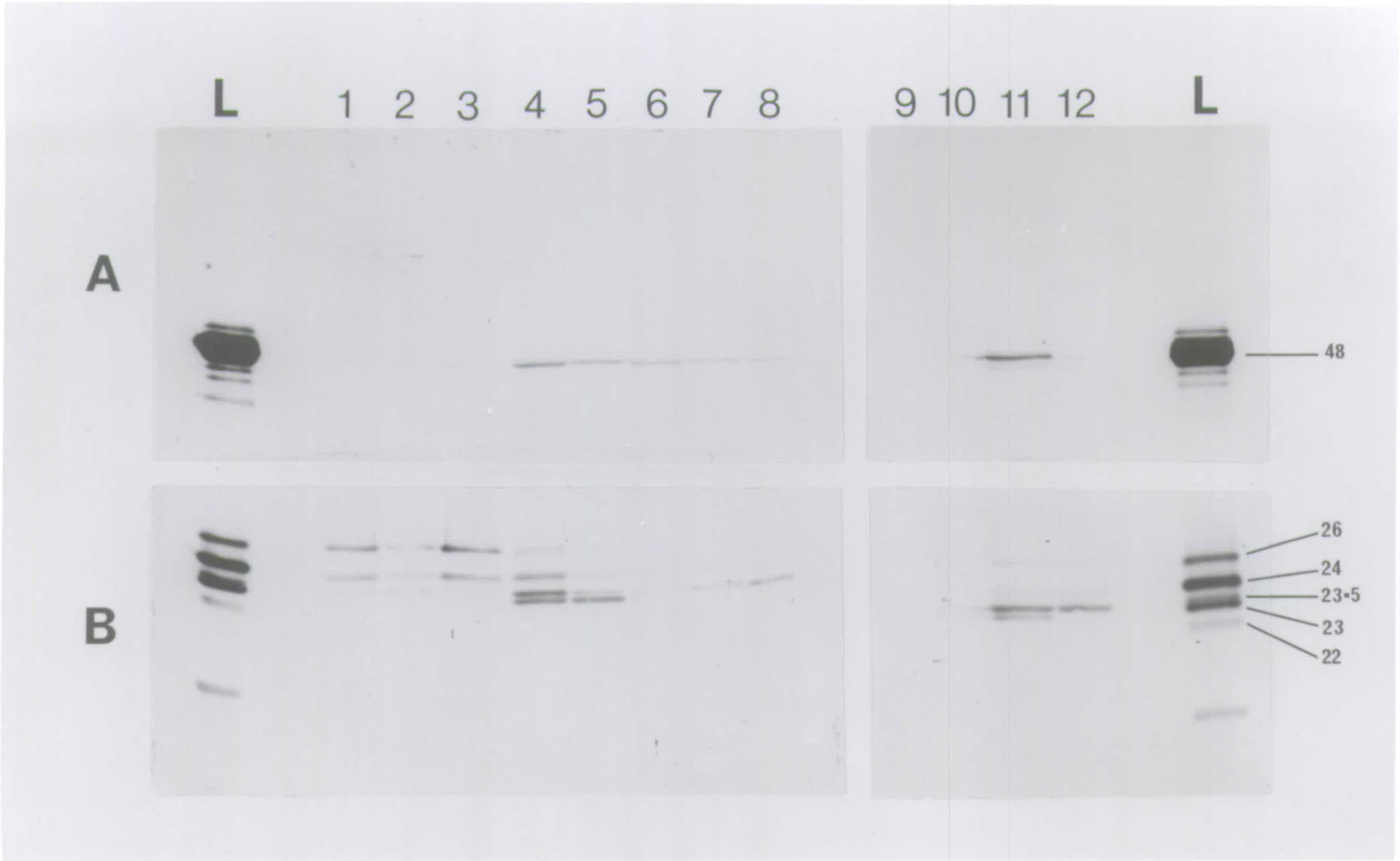

A
B
C
D
L
C
M
R

Fig.2 SDS-PAGE separation of accumulated water soluble proteins from day-old post-hatch chick lens (L), total retina (1), optic nerve and retinal area 2mm. in diameter containing the optic nerve insertion (2), total retina with the area in 2 above removed (3), iris (4), cornea (5), cerebrum (6), mid-brain (7), optic lobe (8), striated muscle (9), heart (10), kidney (11) and liver (12). These were analysed by Western blotting with a monospecific anti-δ-crystallin antiserum (A) and an anti-ß-crystallin antiserum (B). Lens protein: 10µg/lane, all other lanes 100µg. Molecular weights are given for lens crystallins in kilodaltons.


L
1
2
3
4
5
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11
12
L
A
B
48
26
24
23·5
23
22

Fig.3 Hybridisation of the ßA3/A1-crystallin (pCß19/26Cr41) (A) and ßB2-crystallin (026) (B) probes to Northern transfers of total cellular RNA from 8 day embryo neural retina (1), 16 day embryo neural retina (2), day-old post-hatch neural retina (3), and day-old post-hatch lens (4). Transfers in A show 20µg RNA/lane and were exposed for 12 days while those in B show 15µg RNA/lane and were exposed for 4 days. The positions of the 28S and 18S ribosomal subunits are indicated.

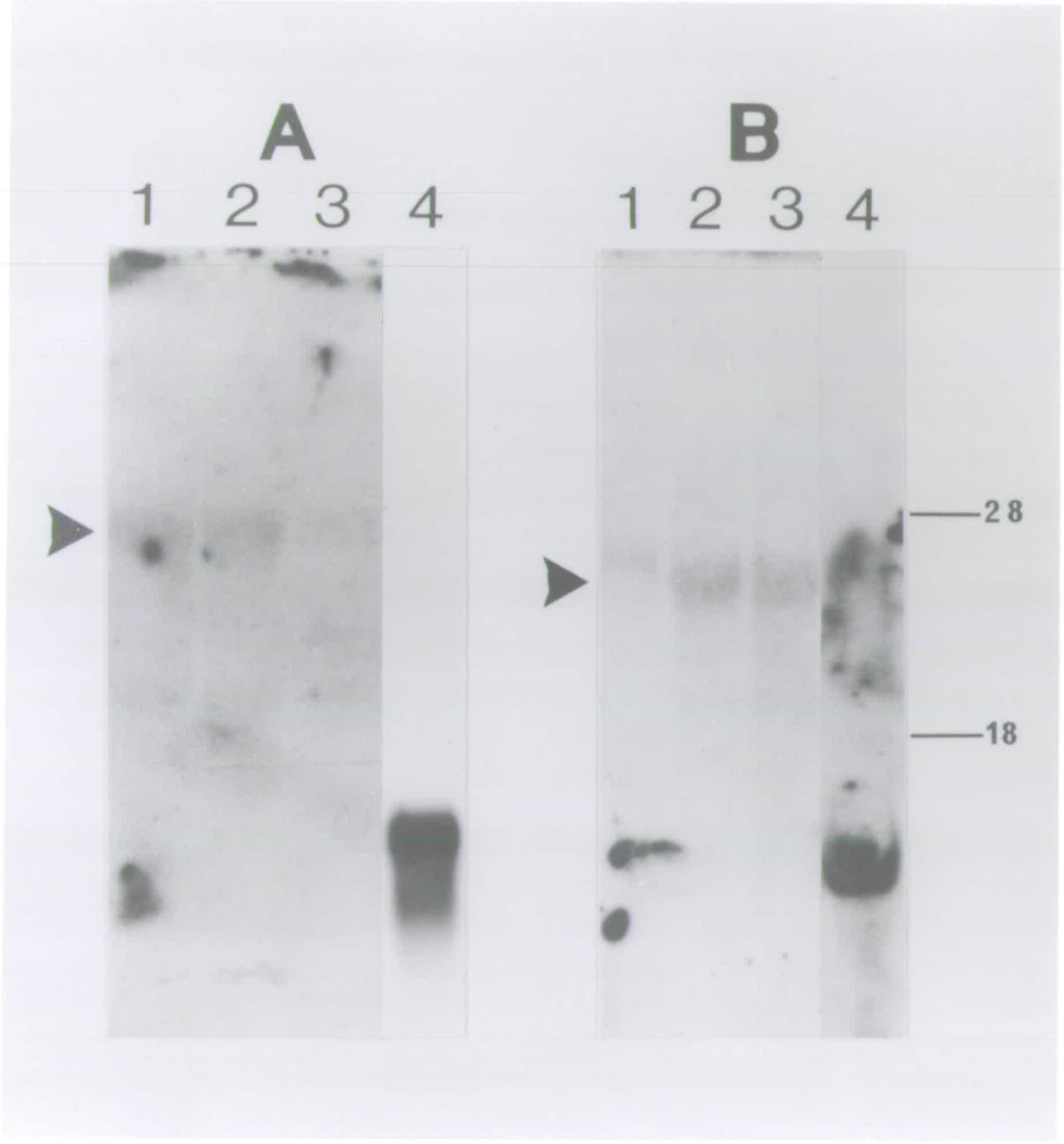

A
1 2 3 4
B
1 2 3 4
28
18

Fig.4 In situ hybridisation of ßB2-crystallin cDNA probes to squashes of freshly dissected 8 day embryo neural retina (pCß25Cr61) A, 14 day embryo neural retina (Pst 1 insert of 026) B, 8 day embryo neural retina (pBR322, the plasmid vector with no cDNA insert) C, and 14 day embryo lens (Pst. 1 insert of 026). The change in intracellular location of ßB2-crystallin RNA in positive neural retina cell clusters can be seen. Scale bar represents 10µm.

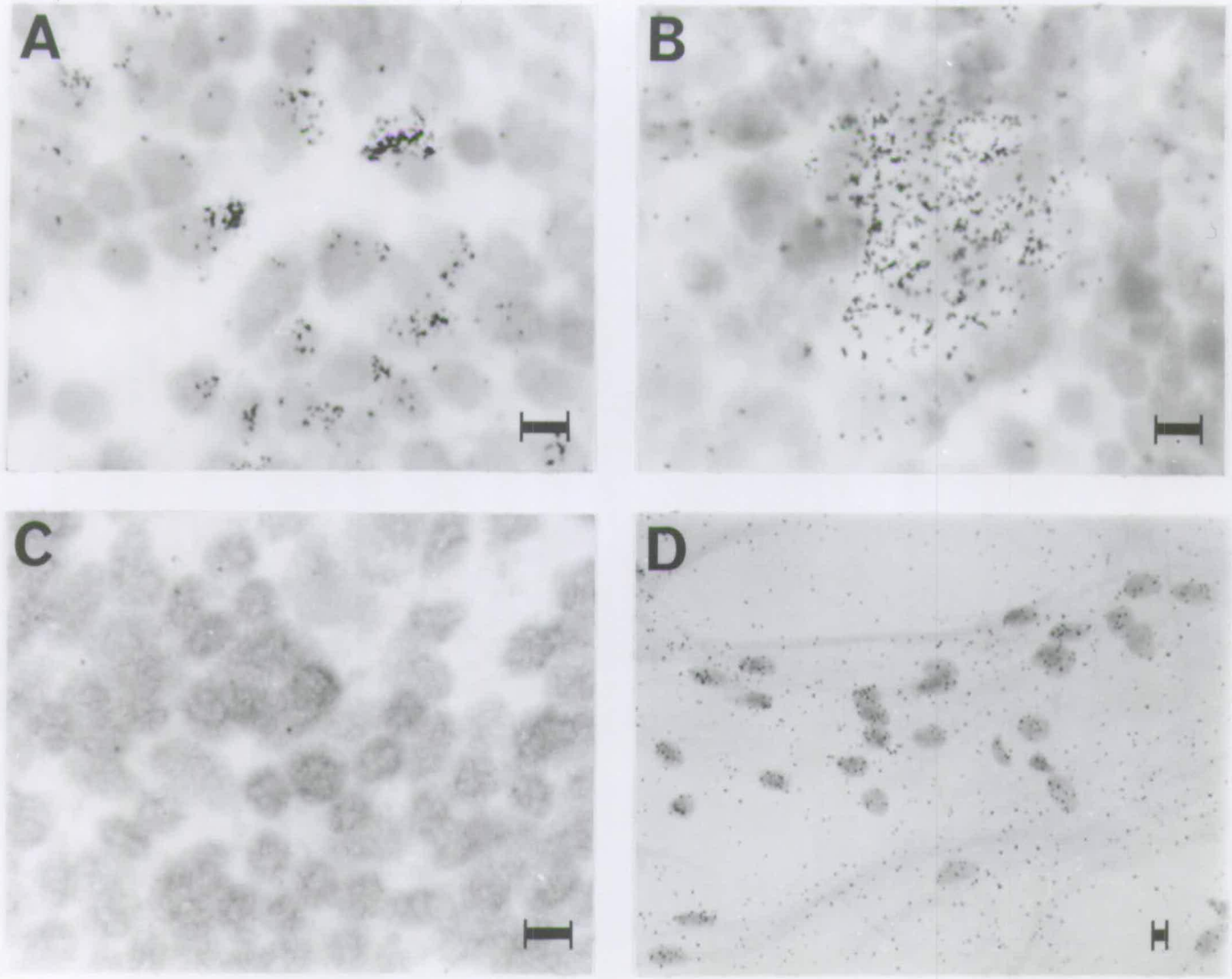

A
B
C
D

Fig.5 SDS-PAGE separations of water-soluble proteins from day-old post-hatch chick lens (1 and 4), neural retina from 8 day embryos (2), and day-old post-hatch chicks (3), visualised by Coomassie Blue staining, (A), or Western blot analysis with an anti-ß-crystallin antiserum (B). The presence of low levels of the 26kD ß-crystallin polypeptide is seen in the retina at both stages and the appearance of the 24kD ß-crystallin polypeptide is seen at the day-old post-hatch stage. Lens protein 10µg/lane, neural retina protein 100µg/lane.

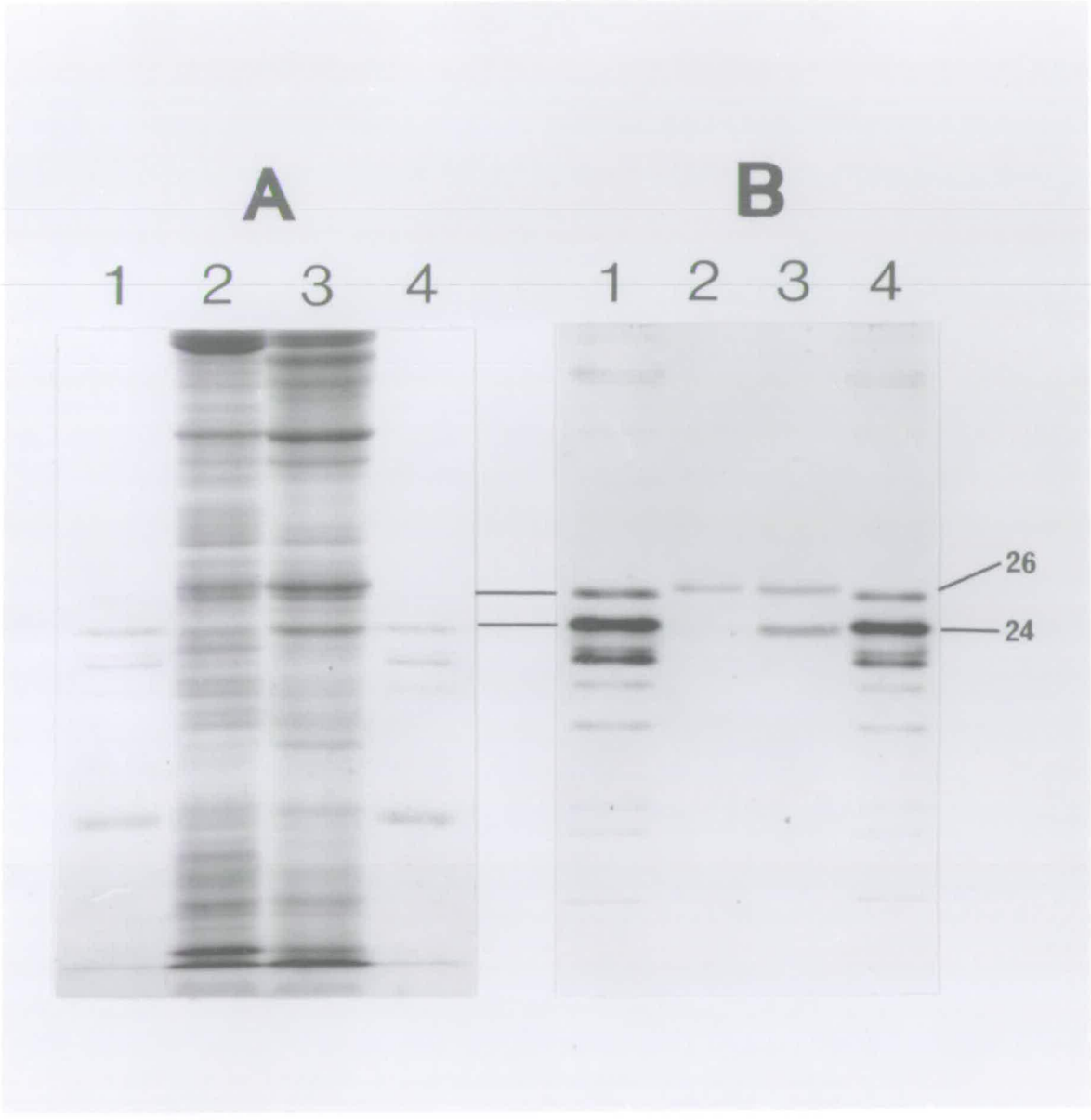

A
1 2 3 4
B
1 2 3 4
26
24

Fig.6 Dot-blot hybridisation of crystallin cDNA probes to total cellular RNA, extracted from fresh embryonic lens (A, B and C) and cultured 8 day embryo neural retina (D, E and F), showing the early appearance and rapid accumulation of relatively high steady state levels of ßB2-crystallin RNA during transdifferentiation. Initial dots: 0.25µg lens RNA and 2.5µg neural retina RNA, with doubling dilutions. Days of embryonic development for lens, and days in culture for neural retina are shown. 0 indicates freshly dissected 8 day neural retina. Probe specificities were as follows: A and D, δ-crystallin clone M56, B and E ßA3/A1-crystallin clone pCß19/26Cr42, C and F ßB2-crystallin clone pCß25Cr61. Blot A exposed for 3 days, all other blots exposed for 18 days.

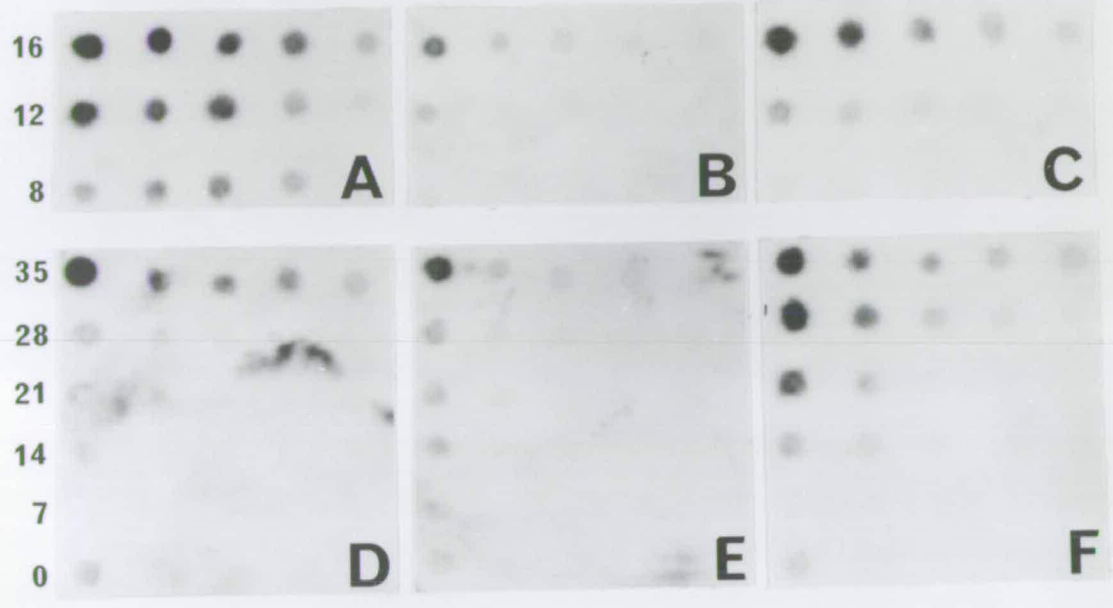

16
12
8
A
B
C
35
28
21
14
7
0
D
E
F

Fig.7 In situ hybridisation of the ßB2-crystallin probe (pCß25Cr61) to 8 day embryo neural retina cell cultures grown for 21 days (A) and 28 days (B). A control hybridisation at the 28 day stage using the plasmid vector pBR322 with no cDNA insert (C) is shown to indicate the low experimental background level of silver grains. Scale bar represents 50µm.

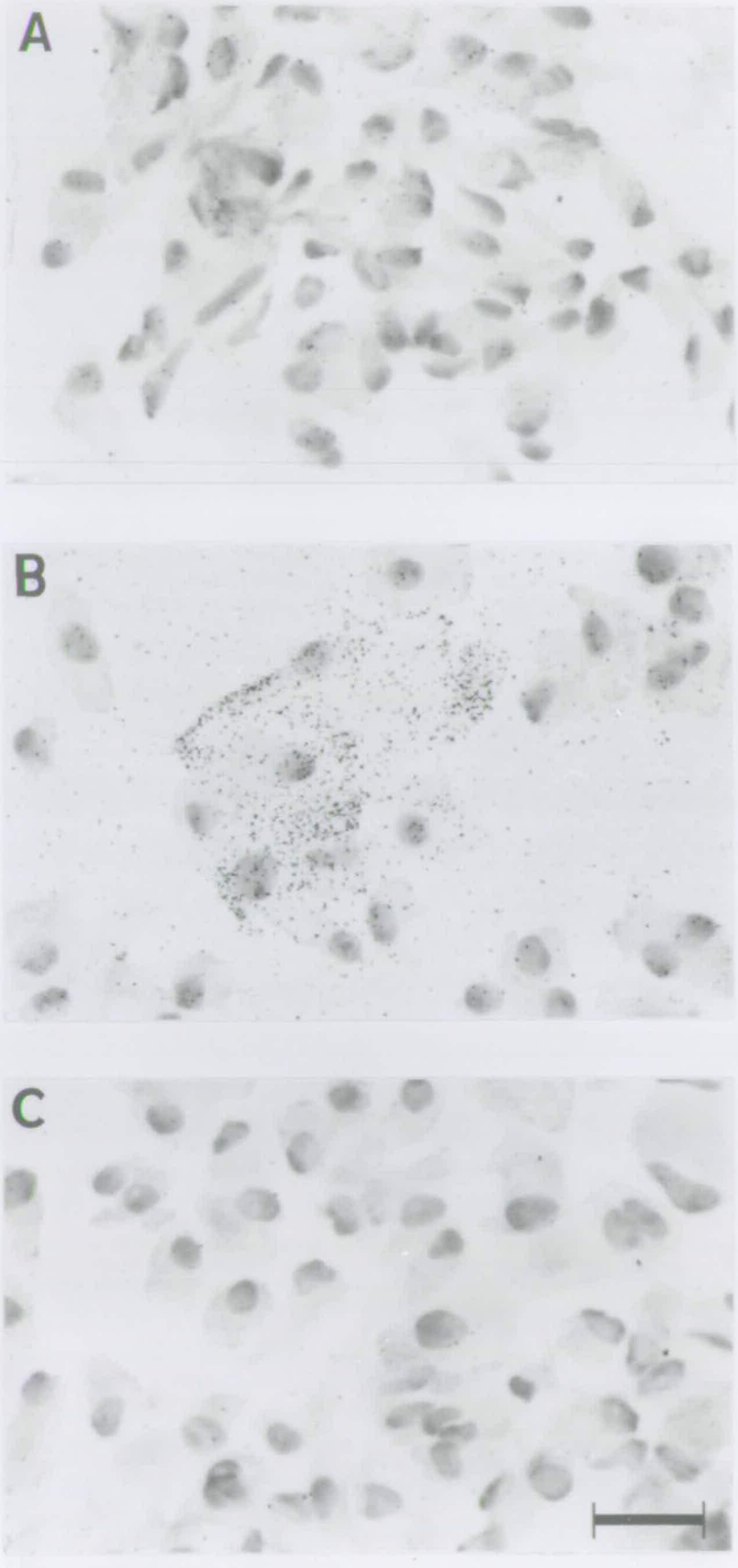

A
B
C

Fig.8 Pattern of crystallin accumulation during embryonic lens development (A) and during transdifferentiation of neural retina (B) by Western blotting of an SDS-PAGE separation, using 50µg protein per lane, and an anti-chick lens protein antiserum. Days of development are shown for lens and days in culture for neural retina cells. D/O and L indicate day-old post-hatch chick lens protein. Molecular weights are given for lens crystallins in kilodaltons.

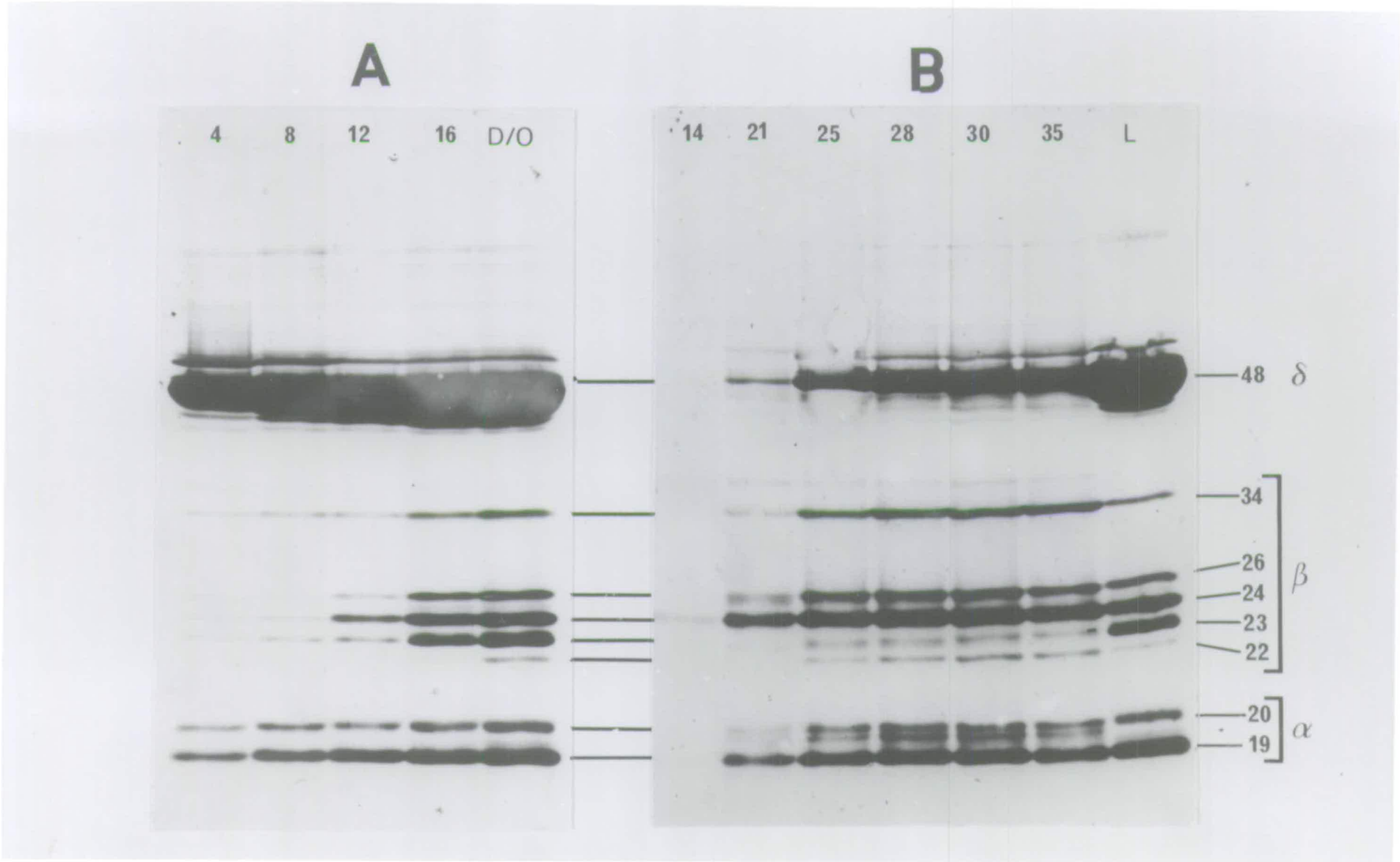
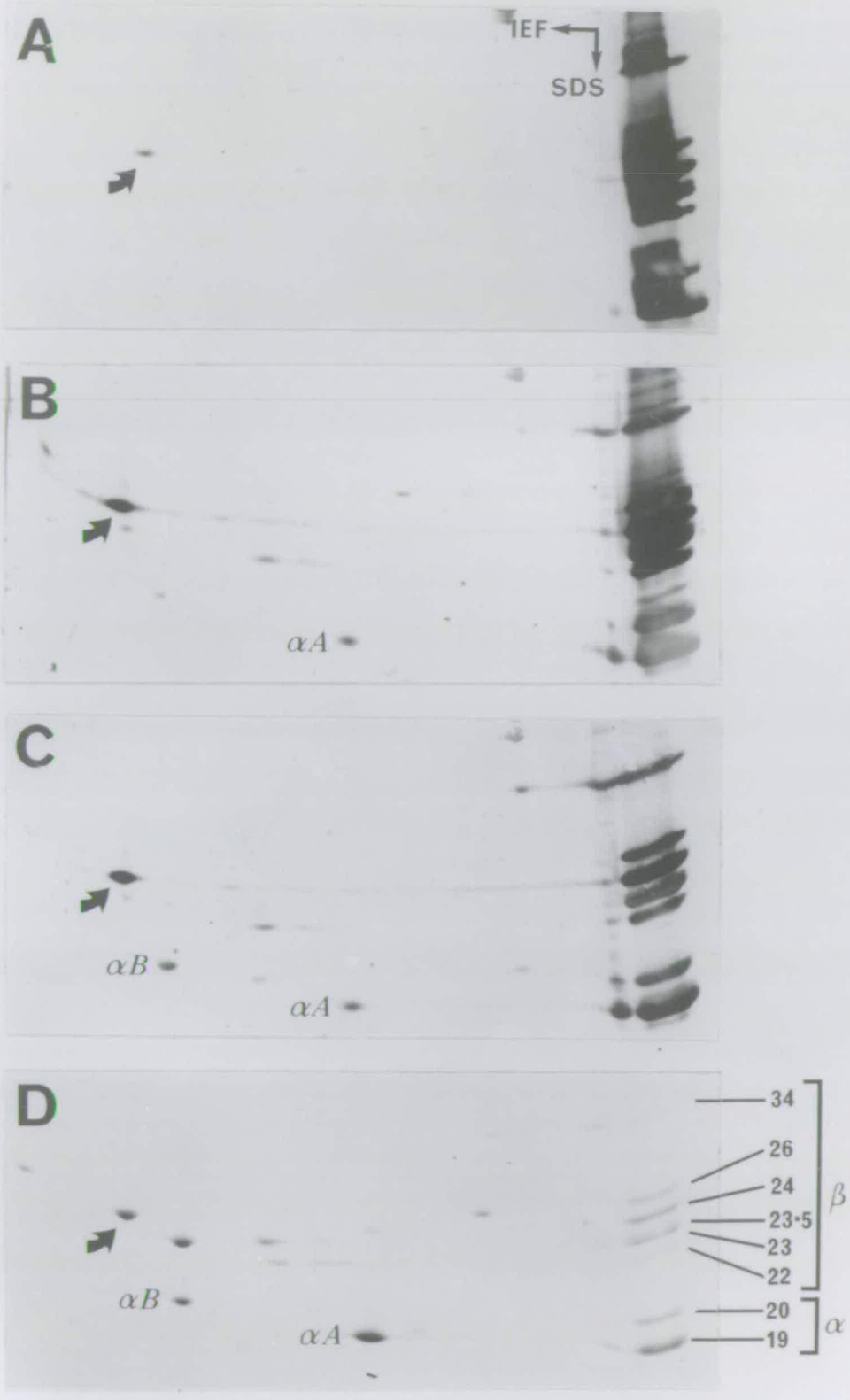

A
4
8
12
16
D/O
B
14
21
25
28
30
35
L
48
δ
34
26
24
23
22
β
20
19
α

Fig.9 Pattern of crystallin accumulation during transdifferentiation of neural retina by Western blotting of 2D (IEF/SDS)-PAGE separation of 200µg water-soluble proteins after 14(A), 21(B) and 28(C) days in culture using an anti-chick lens protein antiserum. D shows the total protein of the day-old post-hatch lens stained with Coomassie Blue. Each blot and gel has a reference lane of 50µg chick lens protein separated by SDS-PAGE only. The horizontal arrow indicates the direction of the acidic to basic pH gradient of the IEF separation. Molecular weights are given for lens crystallins. The position of the early appearing 24 kilodalton ß-crystallin is marked with an arrow.


A
IEF
SDS
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C
αB
αA
D
αB
αA
34
26
24
23·5
23
22
β
20
19
α

IDENTIFICATION AND LOCALISATION OF ßB2-CRYSTALLILN IN THE MAMMALIAN RETINA

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ABSTRACT

ß-crystallins are abundant lens proteins in mammalian, as well as sauropsidan species. We have recently reported the presence of low levels of ß-crystallins in chick non-lens tissues, both ocular and extra-ocular including, the expression of ßB2-crystallin in the retina. Here we report that extralenticular ß-crystallin expression is also found in mammals. ßB2-crystallin is expressed in mouse and cat neural and pigmented retinas and in cat iris. Although present at levels lower than those found in the lens, the appearance and accumulation of ßB2-crystallin in the neural retina coincides with the functional maturation of this tissue. The distribution of ßB2-crystallin RNA and protein in the retina is consistent with its expression in retinal glial cells and localisation in the termini of their apical and basal extensions.

INTRODUCTION

Vertebrate lens fibre cells contain high levels of limited numbers of water soluble proteins collectively termed crystallins. Two major gene families, the α- and ß/γ-crystallins, are represented in all species so far examined, whereas the δ-, ε-, τ- and ρ-crystallins have a restricted species distribution (reviewed by Wistow and Piatigorsky 1988). The protein composition of succesively formed lens fibre cells and, therefore, the overall protein composition of the lens itself changes during development. For this reason the sequence of developmental changes in the representation of the crystallins provide an attractive model for investigations of the mechanisms involved in the regulation of gene expression (reviewed Piatigorsky 1987).

Crystallins have long been regarded as lens specific, but there has also been evidence for extralenticular expression at low levels (see Clayton et al 1968, 1979, 1986a,b). δ-crystalllin, the most abundant embryonic chick lens protein, is also expressed at lower levels in several embryonic and post-hatch chick tissues (Agata et al 1983; Bower et al 1983a,b; Jeanny et al 1985; Ueda and Okada 1986; Clayton et al 1986a,b). δ-crystallin performs a structural role at high concentrations in the chick lens but it functions at low concentrations in extralenticular tissues as the urea cycle enzyme, argininosuccinate lyase (Piatigorsky 1988; de Pomerai et al 1991). These two roles appear to be differentially apportioned between the two δ-crystallin genes (Kondoh et al 1991) which exhibit reciprocal relative mRNA abundance levels when lens and non-lens tissues are compared (Thomas et al 1990; Head et al 1991). Other crystallins of restricted species distribution have also been found to be identical, or similar to metabolic enzymes (reviewed by de Jong et al 1989; Piatigorsky and Wistow 1989). This pattern of evolutionary relationships between crystallins and metabolic enzymes does not appear to hold true for the α- and ß/γ-crystallin. α-crystallin shares sequence homology with the ubiquitous small heat shock proteins (shsp) but also with protozoan and prokaryote antigens (see de Jong et al 1989). Of the two α-crystallin genes, αB-crystallin is expressed in several extralenticular non-lens tissues (Duguid et al 1988; Bhat and Nagineni 1989; Dubin et al 1989; Iwaki et al 1989, 1990) but the αA-crystallin gene is transcribed in the embryo chick retina (Agata 1985; Errington et al 1985; Clayton et al, submitted for publication) and both polypeptides appear to be present in the cat retina (Lewis et al 1988). The evidence to date suggests that extralenticular αB-

crystallin performs a structural role but that it is also inducible under conditions of cellular stress (reviewed in Clayton et al, submitted for publication). The ß/δ-crystallin gene family shares structural features with the spore coat protein, protein S, of Myxococcus xanthus (Wistow et al 1985), the encystment protein, spherulin 3a of Physarum polycephalum (Wistow 1990) and the c-myc protein (Crabbe 1985).

We have recently re-examined the earlier findings of ß-crystallin antigens in extralenticular chick tissues including retina (Clayton et al 1968; de Pomerai et al 1977; Clayton et al 1979) and reported the presence of ßB2- and ßA3/A1-crystallin RNA in the chick retina and the increasing abundance of the 24KD ß-crystalllin polyeptide (ßB2) in this same tissue during embryonic development (Clayton et al 1988; Head et al, in press). In order to determine whether extralenticular expression of ßB2-crystallin is a phenomenon restricted to the retina of the chick, we have examined its expression in ocular tissues of the mouse, (at several developmental stages) and in cat and cow (in adult animals only). We report here the presence of ßB2-crystallin in mouse and cat retina and that during the maturation of mouse retina the level of ßB2-crystallin immunoreactivity increases and its distribution widens.

MATERIALS AND METHODS

Antibodies

Chick ß-light ($ß_L$)- and δ-crystallins were purified by gel filtration as described by Thomson et al (1978) and polyvalent antibodies raised in rabbits by the Science Faculty Animal Area staff (University of Edinburgh). The anti-rat ßB2-crystallin antibody was was a generous gift from Drs. J.G.G. Schoenmakers and N.H. Lubsen (Department of Molecular Biology, University of Nijmegen, The Netherlands) and was produced as follows: the rat ßB2 cDNA clone described in Aarts et al (1989) was inserted 3' to the T7 promoter of the pET3a expression vector and used to transform competent E. coli. The ßB2-crystallin expressed in E. coli was purified by SDS-PAGE, the band elueted from the gel and used to inoculate rabbits (Lubsen, pers. comm.).

Immunoblotting

Ocular tissues were dissected from mice of selected ages (CBA or C57 strain, ICAPB Animal House, University of Edinburgh), from a cat (10 month-old short-haired domestic variety, kindly provided by K.W.

Head, Department of Veterinary Pathology, University of Edinburgh) and from cows (Gorgie Abattoir, Edinburgh). Bovine and feline eyes were transported on ice. Tissues were dissected, the integrity of the lens capsule checked, washed in phosphate buffered saline (PBS) and stored under liquid nitrogen. Water soluble protein preparation and analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was as described in Patek and Clayton (1985). Duplicate gels were either stained with Coomassie brilliant blue (CBB), according to Thomson et al (1978) or processed for Western blot analysis by a modification of the method of Towbin et al (1979). SDS-PAGE gels for Western analysis were pre-equilibrated for 30 minutes in transfer buffer, (25mM Tris, 192mM glycine, 20% methanol) and transferred to 0.45um nitrocellulose filters (Schleicher and Scheull) at 30V for >18 hours using a Transblot cell (Bio-Rad laboratories) in pre-chilled transfer buffer at 4°C. Remaining active sites were blocked by several changes of 1% Tween 20 in TBS (10mM Tris-HCl pH7.4, 0.9% NaCl), over a period of 1 hour. Incubation in the primary antibody was carried out at 1:250 dilution in antibody buffer (0.2% Tween 20 in TBS) containing bovine serum albumen (BSA) at 1 mg/ml for 24 hours at 4°C. After three 5 minute washes in antibody buffer, blots were incubated for 2 hours in a 1:250 dilution of donkey anti-rabbit IgG conjugated to horseraddish peroxidase (HRP) (kindly provided by the Scottish Antibody Production Unit, Carluke Hospital) in PBS containing 1% BSA. After washing, as described above, immune complexes were visualised using the HRP/H_2O_2/4-chloro-1-naphthol colour reaction of Hawkes et al (1982).

Native protein spot blots were prepared by vacuum filtration (transit time >5 minute, volume 100µl), of proteins diluted in 10mM sodium phosphate buffer pH7.2, 10mM 2-mercaptoethanol on to pre-equilibrated nitrocellulose filters using a Hybri-Dot manifold (Gibco-BRL). All remaining steps starting with the blocking of remaining active sites were as described for Western blots.

Immunohistochemistry

Eyes from mice of selected ages were removed, embedded and frozen sections cut, as described in Bower et al (1983a). The peroxidase anti-peroxidase (PAP) method of Sternberger (1979) was used, which employed methanol/acetic fixation, H_2O_2 inhibition of endogenous peroxidase activity, pre-immune donkey serum to reduce non-specific interactions and the H_2O_2/diaminobenzidine colour reaction. The anti-rat ßB2- and anti-δ-crystallin antibodies were used diluted 1:500 and

incubation performed for 24 hours at 4°. Immunostained sections were photographed using an Olypus Vanox microscope and Nomarski interference contrast optics. Representative slides were stained with haematoxylin/eosin/orange G (as described in Bower et al 1983a) and photographed under transmitted light. The pre-immune donkey serum, donkey anti-rabbit IgG and rabbit peroxidase anti-peroxidase complex were all kindly provided by the Scottish Antibody Production Unit (Carluke Hospital).

In Situ Hybridisation

The insert fragment of the rat ßB2-crystalin cDNA clone (described in Aarts et al 1989) was labelled by random primed synthesis using 10ng template cDNA, 1.85MBq ^{3}H-dCTP (>1.85TBq/mmol, Amersham) and the procedure described in Sambrook, Fritsch and Maniatis (1989). Labelled DNA was purified by gel filtration and ethanol precipitation after addition of the nucleic acid components of the hybridisation mix to act as carrier. In situ hybridisation was by the method of Bower et al (1983a), but a modified hybridisation buffer was used, consisting of 50% formamide, 0.6M NaCl, 10mM Tris-HCl pH7.0, 1mM EDTA, 1x Denhardt's solution, 1% dextran sulphate, 250µg/ml heat sheared herring sperm DNA, 500µg/ml yeast tRNA and ^{3}H-labelled probe present at 0.1µg/ml.

RESULTS

Coomassie Blue stained SDS-PAGE of mouse lens protein shows three abundant polypeptides of 20kD, 23kD and 27kD (Fig. 1A lane 1 and Fig. 2A lane 1). The 27kD band is recognised in Western blots both by the anti-chick ß-crystallin antibody (Fig. 1B lane 1) and by the anti-rat ßB2-crystallin antibody (Fig. 2B lane 1). The reaction of anti-rat antibody to the 27kD (ßB2-crystallin), is stronger than that of the anti-chick antibody under these conditions, but the former also gives a reaction with several bands, of both slightly higher, and slightly lower molecular weights than the 27kD ßB2-crystallin (Fig. 2B, lane 1).

The anti-chick ß-crystallin antibody gives a reaction in Western blots with a 27kD polypeptide in mouse neural and pigmented retina (Fig. 1B, lanes 2 and 3). The anti-rat ßB2-crystallin antibody similarly gives a reaction with a 27kD polypeptide in Western blots of neural retina protein, but also recognises other retinal polypeptides in the 30kD-77kD molecular weight range (arrowed in Fig. 2B, lane 2).

Both the anti-chick and anti-rat ßB2-crystallin antibodies give a strong reaction with mouse lens protein in the native spot assay, and also react with mouse neural retina, but not with mouse brain, heart or liver (Fig. 3). The anti-chick ß-crystallin antibody gives an equally strong reaction when the embryonic and juvenile neural retinas are compared, but the anti-rat ßB2-crystallin antibody gives only a weak reaction with the embryonic neural retina and a stronger one with the juvenile retina (Fig. 3).

In situ hybridisation with a rat ßB2-crystallin cDNA to sections of eyes from 7 day post-partum mice results in dense labelling of the outer cortical lens fibre cells in their central area, which contain the fibre cell nuclei. A gradient of labelling is evident, declining towards the lens nucleus (Fig.4B and D). A lower level of labelling is also seen distributed throughout the inner nuclear layer of the retina (Fig. 4A and C), although some cells are not labelled, certain cells are labelled in a perinuclear location (arrowed in Fig. 4C).

Peroxidase anti-peroxidase immunohistochemistry with the anti-rat ßB2-crystallin antibody identifies a changing pattern of immunostaining of the mouse retina during post-natal development (Fig. 5B, E and H). No discernable immunostaining is seen in this same tissue with an anti-δ-crystallin antibody (Fig. 5C, F and I). When compared to haematoxylin/eosin stained sections (Fig. 5A, D and G) the pattern of the anti-rat ßB2-crystallin localisation in the retina changes from the outer plexiform layer only, at 7 days post-partum, (arrowed in Fig. 5B), to localisation in the outer and inner plexiform layers at 21 days post-partum (arrowed in Fig. 5E), and at the 2 month-old stage, (arrowed in Fig. 5H) to the localisation spreads to include the photoreceptor outer segments and individual inner nuclear layer cells, The choroid is stained at each of these stages.

The lens protein of mouse, cat and cow exhibits a prominent band of approxiamtely 27kD in Coomassie Blue stained SDS-PAGE. This abundant lens protein, or a protein with an identical mobility, is recognised by both the anti-chick ß-crystallin and anti-rat ßB2-crystallin antibodies (Fig. 6A, B and C, lanes 1, 3 and 7). A 27kD polypeptide is also recognised by both antibodies in mouse neural retina (Fig.6B and C, lane 2), cat neural retina, retinal pigmented epithelium, and iris (Fig. 6B and C lanes 4, 5 and 6). No such bands were evident in samples of bovine neural or pigmented epithelium, or of choroid (Fig. 6B and C, lanes 8 and 9).

DISCUSSION

Lens ß-crystallin is an oligomeric protein composed of the related polypeptide products of at least six genes and is represented in all vertebrate species so far examined (reviewed by Lubsen et al 1988; Wistow and Piatigorsky 1988). Unlike some of the lens crystallins which exhibit a species-restricted distribution, the ß-crystallins are not known to be structurally or functionally related to metabolic enzymes, and it has been generally assumed that their evolution has involved specialisation for the lens environment and that their expression is lens-specific (Wistow and Piatigorsky 1988).

We have recently re-examined the earlier reports of low levels of ß-crystallin antigenicity in extralenticular tissues, including the retina, of Xenopus and chick (Clayton et al 1968; de Pomerai et al 1977; Clayton et al 1979) and detected ßB2- and ßA3/A1-crystallin expression in the embryonic and day-old post-hatch chick retina (Head et al, in press).

Here we report that the extralenticular expression of ßB2-crystallin is not a phenomenon restricted to the retina of the chick. A polypeptide showing immunoreactivity and electrophotic mobility of the major 27kD ßB2 lens crystallin of mouse and cat in Western blots, is also detectable at low levels in the neural and pigmented retina of these two species. There are two related questions implicit in the data: first, whether the 27kD polypetide detected by Western blots of mouse retina is a ß-crystallin and second, the nature of the high molecular weight components detected in retina by both the anti-chick and the anti-rat ß-crystallin antibodies.

If the 27kD component of retina is not a crystallin but comigrates with the lens 27kD ßB2-crystallin the detection of both this component and of the high molecular weight components might be explained by one of two possible mechanisms. The first would be a reaction to an contaminant or contaminants found both in the chick $ß_L$-crystallin chromatographic fractions and in the E. coli in which the rat ßB2-crystallin was expressed. This seems unlikely. Alternatively, the retina reactions are due to a ß-crystallin epitope also found in certain non-crystallin proteins. We have already discussed the possibility that the chick ß-crystallin antibody is reactive to such epitopes and have suggested that examination of the full data from Western blots of chick lens, retina and other tissues using this antiserum makes this implausible as an explanation since it would

require that the anti-chick ßL-crystallin antiserum recognises four distinct non-crystallins each indistinguishable by molecular size and antigenicity from one of the four major ß-crystallin polypeptides (Head et al, in press) . We cannot eliminate this possibility with respect to the anti-rat-ßB2 crystallin antibody, but note that the two antibodies , the anti-rat antibody and the anti-chick antibody raised by quite different procedures, both detect a mouse retina polypeptide with the mobility of lens ßB2-crystallin.

A third explanation of the cross-reactions of the anti-crystallin antibodies with retina is that the main 27kD retina component is actually ßB2-crystallin, and that the high molecular weight polypeptides may represent a proportion of the ß-crystallin, or antigenic fragments of ß-crystallin, which are bound to other cellular components. It may be relevant that the association of ß-crystallin with cytoarchitectural components of lens has been demonstrated (Bloemendal et al 1984).

The suggestion that the mouse retina expresses ßB2-crystallin is strengthened by the detection of ßB2-crystallin RNA in Northern transfers of mouse retina RNA (N.H. Lubsen, personal communication). The ßB2-crystallin RNA was detectable only in 2 month-old mouse retina and not at the embryonic, or newborn stages and an increase in protein abundance is also seen in similar comparisons made here by native spot blot (Fig. 3B) and PAP immunohistochemistry (Fig,. 5) using the anti-ßB2-crystallin antibody, and is consistent with our earlier finding in the chick retina (Head et al, in press). In chick, polypeptides which co-migrate with two lens ß-crystallins are found in Western blots of retina, and ß-crystallin RNA is detectable in retina by Northern and in situ hybridisation (Clayton et al 1988, Head et al, in press).

An increasing level of expression, both at the RNA and protein levels during development, suggests a functional cellular component. The in situ hybridisation and immunohistochemical data are consistent with the expression of ßB2-crystallin in retinal glia cells, localised mainly in their apical and basal extensions into the inner and outer plexiform layers.

Speculation as to the function of extralenticular ß-crystallin may be made on the basis of the physical properties of lens ß-crystallin but also on the basis of the functions of other polypeptides with which they share common evolutionary origin. Lens ß-crystallins self-aggregate but can also form associations with cytoarchitectural elements (Bloemendal et al 1984) and ßB2-crystallin, has been shown to

be heat stable like α-crystallin but unlike the other ß-crystallin polypeptides (Maiti et al 1988). Thermal stability and the Ca^{2+} binding capacity of ß-crystallins (Sharma et al 1989) are both useful properties under conditions of cellular stress. It is suggested that this represents a common fearture of those proteins recruited for high level expression in the lens (de Jong et al 1989) and it may be relevant that ß-crystallins share structural features with the Ca^{2+} binding spore coat protein S of Myxococcus xanthus (Wistow et al 1985) and spherulin 3a, an encystment protein of Physarum polycephalum (Wistow 1990).

The extralenticular expression of different ß-crystallins in the chick is dependent on the stage of development and the tissues examined (Head et al 1991, in press). Roth et al (1991) failed to detect ßB1-crystallin RNA in the embryonic chick liver, heart, brain and skeletal muscle at 13 days of development, although embryonic chick neural retina cells accumulate ßB1-crystallin during the in vitro process of transdifferentiation we have no evidence of its expression outside the lens in vivo (Head et al, in press). However, it may be premature to conclude that it is lens-specific until further tissues are examined, and later stages of development.

It would be of considerable interest to know whether the ßA3/A1-crystallin gene is expressed in the mammalian retina, as it is in the chick retina (Head et al 1991, in press), since ßA3-crystallin is implicated in ß-crystallin multimerisation (Slingsby and Bateman 1990).

We are currently assessing the role of extralenticular ß-crystallins using antisense oligonucleotides to selectively inhibit their synthesis in retina cells in vitro.

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Fig.1 Analysis of water soluble proteins, by SDS-PAGE from 2 month old mouse (CBA stain) lens (1), neural retina (2) and retinal pigmented epithelium (3). A shows a gel stained with Coomassie brilliant blue and B shows the corresponding immunoblot (Western), using the anti-chick ß-crystallin antibody. 10µg/lane for lens protein, 100µg/lane for retina protein. Molecular weights are indicated in kilodaltons.

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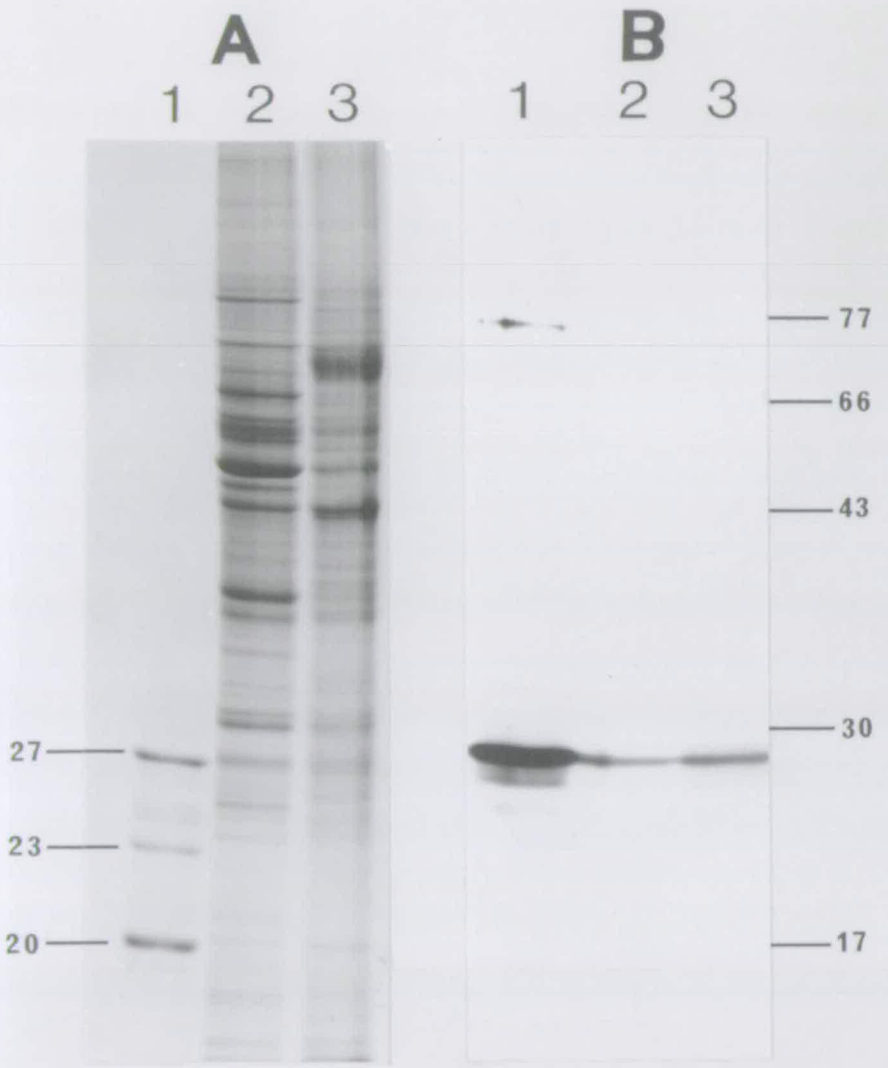
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Fig.2 Analysis of water-soluble protein by SDS-PAGE from lens, 10µg/lane (1) and neural retina, 100µg/lane (2) from 2 month old CBA strain mice. A shows a Coomassie blue stained gel and B shows the corresponding immunoblot (Western) using the anti-rat ßB2-crystallin antibody. Arrows indicate the positions of positively stained bands, at the position of lens ßB2-crystallin (large arrow) and also reactions with retinal proteins of higher molecular weight (small arrows).

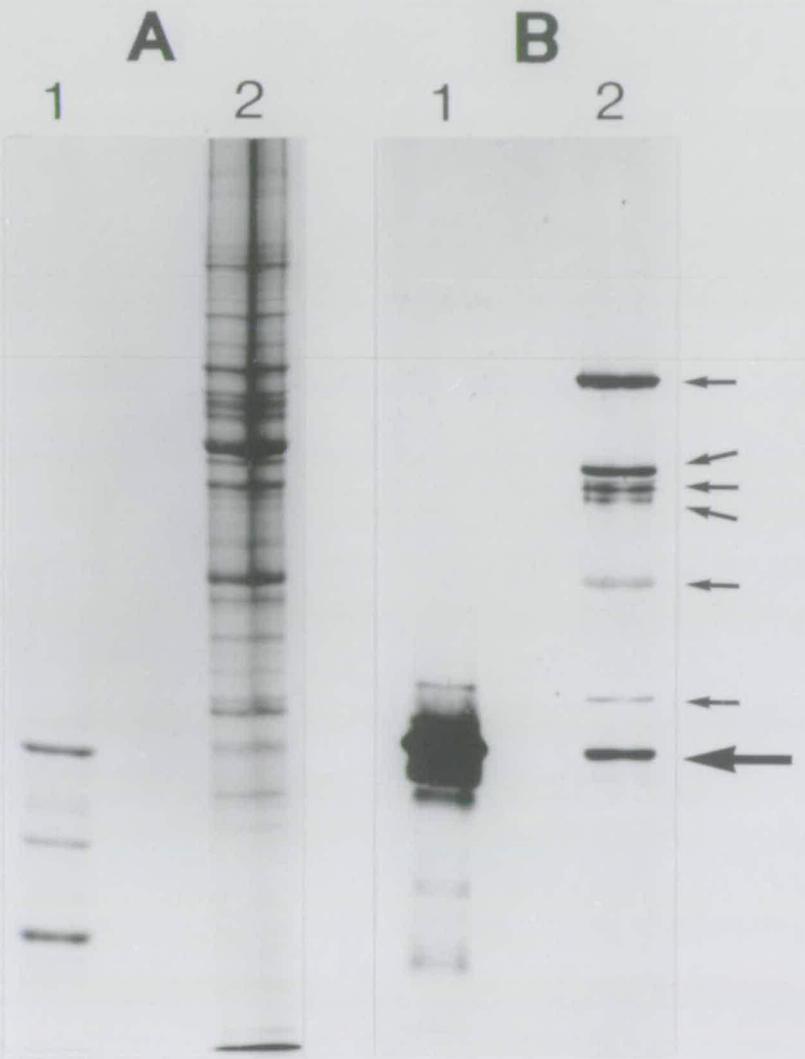
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Fig.3 Immuno-dot blot assay using the anti-chick ß-crystallin antibody (A) or the anti-rat ßB2-crystallin antibody (B) and 10µg native protein from 2 month old C57 strain mouse lens (1), neural retina (3), brain (4), heart (5), liver (6), and 18 day mouse embryo, C57 strain, neural retina (2).

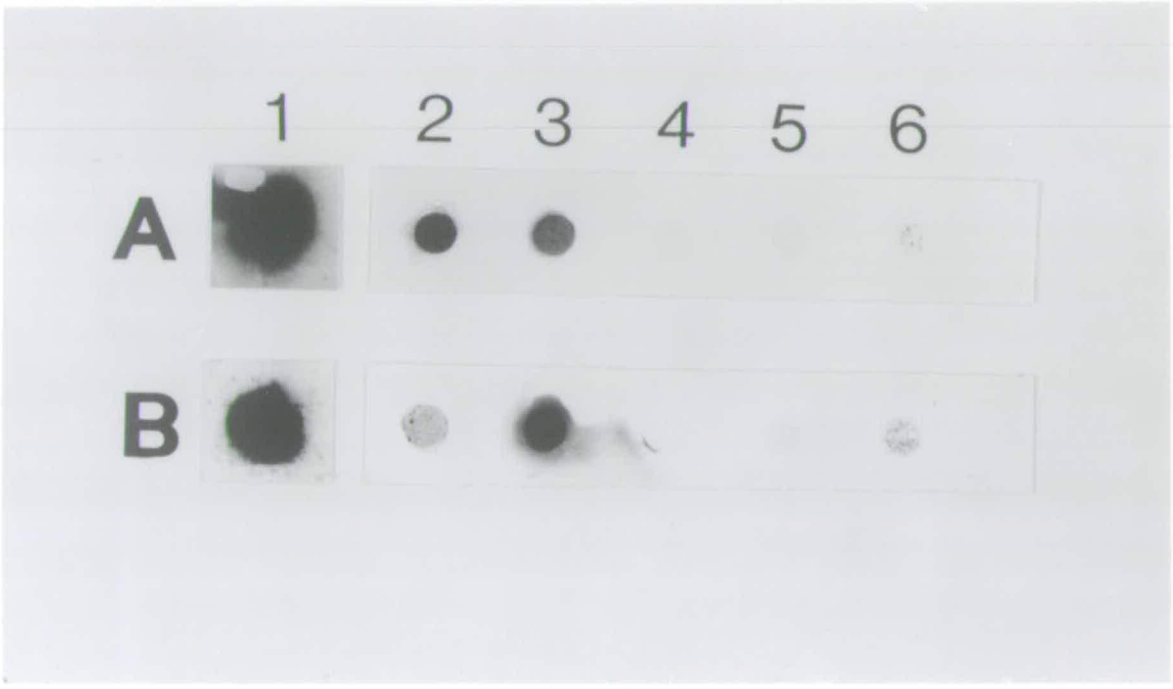
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Fig.4 Localisation of ßB2-crystallin RNA by *in situ* hybridisation in sections 7 day old mouse retina (A, C) and lens (B, D). The pigmented epithelium (PE), outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer (GCL) are indicated in A. An INL cell with perinuclear label is arrowed in C. Scale bars represent 50µm.

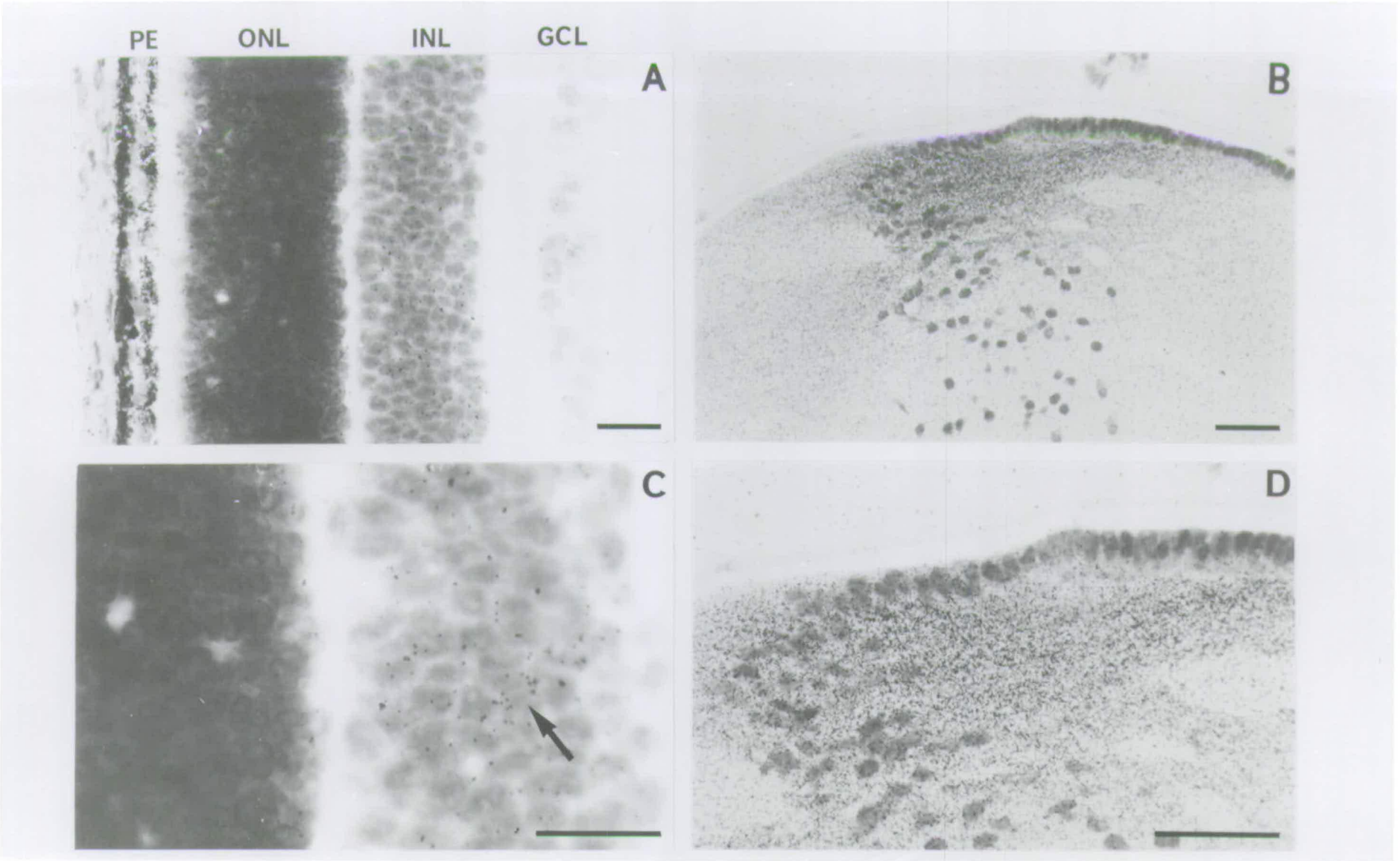
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Fig.5 Immunohistochemical (PAP) localisation of ßB2-crystallin in retina sections from mice of 7 days (A, B, C), 21 days (D, E, F) and 2 months of age (G, H, I). A, D and G were stained with haemaloxylin/eosin/orange G. B, E and H were immunostained with the anti-ßB2-crystallin antibody and C, F and I immunostained with the anti-δ-crystallin antibody. The layers of the retina are marked Ch (Choroid), PE (pigmented epithelium), PhS (photoreceptor segments), ONL (outer nuclear layer), OPL (outer plexiform layer), INL (inner nuclear layer), IPL (inner plexiform layer) and GCL (ganglion cell layer). Arrows indicate the positive immunostained layers (full arrows) and individual cells (arrow heads only). Scale bar represents 100µm.

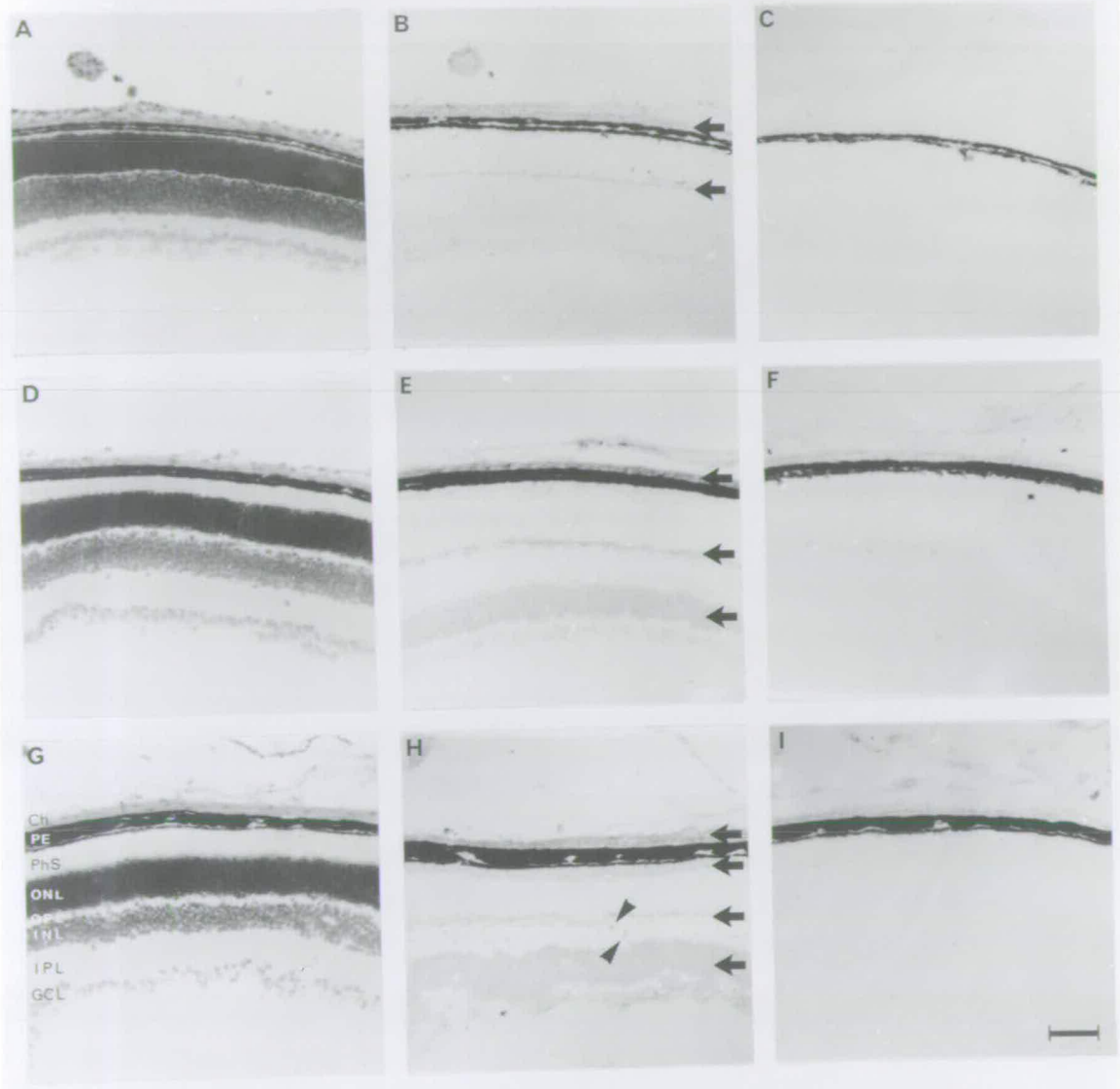
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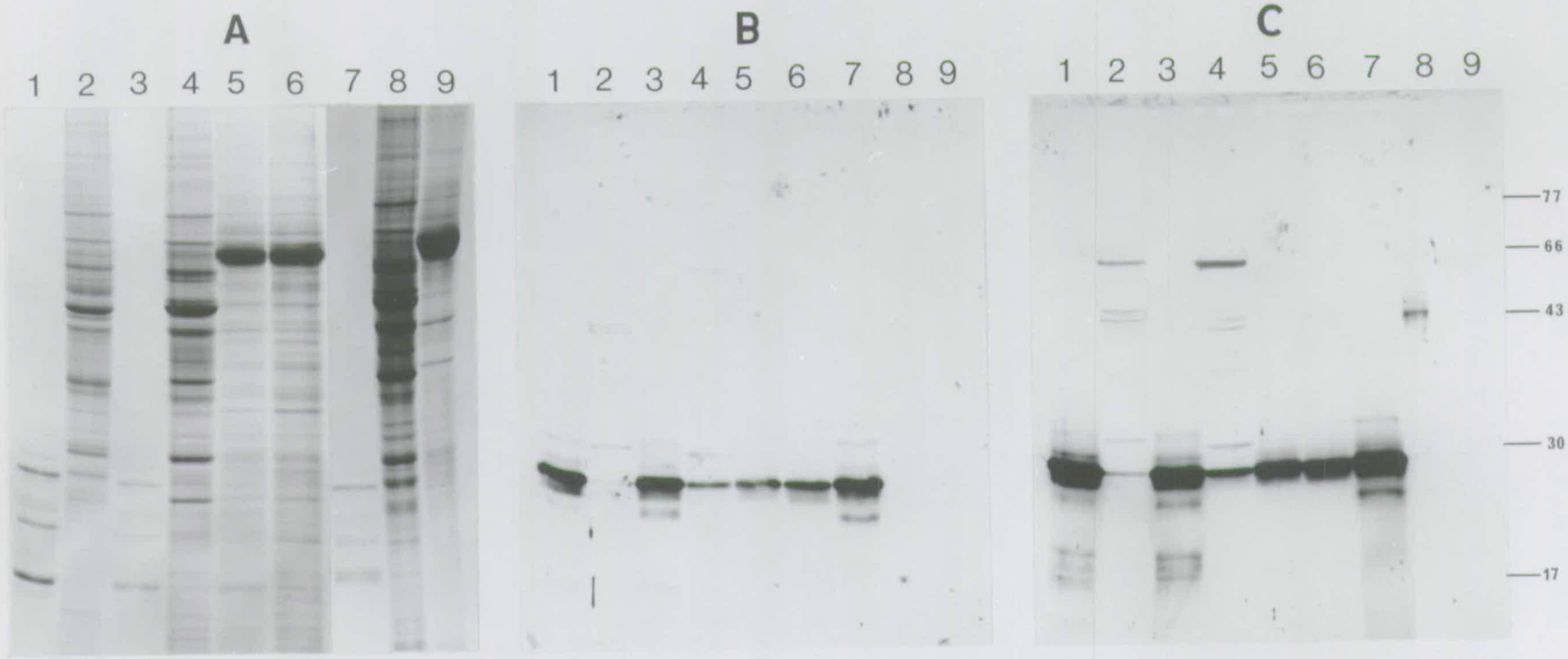
Fig.6 Analysis of water soluble proteins by SDS-PAGE from mouse (2 month old C57 strain) lens (1), mouse neural retina (2), cat lens (3), cat neural retina (4), cat retinal pigmented epithelium (5), cat iris (6), bovine lens (7), bovine neural retina (8), and bovin retinal pigmented epithelium and choroid (9). (A) shows a gel stained with Coomassie brilliant blue, B and C show corresponding immunoblots (Western), using the anti-chick ß-crystallin antibody (B) and the anti-rat ßB2-crystallin antibody (C). 10µg/lane for lens samples, 100µg/lane for non-lens samples. Molecular weights are indicated in kilodaltons.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αA-CRYSTALLIN IN EMBRYONIC CHICK LENS AND RETINA : ANTISENSE OLIGONUCLEOTIDE MEDIATED INHIBITION OF SYNTHESIS AND mRNA LOCALIZATION.

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Short title: Inhibition of α-crystallin expression

Key words: αA-crystallin; lens; retina; antisense oligonucleotides; gene expression.

With 5 figures and 59 references.

Abstract

α-Crystallin is a vertebrate lens protein composed of the two related αA- and αB-crystallin polypeptide subunits. In the chick αA-crystallin mRNA is present throughout embryonic lens development but it is also detectable at lower levels in the developing retina. We find αA-crystallin RNA localizes to a subset of putative glial cells in the posterior retina. In order to investigate the role of lenticular and extra-lenticular α-crystallin we have blocked its expression in very early embryonic lens and retina *in vitro* using antisense oligonucleotides. The resultant pattern of protein synthesis seen in both lens and retina is disturbed suggesting that α-crystallin is required for the correct differentiation of cells in which it is expressed.

Abbreviations: ASL, argininosuccinate lyase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; IEF, isoelectric focusing; dCTP, deoxycytidine triphosphate; kD, kilodaltons; pI, isoelectric focusing point.

Introduction

Crystallins are superabundant in the lens but several crystallins or their RNAs have been detected at lower levels in certain non-lens tissues, both ocular and extra-ocular (Clayton et al 1968; Clayton et al 1979; Agata et al 1983; Bower et al 1983a,b; Agata 1985; Errington et al 1985; Bhat and Nagineni 1989; Dubin et al 1989; Iwaki et al 1989; Head et al 1991). Localization techniques have shown that these tissues are heterogeneous with respect to crystallin expression (Jeanny et al 1985; Takagi 1986; Ueda and Okada 1987; Clayton et al 1988) so that the low levels in the total tissue represent subpopulations of cells which express appreciable levels of crystallin RNAs or proteins, and which, in some cases, have been found in restricted locations (Bower et al 1983a; Linser and Irvin 1987) or in specific cell types (Lewis et al 1988; Iwaki et al 1990a). These data imply non-random expression.

Some of the species-restricted crystallins, such as δ-, ε- or τ-crystallins are enzymes (reviewed by Piatigorsky and Wistow 1989; de Jong et al 1989) which function as such at low concentrations, but as lens structural proteins at high concentrations. Although chick δ1-crystallin has lost the argininosuccinate lyase (ASL) activity associated with the expression of the δ2-crystallin/ASL gene both δ1-

and δ2-crystallin RNAs are found in several ocular and extraocular non-lens tissues in the embryonic and post-hatch chick (Thomas et al 1990; Head, et al 1991).

Members of the α- and ß-crystallin families, structural proteins common to all vertebrate lenses, have been found in extra-lenticular tissues in *Xenopus* (Clayton et al 1968), chick (Clayton et al 1968; Moscona et al 1985; Head et al 1991,in press), rodents (Bhat and Nagineni 1989; Dubin et al 1989; Head and Clayton, unpublished observation), and cat (Lewis et al 1988; Head and Clayton, unpublished observation). In rodents, the expression of the αA-crystallin is reported to be lens specific, but both αB-crystallin RNA and protein are detectable in several non-lens tissues (Dubin et al 1989; Bhat and Nagineni 1989). However both αA- and αB-crystallin subunits appear to be present in adult cat retina (Lewis et al 1988) and the αA-crystallin RNA is transcribed in the embryonic chick retina (Agata 1985; Errington et al 1985). This widespread species distribution of extralenticular crystallins implies evolutionary persistence and therefore a possible non-lens function for these proteins. Their known sequence points to a prevertebrate history (Ingolia and Craig 1982; Wistow 1990) and it is likely, therefore, that if they have a current extra-lenticular function, it antedates the evolution of the vertebrate lens (Clayton et al 1986; Clayton 1990) and, presumably, also antedates the evolution of control mechanisms permitting the quantitatively distinct tissue specific levels of expression.

We have begun an examination of the expression and cellular roles of extra-lenticular crystallins, and report here on preliminary studies of the effects of blocking α-crystallin expression in the early embryonic chick lens and retina. In these experiments short oligonucleotides designed to hybridize specifically to the translation initiation region of αA-crystallin RNA were added to explant cultures of developing lens and retina with the intention of preventing translation either by direct obstruction of the translational machinery or by RNase H mediated mRNA degradation (reviewed by van der Krol et al 1988). Since oligonucleotides present at concentrations sufficient to exert a specific regulatory effect on gene expression may be non-specifically cytotoxic to cells in culture, we have conducted control experiments using a sequence designed to hybridize to the equivalent region of the δ-crystallin mRNAs which are abundant in embryonic mammalian lenses but are absent from avian species.

Both αA- and δ-crystallin RNAs have been detected by Northern blotting in early chick retina before the differentiation of the characteristic adult retinal structure (Agata et al 1983; Bower et al 1983a; Agata 1985; Errington et al 1985). δ-crystallin becomes restricted to a subpopulation of retina cells (Jeanny et al 1985) but the location in the differentiated retina of these two crystallins is unknown. Moscona et al (1985) used an multivalent antiserum to total α-crystallin, which detected both αA- and αB-crystallins and five or six other lens components, and which labelled all the Muller glia cells. We have therefore examined the 14-day retina by *in situ* hybridization with cloned αA- and δ-crystallin probes in order to determine the answer to this question.

Results

αA-crystallin targeted antisense oligonucleotides at high concentration have specific effects on protein synthesis in both lens and retina, (Fig 2D,L) since no effects can be seen in the case of γD-crystallin antisense sequences (Fig. 2H,P). In the developing lens the synthesis of all the major components including αA, αB and δ-crystallin are severely reduced and the synthesis of two polypeptides of approximately 66kD and 25kD increases (Fig. 2D). Exposure of embryo eye cup to the αA-crystallin antisense sequence, at the same concentration, leaves the pattern of protein synthesis relatively unaffected, apart from the appearance of a polypeptide with a molecular weight of approximately 25kD (Fig. 2L). In both cases exposure to the αA-crystallin complementary sequence at the lower concentrations of 50μg/ml(Fig. 2C,G) or 5μg/ml(Fig. 2B,F) rather than 500μg/ml(Fig. 2K,L) produced protein profiles virtually identical to those of the controls (Fig. 2A,E).

The fate of the oligonucleotide in the explant assay was assessed by autoradiographic detection of ^{3}H-labelled oligonucleotides to eliminate the possibility of sequence or cell type specific differential uptake. Heavy labeling is evident in all lens cells exposed to either the αA- or the γD-antisense oligonucleotide and in all eye cup cells exposed to the γD-crystallin antisense oligonucleotide (Fig. 3A,B,D). Labeling is most pronounced in the peripheral cells of the eye cup exposed to the αA-crystallin oligonucleotide (Fig. 3C). In all cases the label is largely nuclear in location (Fig. 3E, F).

In situ hybridization of the αA-crystallin genomic sequence and

the δ-crystallin cDNA to sections of 14-day embryo eyes confirms that the abundance of both RNAs is far higher in lens than in retina, but that both RNAs are transcribed in the region of glial cell bodies in the inner nuclear layer of the retina (Fig. 4). However the αA-crystallin transcripts were found in the posterior region of the retina (Fig. 4B), while the δ-crystallin transcripts were found in the region of maximum curvature, at the rim of the eye socket (Fig. 4F). Hybridization to other regions of sectioned retina with both the αA- and δ-crystallin probes gave negative results (Fig. 4C,E).

When the proteins undergoing synthesis in the 21 day embryo retina are labelled with ^{14}C amino acids *in vitro* and analyzed by 2D-PAGE with a ten fold excess of day-old post-hatch chick lens protein as cold carrier no radioactive incorporation can be seen in the positions of either the αA- or the αB-crystallin polypeptides. Incorporation is detectable in a minor polypeptide of the same molecular weight as αA-crystallin but a slightly more acidic pI (arrowed in Fig. 5A,B). Separate analysis of the lens sample by 2D-PAGE and a highly sensitive silver stain shows the major αA- and αB-crystallin polypeptides flanked by minor spots of more basic and more acidic pIs (arrowed Fig. 5C). A series of spots occupying the same positions as the αA-crystallin and flanking spots are just visible in the sample of fresh 21 day embryo retina (bracketed in Fig. 5D) but no such spots are seen in the corresponding positions of αB-crystallin and flanking spots.

Discussion

Numerous genetic conditions affect both lens and retina either as a consequence of developmental interdependence, or by their expression of shared gene products (reviewed in Clayton 1985). Antibodies to α-crystallin but not ß- or δ-crystallin have adverse effects on lens, retina and brain development in the very early chick embryo (Langman and Maisel 1962). Targeted lens ablation studies in transgenic mice using αA-crystallin promoter driven constructs result in retina as well as lens abnormalities, which the authors interpreted as secondary effects of lens ablation (Landel et al 1988; Kaur et al 1989). We have targeted the αA-crystallin transcripts in separated lens and retina rudiments, cultured at a stage when αA-crystallin RNA is expressed in both tissues (Agata 1985; Errington et al 1985).

In the assay we describe here we have demonstrated the access of oligonucleotides to virtually all cells of eye rudiments in organ culture (Fig. 3). We suggest that the nuclear localization of label

(Fig. 3) probably results from cytoplasmic degradation of labelled oligonucleotides, their entrance into the nucleotide pool and subsequent incorporation into nuclear DNA. The short intracellular half-life of oligomers has been noted by other workers (Thinakaran and Bag 1991). Since the αA-crystallin complementary sequence affects protein synthesis in both tissues while the ϑ-crystallin complimentary sequence has no effect (Fig. 2), we conclude first, that the blocking is specific, second, that retina as well as lens is affected, and third, that α-crystallin is required for the correct differentiation of cells in which it is expressed.

Although the αA-crystallin antisense oligonucleotide hybridizes only to the αA-crystallin RNA in Northern transfers, we cannot exclude the possibility that under intracellular conditions both αA- and αB-crystallin transcripts become targets. The potential for cross-hybridisation to the homologous region of the αB-crystallin RNA remains unknown, as this chicken sequence has not yet been published. The disparity in the level of expression of αA-crystallin RNA in lens and retina and the observation that the αA-crystallin RNA in 3.5 day embryo retina appears in the form of high molecular weight RNA (Agata 1985) suggests that if any αA-crystallin protein is present in retina it must be at trace levels. Transcription and translation of crystallin genes in non-lens tissues is characterised by cellular heterogeneity (reviewed in Clayton 1990) so that detection of trace levels of a particular crystallin overall may yet be compatible with considerable levels in a sub-population of cells. Immature Muller glia cells of the chick retina display heterogeneity with respect to the expression of αA-crystallin (this report), ß-crystallin (Clayton et al 1988; Head et al 1991, in press) and δ-crystallin (Bower et al 1983a; Jeanny et al 1985; and this report). This heterogeneity within an apparently homogeneous cell population may account for the differential susceptibilities of retina cells seen when treated *in vitro* with anticonvulsant drugs or metallic salts (Sedowofia et al; Sedowofia and Clayton, in preparation).

The finding reported here that neither αA-crystallin nor δ-crystallin are randomly distributed in the differentiated chick retina but are each expressed in the position of glial cell bodies in a specific region implies a function for retinal crystallins. The restricted location of ßB2-crystallin in chick retina carries a similar implication (Head et al 1991, in press). Although Moscona et al (1985) found α-crystallin reactivity in all glial cells in chick

retina, the αA-crystallin probe detects cells in a restricted area of the retina, suggesting that other glial cells may express αB-crystallin, or even, from the characteristics of the antibody they used, other lens components. The proportion of glial cells which we find expressing αA-crystallin is low, and these are in the posterior retina. The proportion of cells expressing δ-crystallin is slightly higher and they are at the region of maximum retinal curvature, at the rim of the eye socket. Linser and Irvin (1987) reported a small number of δ-crystallin expressing glial cells around the chick embryo optic nerve insertion but we have not yet been able to detect elevated levels of δ-crystallin RNA in retina or optic nerve cells in this region.

The retina from 21 day embryo chicks synthesizes a polypeptide which co-migrates in 2D-PAGE with a minor lens polypeptide of the same apparent molecular weight as αA-crystallin but a slightly more acidic pI. This acidic 19kD polypeptide is a consistent but minor component seen in our gels of chick lens and is also seen in chicken lens samples analysed in other laboratories (for example see Voorter et al 1989). Although this polypeptide is represented in the α-crystallin fraction as prepared by gel filtration chromatography (see Errington et al 1985) we do not yet know whether it, or any of the other polypeptides which can be seen to flank the αA- and αB-crystallins in 2D-gels of lens protein, represent post-translational modifications of α-crystallin polypeptides.

We have demonstrated that blocking the expression of α-crystallin in the lens profoundly affects protein synthesis, and, in the retina, produces a detectable change in protein synthesis. The molecular weights of the two polypeptides with increased synthesis in the lens are consistent with the known molecular weights of two chicken stress proteins as reported by Carr and de Pomerai (1985). One of these two also appears in the retina. The virtual cessation of crystallin protein synthesis in the lens treated with the antisense αA-crystallin oligonucleotide suggests that α-crystallin is required for correct lens differentiation at this developmental stage and for the coordinated regulation of crystallin gene expression seen during lens differentiation. Unlike δ-crystallin, at least some α-crystallin may be essential for lens fiber morphology and transparency (see Clayton et al 1986). The less pronounced effects seen in the developing retina may be because there are fewer cells expressing α-crystallin, or because of the nature of their requirement for this protein.

During the development of the blind mole rat *Spalax ehrenbergi* a rudimentary lens forms and then degenerates, but the retina retains both its characteristic morphology and biochemistry, suggesting that it performs an additional function other than visual transduction (Sanyal et al 1990). The αA-crystallin gene sequence of this rodent has diverged considerably from the highly conserved sequences seen in other vertebrates, but less than expected for a gene free of all selective constraints, leading to the suggestion that αA-crystallin has an additional and possibly extra-lenticular role in the blind mole rat and other species (Hendriks et al 1987; de Jong et al 1988). The identification of a subset of chick retina cells containing αA-crystallin RNA reported here is entirely consistent with this hypothesis.

αB-crystallin RNA and protein have a wide tissue distribution, including retina, in all the mammalian species so far examined (Lewis et al 1988; Duguid et al 1988; Bhat and Nagineni 1989; Dubin et al 1989; Iwaki et al 1989; Iwaki et al 1990a,b). Evidence for the extra-lenticular expression of αA-crystallin is, so far, limited to the detection of αA-crystallin RNA in the embryonic chick retina (Agata 1985; Errington et al 1985; and this report) and possibly the retina of the cat (Lewis et al 1988). This would suggest that the evolution of the two α-crystallin genes has not only involved the acquisition of regulatory mechanisms facilitating high levels of expression of both polypeptides in the lenses of many, or probably all, vertebrate species but also mechanisms facilitating lower level extra-lenticular expression which are sub-unit-, cell type- and species-specific.

Studies of crystallin proteins which exhibit both lenticular and extralenticular expression and comparisons between crystallins and related non-lens proteins both suggest that the recruitment of proteins as lens crystallins may initially result from intrinsic properties useful in relation to cellular and metabolic stress as well as an ability to pack closely at high concentration (de Jong et al 1989). α-crystallin displays several properties *in vivo* and *in vitro*, such as high thermal stability (Maiti et al 1988), trypsin inhibition (Sharma et al 1987), affinities for plasma membrane proteins (Mulders et al 1985), plasma membrane lipids (Ifeanyi and Takamoto 1991) and actin (del Vecchio et al 1984; Chiesi et al 1990), all of which may relate to its ancestral relationship with heat shock proteins and indicate possible functions for α-crystallins in non-lens tissues.

αB-crystallin is a normal constituent of many tissues including

rodent heart, brain and kidney (Bhat and Nagineni 1989; Iwaki et al 1989; Iwaki et al 1990a; Longoni et al 1990) but its synthesis is also inducible in response to heat shock and Cd^{2+} exposure (Klemenz et al 1991) and αB-crystallin mRNA levels are elevated in scrapie infected brain (Duguid et al 1988). These responses may reflect αB-crystallins involvement in a coordinated adaptive response to cellular stress, involving other recognized stress proteins, but αB-crystallin aggregation and insolublization is also implicated in the apparently irreversible pathogenic changes seen in Alexander's brain disease (Iwaki et al 1989), diffuse Lewy body disease (Lowe et al 1990) and during heart ischaemia (Chiesi et al 1990).

Preliminary reports suggest that αB-crystallin is also expressed in embryonic chick brain and kidney (Agata et al 1990). Further comparative studies of the pattern of extralenticular expression of αA- and αB-crystallin and of the effects of selectively blocking their synthesis, as described here, will help to resolve the question of the different roles of these two related polypeptides in lens and non-lens cells.

Materials and Methods

Oligonucleotides

Antisense oligonucleotides complimentary to the first 20 bases of coding sequence, including the initiation codon, of the chicken αA-crystallin gene (Thompson et al 1987) and mouse δD(δ1)-crystallin gene (as reported in Murer-Orlando et al 1987), were synthesised by Oswel DNA Service (Department of Chemistry, University of Edinburgh), using an Applied Biosystems 380B automatic DNA synthesizer (see Fig. 1 for sequences). The oligonucleotides were fully deprotected with -OH 5'and 3' ends, purified by gel-filtration on a Sephadex G-25 NAP-10 column (Pharmacia) and the DNA concentration estimated by measurement of the OD_{264} in 1ml of distilled water. Samples were lyophilized to dryness prior to solution in culture medium.

The specificity of the αA-crystallin oligonucleotide was assessed by hybridisation to a Northern transfer of chick lens RNA using the conditions described in Head et al (1991). The probe hybridized to a single RNA species of approximately 1.6Kb. (data not shown) consistent with the size of the mature αA-crystallin RNA (Errington et al 1985, Agata 1985).

Tissue Culture

Chicks of the N-J chicken (described in Patek and Clayton 1988)

were used throughout. Lenses and eye cups (future retina) were removed separately from 3.5 day embryos and cultured individually in multiwell plates for 24 hours, using the conditions of de Pomerai et al (1977). In these experiments the 10% foetal calf serum was replaced by 2% Ultroser G (Gibco/BRL), heat treated at 65°C for 1 hour to inactivate nucleases and oligonucleotides dissolved at final concentrations of 500, 50, or 5μg/ml. After 21 hours, half the volume of culture medium was replaced with fresh medium containing the appropriate oligonucleotide, and L-[^{35}S] methionine (> 37TBq/mmol, Amersham, UK) added to a final concentration of 3.7 MBq/ml. After a further 3 hours incubation, the eye rudiments were washed twice with ice-cold physiological saline containing 10mM 2-mercaptoethanol and stored frozen at -70°C in 10mM sodium phosphate buffer pH 7.2 containing 10mM 2-mercaptoethanol.

The retina, including both neural and pigmented layers, from 21 day embryo chicks was cultured as small tissue fragments for 24 hours in medium containing 2% Ultroser G (Gibco/BRL) and 0.37MBq/ml L[U-^{14}C] amino acid mixture (Amersham, UK) using a rotation plate for gentle agitation. Tissue fragments were washed and stored at -70°C.

Protein Analysis

Protein samples were prepared for SDS-PAGE by several cycles of rapid freeze/thaw lysis, addition of 50μg of cold carrier chick lens protein and boiling for 5 minutes in an equal volume of 2X SDS-PAGE sample buffer. The 12,000g supernatant was analyzed by SDS-PAGE and stained with Coomassie brilliant blue according to Patek and Clayton (1985). Gels were impregnated with Amplify fluorographic reagents (Amersham, UK) and exposed to Curix RP1 film (Agfa) at -70°C for 3-6 weeks. Labelled crystallin polypeptides were identified in fluorograms of cultured lenses by their exact co-migration with cold carrier lens polypeptides. The molecular weight of unknown polypeptides was estimated by reference to the migration of co-electrophoresed and stained cold carrier crystallin polypeptides of known molecular weight.

2D(IEF/SDS)-PAGE was by isoelectric focusing in the first dimension (Cuthbert et al 1978) followed by SDS-PAGE of rod gels pre-equilibrated in 2X SDS-PAGE sample buffer and electrophoresis according to Patek and Clayton (1985). Staining with Coomassie brilliant blue and fluorography were as described above. Silver staining was by the polychromatic method of Sammons et al (1981).

Autoradiographic Detection of ^{3}H-labelled Oligonucleotides

In order to determine whether the oligonucleotides were equally well taken up by these tissues we repeated the explant assay with ^{3}H-labelled oligonucleotides present at 5μg/ml. Oligonucleotides were labelled to >1x10^7 dpm/μg with 1.85MBq ^{3}H-dCTP (>1.85TBq/mmol, Amersham, UK) using 30U of calf thymus terminal transferase (BCL) and purified by chromatography using Sephadex G25 (Pharmacia) and ethanol precipitation. After treatment explants were washed three times in saline, embedded in OCT (Miles Scientific), sectioned at 8μm, fixed in 4% paraformaldehyde in PBS (phosphate buffered saline) for 20 minutes and washed twice for 5 minutes in PBS. Autoradiography and staining were as described in Bower et al (1983a).

In Situ Hybridization

Eyes from 14-day embryo chicks were removed, fixed, sectioned and prepared for *in situ* hybridization according to Hafen et al (1983). Sections were hybridized with the Pst1 insert of the chick δ-crystallin cDNA clone M56 (Bower et al 1983a,b) or the EcoR1 fragment of the chick αA-crystallin genomic clone L21a (Errington et al 1985) labelled with ^{3}H-dCTP (>1.85TBq/mmol, Amersham, UK) by random primed synthesis (Feinberg and Vogelstein 1983). Hybridization was carried out for >16 hours in a buffer consisting of 50% formamide, 0.6M NaCl, 10mM Tris-HCl pH7.0, 1mM EDTA, 1X Denhardt's solution, 1% dextran sulfate, 250 μg/ml heat sheared herring sperm DNA, 500μg/ml yeast tRNA and ^{3}H-labelled probe DNA present at 1μg/ml. Washing, autoradiography and staining were as described in Bower et al (1983a). *In situ* hybridization slides were photographed in color using an Olympus Vanox microscope and Nomarski interference contrast optics.

Acknowledgments We thank AFRC, Roslin, for the provision of fertile N-J eggs, A. Edgar and C. Nicolson for technical assistance, F. Johnstone and G. Brown for their photographic skills and M. Ramsay and P. Robinson for preparing the manuscript.

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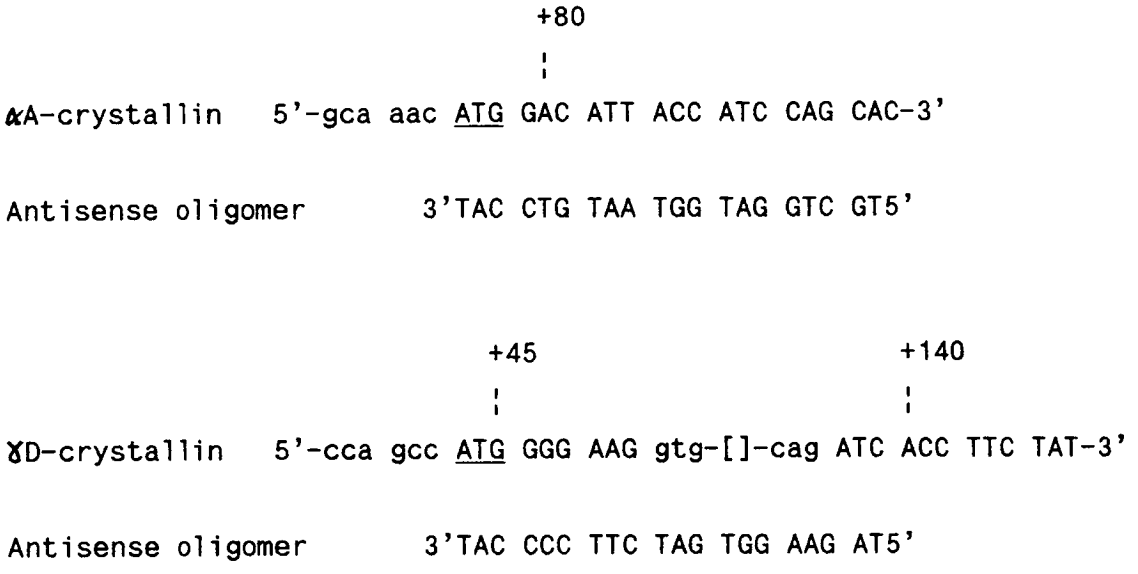


Fig. 1 5' regions of the chick αA-crystallin gene and mouse γD-crystallin gene including the translation initiation codon which is underlined. Numbers identify the position relative to the transcription initiation site. Translated codons and complimentary oligonucleotide sequences are shown in capital letters.

Fig. 2 Pattern of protein synthesis by SDS-PAGE and flourography of ^{35}S-methionine labelled protein of individual 3.5 day embryo lenses (A-H), and eye cups (I-P) cultured for 24 hours in control medium (A,E,I,M), or exposed to antisense αA-crystallin oligonucleotides (B-D, J-L), or γD-crystallin olgigonucleotides (F-H, N-P) at 5μg/ml (B,F,J,N), 50μg/ml (C,G,K,O) or 500μg/ml (D,H,L,P). Arrows indicate the 25kD and 66kD polypeptides synthesised in response to the antisense αA-crystallin oligonucleotides at the highest concentration.

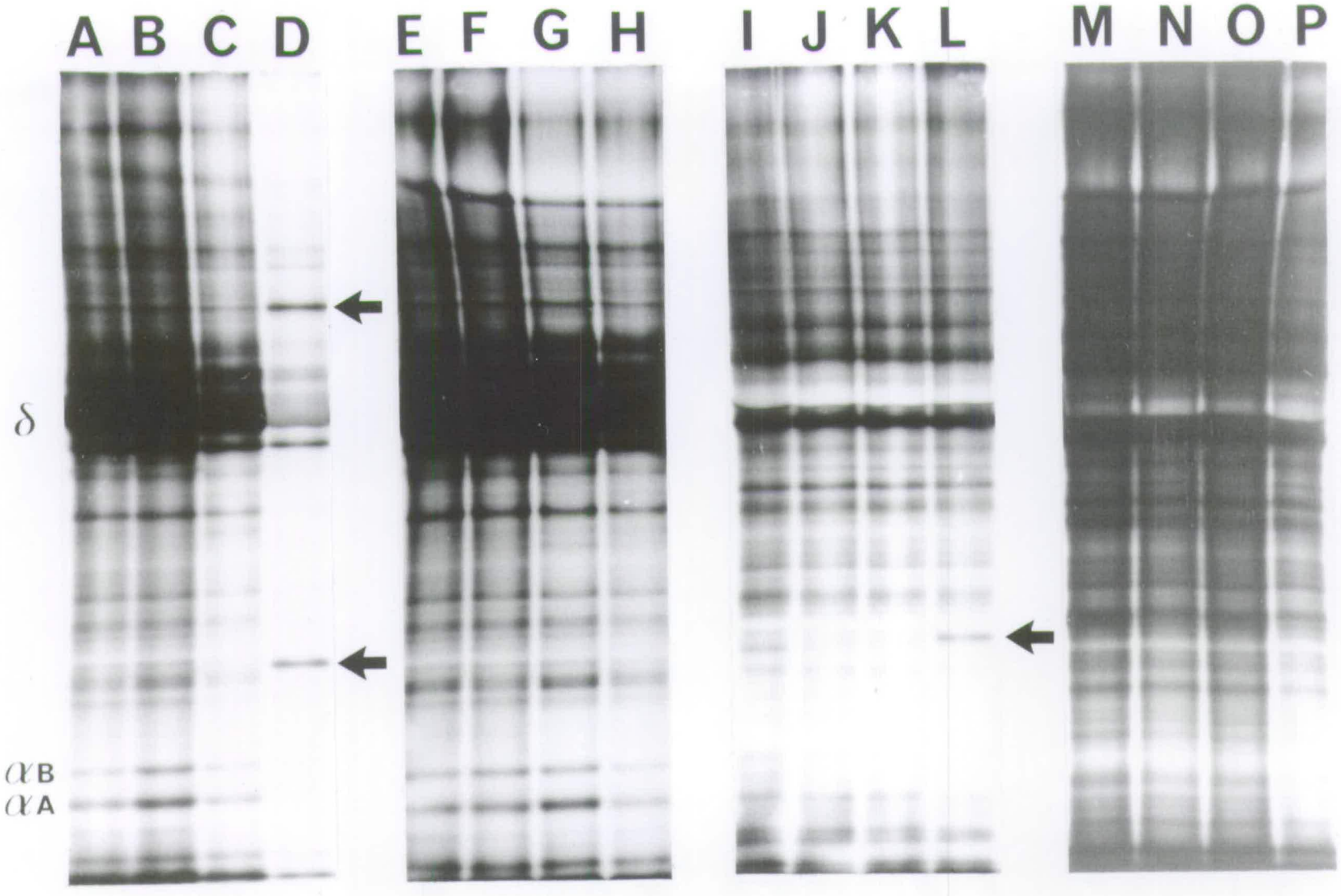
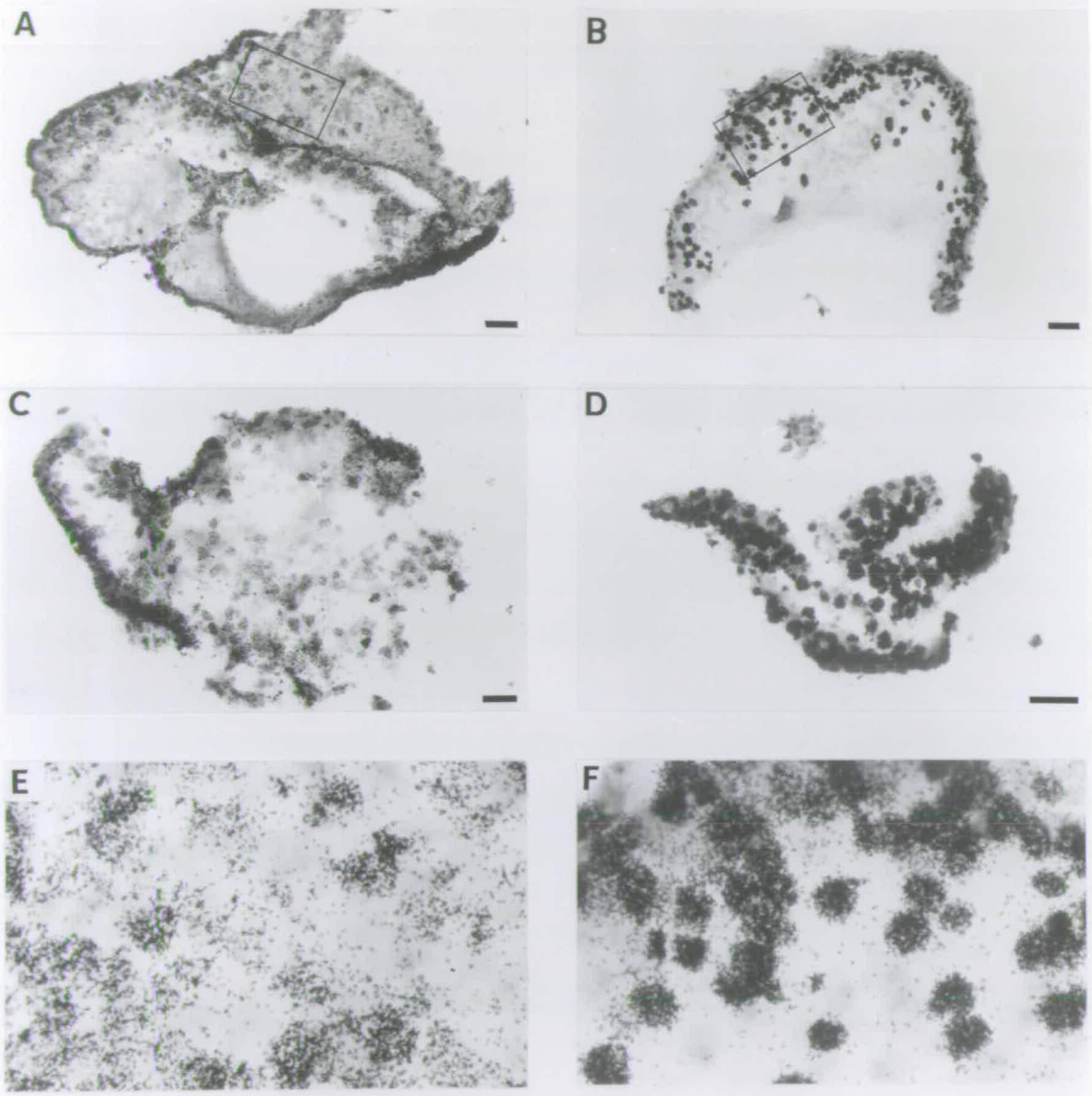

A B C D
E F G H
I J K L
M N O P
δ
αB
αA

Fig. 3 Autoradiographic localization in 3.5 day embryo lenses (A,B) and eye cups (C,D) of ^{3}H-labelled antisense αA-crystallin oligonucleotides (A,C) and antisense γD-crystallin oligonucleotides (B,D). The boxed areas in A and B are shown at high magnification in E and F where heavy labeling with silver grains is evident over weakly haematoxylin stained nuclei. Scale bars represent 30μm.


A
B
C
D
E
F

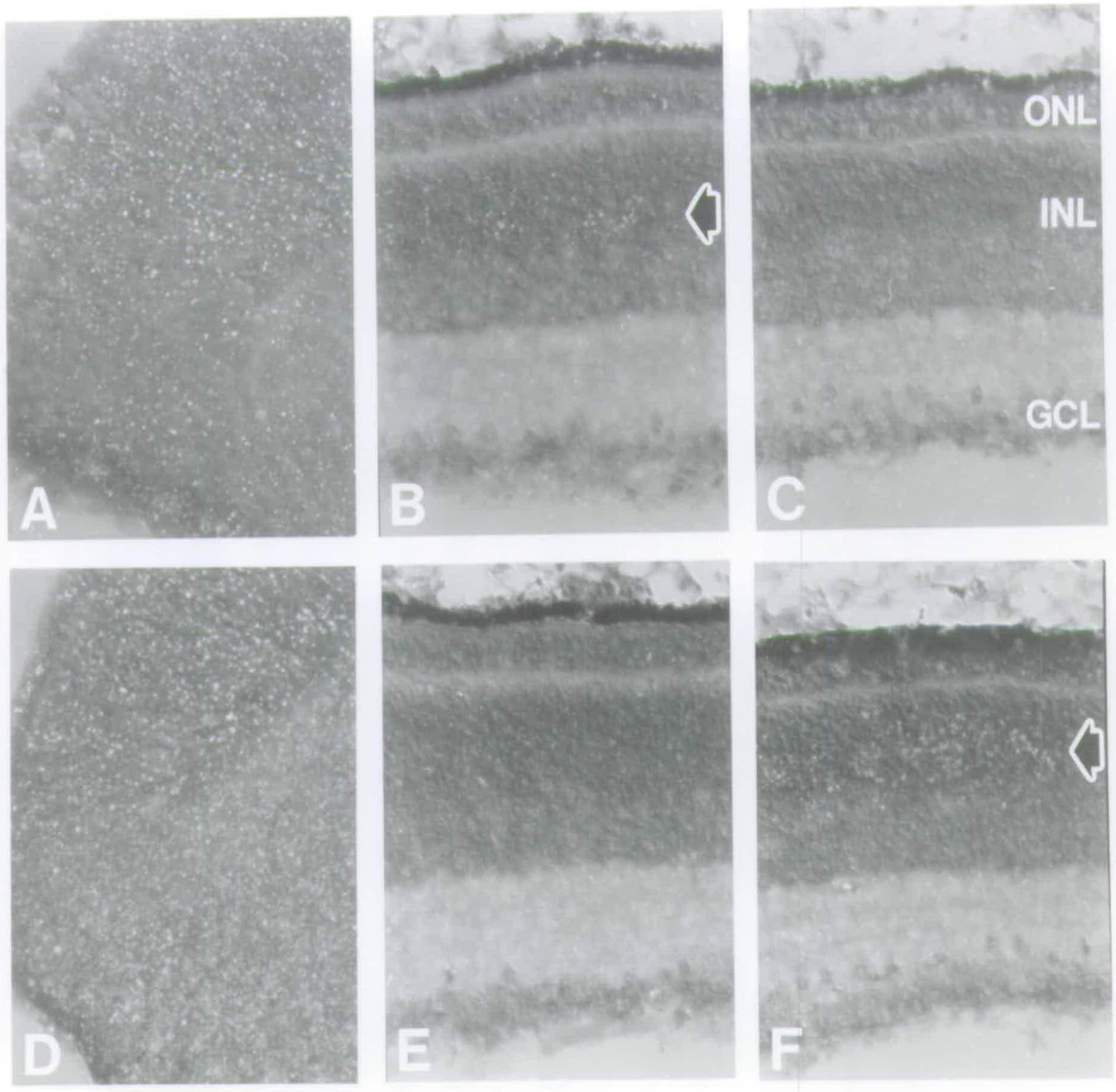
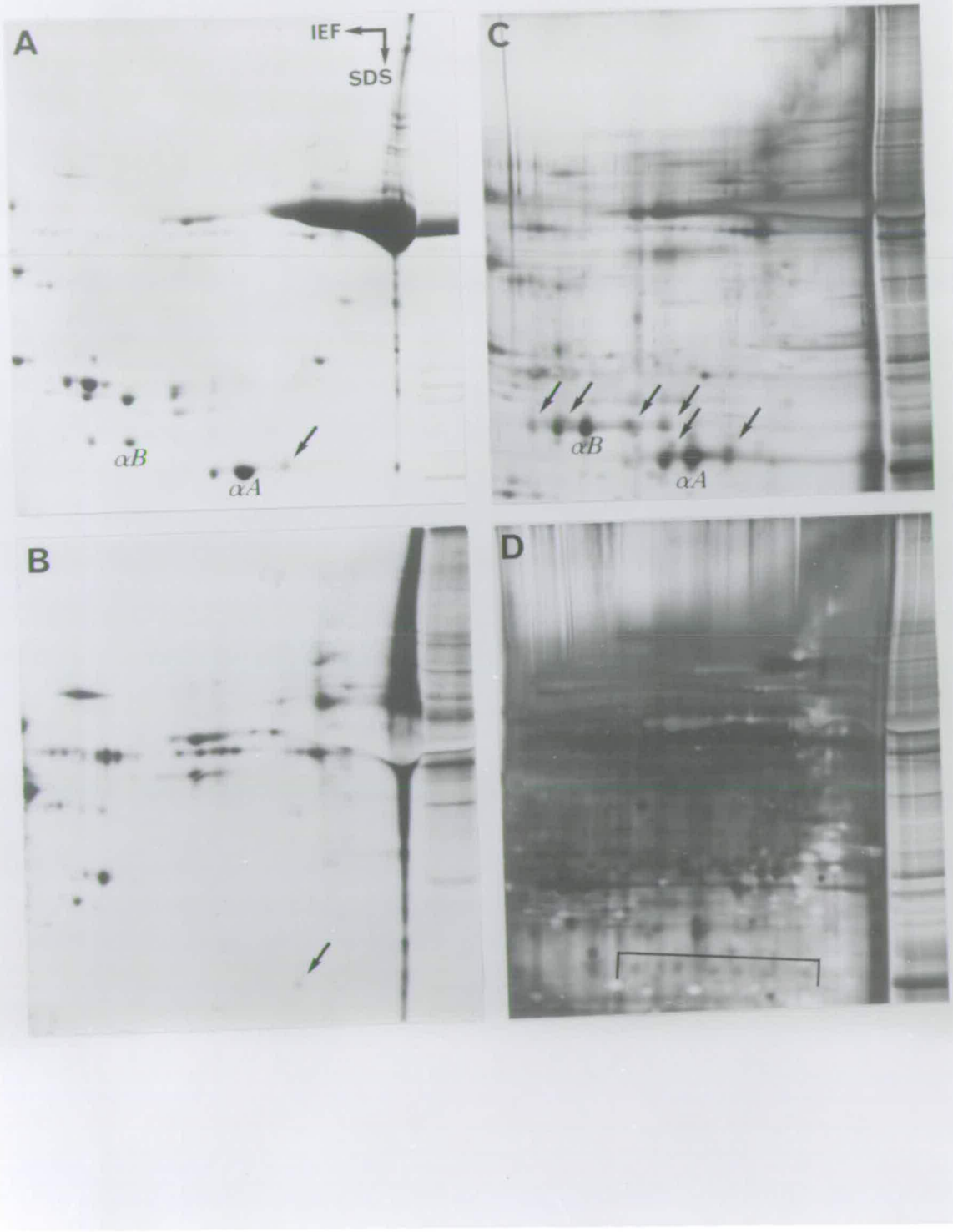

A
B
C
ONL
INL
GCL
D
E
F

Fig. 4 *In situ* hybridization of αA-crystallin (A,B,C) and δ-crystallin (D,E,F) probes to sectioned 14-day embryo lens (A,D) and retina (B,C,E,F). The outer nuclear (ONL), inner nuclear (INL) and ganglion cell (GCL) layers are marked and the position of crystallin RNAs are indicated in the INL with arrows. The negative regions shown in C and E are from the equivalent regions shown in F and B respectively. Scale bar represents 30μm.

The fields shown are from a series of sections prepared from a single eye and therefore these RNA localizations should be regarded as provisional until a more complete series is prepared from the eyes of several individual chicks.

Fig. 5 2D (IEF/SDS)-PAGE stained with Coomassie brilliant blue (A) and the corresponding fluorogram (B) or stained with silver (C, D). A shows 300µg day-old chick lens cold carrier protein with 3×10^4 ^{14}C dpm (34µg) labelled 21 day embryo retina protein. The marker lane shows 30µg of this same protein mixture separated in the SDS dimension only. 40µg of day-old chick lens protein (C) and 400µg of fresh 21 day chick retina protein (D) are shown with a marker lane loaded with 5µg of day-old chick lens protein. The horizontal arrow in A denotes the acidic to basic pH gradient of the IEF separation. The position of a 19kD acidic polypeptide accumulated in the lens sample (A) and sythesized in the retina (B) is marked with an arrow. The spots seen to flank lens αA- and αB-crystallin are arrowed in C and the position corresponding to the αA-crystallin region is bracketed in D.


A
IEF
SDS
αB
αA
C
αB
αA
B
D

DISCUSSION

1. Crystallin Evolution and Expression

The evolution of the vertebrate eye must have involved the co-ordinated evolution of two major developmentally separate but functionally integrated structures, lens and retina, which exhibit apparently entirely novel properties, namely the transparency and refractivity of the lens and the photon capture and signal transduction mechanisms of the retina. Although there is little doubt that a conventional neo-Darwinian stepwise accumulation of visual discrimination from the most rudimentary local light sensitivity would be highly advantageous (discussed in Dawkins 1986) the intermediate morphological steps remain a matter of conjecture (see de Jong 1981).

However, an understanding of the evolution of the lens in terms of its abundant water soluble proteins, the crystallins, has advanced rapidly in recent years with the finding that several of the taxon-specific crystallin classes are related to, or identical with, common metabolic enzymes whereas the ubiquitous crystallins exhibit more distant relationships with stress related proteins (reviewed de Jong et al 1989; Piatigorsky and Wistow 1989).

Based on these data several different evolutionary scenarios may be advanced concerning the mechanism by which non-lens proteins have been recruited as lens crystallins. In certain cases such as those of ϵ- and τ-crystallin, single genes encode both a lens structural protein and a functional enzyme, a phenomenon referred to as gene sharing (Piatigorsky and Wistow 1989). Here recruitment has presumably occurred by the acquisition of mechanisms which facilitate differential tissue-specific gene expression.

The α-, ß- and δ-crystallins, each encoded a gene family, are thought to have been generated by duplication and divergence of different ancestral genes (reviewed Yasuda and Okada 1986; Lubsen et al 1988; Wistow and Piatigorsky 1988). The duplication and divergence of such ancestral crystallin genes allows the specialisation of one sequence as a lens crystallin without compromising the molecular features

important to the non-lens function. The extralenticular expression of αB-crystallin in rodents and the possibility that the δ2-crystallin gene is the avian orthologue of the mammalian argininosuccinate lyase gene have been used to support the suggestion that αA- and δ1-crystallin represent the lens specific paralogues of the αB- and δ2-crystallin genes (de Jong et al 1989; Piatigorsky and Wistow 1989).

Alternatively, the duplications of crystallin genes may solely represent adaptive variations on a molecular theme found to be particularly advantageous in the lens. If this is so then it follows that any observable homologies with other genes could be expected to be weak or extend outside the vertebrates. Crystallin genes derived by this evolutionary route could also legitimately be expected to exhibit strict lens specificity. In the absence of evidence to the contrary the ß-crystallins have been proposed to conform to this model (Lubsen et al 1988; de Jong et al 1989; Piatigorsky and Wistow 1989).

The work described here shows that neither of these two simple models can be wholly correct as applied above since they fail to account for the expression of αA-, ßA3/A1, ßB2-, δ1- and δ2-crystallin in the retina and other embryonic and post-hatch chick tissues.

Since the expression and function of extra-lenticular crystallins has recently become a subject of intensive study by an increasing number of researchers the results of this study will be discussed in relation to those of other recently published reports.

2. α-Crystallin

The available information shows αB-crystallin to exhibit a complex pattern of developmentally regulated expression in rodent tissues. Outside of the lens αB-crystallin is most abundant in the rodent heart (Bhat and Nagineni 1989) comprising up to 5% of the water soluble protein and is found in the form of a homomultimeric 400-650kD protein loosely associated with cytoachitectural elements, such as desmin, and localised in the center of the myofibril I-band at the level of the Z-line (Longoni et al 1990a,b). In other organs the

overall level is lower (Bhat and Nagineni 1989; Dubin et al 1989; Iwaki et al 1989) but expression exhibits cellular heterogeneity and this correlates with a high level of oxidative function (Iwaki et al 1990a).

α-crystallin has been known for some time to have a broad spectrum of affinities for cellular components such as actin (del Vechio et al 1984) and plasma membrane components (Bloemendal et al 1982; 1984; Mulders et al 1985) and an ability to bind and inhibit trypsin (Sharma et al 1987). These properties may relate to the ancestral relationship of α-crystallin and the small heat-shock proteins (Ingolia and Craig 1982) but they may also be indicative of current extralenticular functions of α-crystallin as a constituitively expressed stress protein.

Any passive protective or structural role for α-crystallin in rodent heart has clear and profound limitations since during ischemia αB-crystallin becomes rapidly and irreversibly insolublised, probably as a result of cytoplasmic acidification resulting in the formation of high molecular weight α-heavy (10^7-10^9kD) aggregates and subsequent denaturation (Chiesi et al 1990). Although these changes in cardiac α-crystallin occur within ten minutes of the onset of an ischemic episode whereas cataract formation may occur over a period of years, α-crystallin multimerisation and insolublisation is an unexpected example of an overlap in the molecular pathology of two quite distinct pathological processes.

Further more αB-crystallin shows a variable involvement in the irreversible pathogenic changes associated with the formation of CNS intracytoplasmic inclusions, the Rosenthal fibres of Alexander's disease (Iwaki et al 1989), the Lewy bodies found in Parkinsons disease and to a lesser extent the neurofibrillary tangles found in Altheimer's disease (Lowe et al 1991). The levels of αB-crystallin which accumulate during Alexander's brain disease are much higher than those normally present in CNS astrocytes (Iwaki et al 1989; 1990a) and are found in an insoluble form in conjunction with glial fibrillary acidic protein and ubiquitin in the Rosenthal fibres (Tomokame et al 1991).

αB-crystallin is also inducible in NIH3T3 fibroblasts in vitro in response to heat-shock, Cd^{2+} and sodium arsenite (Klemenz et al 1991a,b) and αB-crystallin RNA levels are elevated in response to scrapie infection of the rodent CNS (Duguid et al 1988). The rapid inducibility of αB-crystallin under classical stress conditions by virtue of a perfect heat-shock promoter element and the intracellular associations and localisation of αB-crystallin during this response suggests that αB-crystallin currently functions as a small heat-shock protein in mammals.

In contrast the expression of αA-crystallin RNA has been shown to be lens specific in mice (Dubin et al 1989) but in the chick αA-crystallin RNA had previously been shown to be expressed in the embryonic chick neural retina (Agata 1985; Errington et al 1985). α-crystallin polypeptides with the electrophoretic mobility of the αA- and αB-crystallin subunits are detectable on Western blots of cat neural retina and these localise to the astrocytes and Muller glia (Lewis et al 1988).

The in situ hybridisation data presented here (Clayton et al, submitted for publication) shows that the αA-crystallin RNA detected in the 8 day embryo chick neural retina (Errington et al 1985) is still present at the 14 day stage but it is restricted to a limited number of cells in the position of Muller cells in the most posterior and central region of the retina.

2D gel electrophoresis failed to detect αA- or αB-crystallin synthesis in the post-hatch retina but this may result from the small proportion of cells engaged in αA-crystallin transcription as judged by the in situ hybridisation data. These experiments did, however, identify the synthesis of a 19kD acid polypeptide in the retina which may be a post-translation modification of αA-crystallin (Clayton et al, submitted for publication).

The requirement of early (3.5 day embryo) chick lens and retina for αA-crystallin was assessed by using antisense oligonucleotides targeted to the translation initiation sequence of the αA-crystallin RNA to selectively inhibit its synthesis (Clayton et al, submitted for publication). The effects on protein synthesis in the lens were

profound including the virtual cessation of αA-, αB- and δ-crystallin synthesis and the induction or elevation of synthesis of two polypeptides with molecular weights close to 66kD and 24kD. Similar treatment of the retina resulted less pronounced effects but included the appearance of the 24kD polypeptide. The level of αA-crystallin RNA in the lens at this stage of development is relatively high compared to that found in the retina (Agata 1985; Errington et al 1985) and results presented here demonstrate that αA-crystallin expression is required for the correct differentiation and co-ordinated regulation of crystallin expression seen in the lens. The level of αA-crystallin RNA found in the retina at this stage is comparatively low but the inhibition of its synthesis does result in a disturbance in protein synthesis suggesting that some αA-crystallin RNA is, in fact, translated in at least some retina cells and may be required for their correct differentiation at this stage. It may be significant that the molecular weights of the polypeptides which exhibit increased synthesis in lens and retina treated with αA-crystallin antisense oligonucleotides are close to those reported for the small and large stress proteins in the chick (Car and de Pomerai 1985) and the pattern of protein synthesis in the treated lens conforms to a classical stress response: cessation of protein synthesis and induction of stress proteins. The less pronounced effects in the treated retina may, in principle, be due to a reduced number of cells actively transcribing αA-crystallin or the nature of the requirement of the cells for this protein.

It is not yet clear whether αA-crystallin, in chick non-lens tissues perform functions similar to those performed by, αB-crystallin in rodent extralenticular sites. It is noteworthy that the affinity of native lens α-crystallin for actin, trypsin and cell membrane components referred to above may be properties of both αA- and αB-crystallin chains. Both, newly synthesised bovine αA- and αB-crystallin chains have affinities for lens plasma membranes (Bloemendal et al 1982). When native αA- and αB-crystallin polypeptides are separately tested with lens plasma membranes — an interaction is seen with αA-crystallin but not with αB-crystallin (Ifeanyi and Takamoto 1990). The micelle structure of native soluble lens α-crystallin proposed by Augustyn and Koretz (1987) results from the strongly amphiphilic nature of the α-

crystallin polypeptide chains (see Puri et al 1983) and also, presumably, accounts for the interaction with the plasma membrane lipid bilayer. Affinities for both lipid and cytoachitectural components such as actin may provide a role for αA-crystallin equally relevant to lens and non-lens cells.

3. ß-Crystallin

A significant proportion of the work presented here is concerned with an examination of the presence of ß-crystallin antigens in extralenticular chick tissues (Clayton et al 1968; de Pomerai et al 1977; Clayton et al 1979). In these earlier studies antisera against total lens proteins were used to assess the tissue specificity of crystallins identified by antigenicity and protein mobility using the Osserman test (Clayton et al 1968), or using antisera raised against purified ß-crystallins to assay changes in ß-crystallin antigen abundance in the developing neural retina by haemagglutination inhibition (Clayton et al 1979). Some, but not all, of the ß-crystallin antigenic determinants were detected in extralenticular tissues (Clayton et al 1968). Even where antisera raised against dissociated individual ß-crystallin subunits are used in the detection of both anodal and cathodal subunits in embryonic neural retina by haemagglutination inhibition the identity of the ß-crystallin subunits detected is not certain since chick ß-crystallin is a complex heteropolymer and its six or more polypeptide subunits are characterised by a series of partially overlapping antigenic determinants (Clayton and Truman 1974; Truman and Clayton 1974).

The proposition that the ß-crystallin antigenicity found in the chick retina results from the expression of the same genes as those involved in chick lens differentiation is considerably strengthened by the study reported here (Head et al, in press) and this has also been extended to the mammalian retina (Head and Clayton, also presented here).

In these studies the identity of the ß-crystallin epitopes in non-lens tissues is addressed firstly at the level of individual ß-crystallin polypeptides, by Western blotting and secondly at the level of their

RNAs using ß-crystallin cDNA probes in Northern, dot-blot and in situ hybridisations. Both the protein and RNA data presented here are internally consistent but a direct correlation between these data must, at present, be regarded as provisional since the exact relationship of ß-crystallin cDNAs to the ß-crystallin polypeptides remains to be demonstrated.

This situation arises from four interrelated problems. Firstly, six of the seven ß-crystallin, polypeptides resolved by the SDS-PAGE system employed here are tightly grouped in the 22-26kD molecular weight range (Patek and Clayton 1985). Secondly the identification of, and distinctions between, primary gene products and possible post-translational modifications has not yet been properly made for the 2D separations employed here. Thirdly, comparisons between SDS-PAGE separations made in different laboratories are made difficult by a variability in resolution which is further complicated when urea, or a urea/SDS mixture is used as the protein dissociating agent. Fourthly the potential for cross-hybridisation between paralogous ß-crystallins results in mixed patterns of hybrid selected in vitro translation products during analysis of cDNA clones (see Hejmancik and Piatigorsky 1983; Errington et al 1986).

Although a single unequivocal assignment of all the ß-crystallin primary gene products according to the generally accepted nomenclature (Berbers et al 1984) cannot currently be made, the following relationships are known, or can be inferred from existing data.

(1) The cDNA clone pcß35cr51 in hybrid-selected in vitro translation studies produces a polypeptide of approximately 35kD (Hejmancik and Piatigorsky 1983) and this is the chick orthologue of the bovine ßB1-crystallin polypeptide (Hejmancik et al 1986). Although ßB1-crystallin, by definition, is the most basic of the bovine ß-crystallin polypeptides (see Berbers et al 1984), its chick orthologue is a highly acidic polypeptide on 2D gel analysis (Voorter et al 1989) and corresponds to the 34kD polypeptide seen using the SDS-PAGE and 2D gel systems used here (Patek and Clayton 1985; Head et al, in press).

(2) Amino acid sequence analysis shows that the highly acidic 28kD chick ß-crystallin corresponds to bovine ßB3-crystallin (Voorter et al 1989). Comparison of their 2D gels and those shown here

(Head et al, in press), indicates that this is the more acidic of the two 26kD polypeptides resolved by Patek and Clayton (1985).

(3) The pCß19/26 Cr42 ß-crystallin cDNA in hybrid selected translation results in two polypeptides of 19kD and 26kD (Hejmancik et al 1983). This can be shown to occur by the alternative use of two in-frame translation initiation codons and more accurate estimations suggests molecular weights of 25kD (ßA3-crystallin) and 23kD (ßA1-crystallin), for these polypeptides (Peterson and Piatigorsky (1986).

(4) The ß-crystallin cDNA clones pCß25Cr61 (Hejmancik and Piatigorsky 1983) and ß026 (Errington et al 1986), both select mRNA encoding a 25kD polypeptide and their relationship of identity is confirmed by cDNA/cDNA hybridisation studies (Head and Clayton, unpublished results). The original lens developmental RNA quantitation data using the pCß25Cr61 probe of Hejmancik et al (1985) were represented in review form, identified as ßB2-crystallin RNA (Piatigorsky 1987) suggesting that the relevant sequencing studies and identification have been carried out in the author's laboratory but have not yet been published.

It should be noted that two separate attempts were made in the course of this study to resolve the question of the correlation between the cDNA probes used in hybridisation studies and the polypeptides resolved in the 2D gel system employed here, but neither attempt was successful. However, it is clear that if the accepted ß-crystallin nomenclature, based on the relative pI values of bovine ß-crystallins polypeptides is extended to the chick ß-crystallins solely on the basis of their pI, their nomenclature would not be an accurate reflection of the evolutionary relationship of orthologous ß-crystallin genes (see Voorter et al 1989).

Although other interpretations of these data cannot be wholly discounted, in the following discussion it is assumed that the two ß-crystallin cDNA clones, pCß25Cr61 and ß026, can be used interchangeably since they both encode ßB2-crystallin which is the major 24 kD basic polypeptide seen in 2D gels, and that the highly basic 26kD ß-crystallin seen in 2D gels is the ßA3-crystallin polypeptide.

ß-crystallin antigenicity in the day-old post-hatch chick retina (neural, pigmented and choroid layers) can be accounted for largely, but perhaps not exclusively, by the presence in Western blots of polypeptides with ß-crystallin antigenicity ad the electrophoretic mobility of the 24kD (ßB2) and 26kD (ßA3) ß-crystallins of lens. Northern transfer and dot-blot hybridisation under conditions of high stringency, similarly demonstrate the presence of the ßB2- and ßA3/A1-crystallin RNAs in this same tissue and stage at between 1 x 10^{-2} to 1 x 10^{-3} of their level in the day-old post-hatch lens. However, neither ß-crystallin is uniformly expressed in the developing retina. The 24kD (ßB2) crystallin is more abundant than the 26kD (ßA3) crystallin when neural retina is assayed alone, whereas the reverse is true when the entire retina, comprising of both the neural and pigmented layers, is assayed. The abundance of both polypeptides is reduced in the region of the optic nerve head and the surrounding area. In situ hybridisation to tissue squashes shows that ßB2-crystallin RNA detectable in Northern transfers of 8 day embryo neural retina is found at high levels in the nuclei of a very limited number of cells. As development proceeds the ßB2-crystallin RNA abundance seen in Northern transfers also increases and it is localised in the cytoplasm of an increasing number of positive cell clusters found largely in the superior retina. The increased abundance of ßB2-crystallin RNA between the 8 day embryo and day-old post-hatch stage is reflected in the appearance of the 24kD (ßB2) crystallin polypeptide in the day-old post-hatch neural retina. Although the level of ßA3/A1-crystallin RNA falls over this same period, the corresponding level of the 26kD (ßB3) crystallin is unaffected.

When iris, cornea, brain, striated muscle, heart, kidney and liver are similarly compared by Western blotting, each of the tissues, except for striated muscle was found to be characterised by a different quantitative or qualitative balance of four polypeptides, each with ß-crystallin antigenicity and electrophoretic mobilities indistinguishable from those of the lens ß-crystallin polypeptides.

It may be argued that the increasing abundance and cellular distribution of ßB2-crystallin RNA and protein during the development of the retina in chicks, indicated that ßB2-crystallin is a functional component in the retina (Head et al, in press). This suggestion is

supported by the finding shown here that low levels of ßB2-crystallin are also a feature of the mammalian retina and here too the levels of ßB2-crystallin increase during development (Head and Clayton, included in this thesis).

However, several features of extralenticular ßB2-crystallin expression in mammals differ from those seen in the chick. Firstly, of those chick tissues assayed, ßB2-crystallin is found to be the most abundant in the chick neural retina, whereas, both mouse and cat pigmented retina accumulates higher levels of ßB2-crystallin than the neural retina. However, ßB2-crystallin could not be detected in either tissue layer in the bovine retina. In the chick, ßB2-crystallin RNA was found to be restricted by retinal region, but the results of a developmental study of ßB2-crystallin localisation in mice, indicates no regional variation, although it did show a changing pattern of distribution throughout the retinal cell layers.

Whether any of these differences reflect genuine functional differences in the regulation of extralenticular ß-crystallin gene expression between species remains to be shown, since a full and complete developmental study on each species was not carried out. The apparent delayed appearance of ßB2-crystallin expression in mouse may be due to the delayed functional maturation of the mouse retina, since chicks are fully sighted at hatch, whereas the eyes of newborn mice are closed.

Suggestions as to the possible role or roles of ß-crystallins in non-lens cells is speculative, evidence being drawn from the biochemical properties of lens ß-crystallin and from evolutionary relationships. In addition to forming a super-gene family with the δ-crystallins (reviewed Lubsen et al 1988), the ß-crystallins exhibit sequence homology with a polypeptide of unknown function, expressed in lens and non-lens tissues in Xenopus and structural homologies with spherulin 3a, an encystment protein of Physarum polycephalum (Wistow 1990) and the Ca^{2+}-binding spore coat protein S of Myxococcus xanthus (Wistow et al 1985). This latter homology may be particularly significant since C^{2+} binding is also a feature of lens ß-crystallin (Sharma et al 1989). Alternatively a cytoarchitectural role may be suggested, since bovine ß-crystallins are known to have an affinity for lens plasma

membrane (Bloemendal et al 1982, 1984) and in the chick lens ß-crystallins are found in association with cytoarchitectural filaments and chains (Ikeda and Maisel 1975). A third possibility is that of a stress related function: ßB2-crystallin is heat stable, like α-crystallin but unlike other ß-crystallins. A stress-related role for extralenticular ß-crystallin would be consistent with the suggestion of Chiou (1988), that certain regions of the α-, ß-, and γ-crystallins and the small and large heat-shock proteins all share a common evolutionary origin. The particular individual significance of the expression of ßA3-crystallin in extralenticular tissues is not known but in the lens it is implicated in the ß-crystallin multimerisation process (Sligsby and Bateman 1990). Crystallins which exhibit both lenticular and extralenticular expression will, presumably, have evolved under multiple constraints. It may, therefore, be noteworthy that the ßA3/A1 gene is highly conserved (Aarts et al 1989b).

4. δ-crystallin.

The mechanisms which regulate δ-crystallin expression in the chick lens are complex, since they must account for the response to lens induction, the extremely high level of expression seen in the embryonic lens fibres and the gradual reduction and cessation of expression in lens fibres formed post-hatch (reviewed by Piatigorsky 1984). These regulatory mechanisms must also account for the low levels and ontogenic changes in δ-crystallin expression in non-lens tissues (see Clayton et al 1986a). It is perhaps not surprising that analysis of the δ-crystallin promoter and enhancer elements suggest that they consist of several functionally distinct modular but interactive elements (Goto et al 1990).

However, the finding that the second δ-crystallin gene (δ2-crystallin) is transcriptionally active in the embryonic lens (Parker et al 1988) and that the δ2-crystallin gene may be the chick orthologue of the mammalian argininosuccinate lyase (ASL) gene (Piatigorsky et al 1988), raises the question of the relative contribution and localisation of these two genes in lens and non-lens tissues during development.

Recently Northern blotting has been used to show that the initiation of δ-crystallin expression must occur much earlier than previously thought and well before lens differentiation, since δ-crystallin RNA is detectable as 0.05% of the poly(A)$^+$ RNA fraction of the entire stage 4 chick embryo (primitive streak stage) and is present in all embryonic tissue layers at stage 10, just prior to overt lens differentiation (Sullivan et al 1991). However, after lens differentiation has begun, the levels of δ-crystallin RNA rise steeply to 3.4% of poly(A)$^+$ lens RNA, whereas, those found in other tissues fall to 0.001% of the poly(A)$^+$ RNA (Sullivan et al 1991). A quantitative measure of the increase in δ-crystallin RNA during early lens morphogenesis had previously been made using solution hybridisation to poly(A)$^+$ RNA extracted from chick embryo heads at selected developmental stages (Shinohara and Piatigorsky 1976). Neither of these studies address the identity of the mRNA, whether δ1- or δ2-crystallin, nor the possibility of differential localisation. The data of Shinohara and Piatigorsky (1976), and their extrapolation of δ-crystallin RNA levels, from stage 12 to stage 18, backwards in time to identify the stage at which transcription is initiated, is particularly difficult to interpret, since Sullivan et al (1991) clearly show that the head ectoderm, mesoderm and neuroectoderm, all of which were assayed along with the lens rudiments by Shinohara and Piatigorsky (1976), could be expected to contribute a significant proportion of δ-crystallin RNA. Furthermore, both of these reports assay the poly(A)$^+$ RNA fraction only, presumably in an effort to enrich for δ-crystallin RNA. Although the extent to which δ-crystallin RNA is polyadenylated in early lens and non-lens tissues is not known, differences occur in RNA processing in the lens during development and between lens and non-lens tissues at later stages of development (Bower et al 1983a,b).

These limitations do not apply to the localisation of RNA by <u>in situ</u> hybridisation and as described here, this technique may be adapted to allow the use of oligonucleotide probes, which can discriminate between mRNAs with a high degree of sequence identity, such as those of δ1- and δ2-crystallin.

<u>In situ</u> hybridisation shows that the accumulation of high levels of δ-crystallin RNA during lens morphogenesis occurs later than previously

thought and is specific to those cells of the posterior lens pit which are in closest apposition to the optic cup (Head et al, submitted for publication, a) suggesting that proximity to the optic cup continues to play an important role in the spatial distribution of δ-crystallin expression. This increase in δ-crystallin RNA abundance in the developing lens can be fully accounted for, by δ1-crystallin RNA with no corresponding increase in δ2-crystallin transcripts (Head et al, submitted for publication, b). During these early developmental stages, δ1-crystallin RNA is highly abundant in the primary and early secondary lens fibres, whereas the lower levels of δ2-crystallin RNA are found in ocular and extra-ocular tissues and furthermore show no bias for the lens fibres as compared to the lens epithelium (Head et al, submitted for publication, b). These results suggest that the δ-crystallin transcripts found in non-lens tissues of very early embryos (Agata et al, 1983; Bower et al 1983a; Sullivan et al 1991) are probably δ2-crystallin transcripts and that these low levels of δ2-crystallin RNA are distributed evenly throughout extralenticular tissues (Head et al submitted for publication, b). During this period, stage 4 (18 hours) to stage 21 (3.5 days), the level of δ-crystallin RNA, as a whole, is falling in non-lens tissues (Sullivan et al 1991) but by 3.5 days of development high levels of nuclear δ-crystallin RNA are found in infrequent cell clusters in non-lens tissues including retina (Jeanny et al 1985) and at later stages of development too, δ-crystallin expression in the retina is restricted to a sub-population of neural retina cells defined both by cell type and location (Linser and Irvin 1986; Clayton et al, submitted for publication and presented here).

The question of the relative levels of δ1- and δ2-crystallin RNA in lens and non-lens tissues is the subject of two independent studies, one described here (Head et al 1991) and the other reported by Thomas et al (1990). These two studies use different techniques and the tissues and stages of development selected also differ but both sets of results clearly show that, irrespective of developmental stage assayed, δ2-crystallin RNA is much less abundant than δ1-crystallin RNA in the lens. Conversely, in all non-lens tissues assayed δ2-crystallin RNA is more abundant than δ1-crystallin RNA.

When the levels of δ1- and δ2-crystallin RNA in the whole lens is

assayed during the embryonic period a small increase in the level of δ1 and a corresponding decrease in the level of δ2 RNA is evident (Head et al 1991) but when the lens epithelia and fibre masses are separately compared two opposing trends are seen, in that, the δ1/δ2 RNA ratio falls in the epithelium but rises in the fibre mass (Thomas et al 1990) to greater than one hundred by the day-old post-hatch stage (Head et al 1991).

No gross changes in the relative abundance of the δ1 and δ2-crystallin RNAs in the neural retina could be detected during the same embryonic period (Head et al 1991). Dot-blot hybridisation indicates that there is a ten to one hundred times excess of δ2-crystallin RNA compared to δ1-crystallin RNA in each non-lens tissue assayed whereas δ1-crystallin RNA predominates in the lens by approximately ten times (Head et al 1991). The levels of δ-crystallin RNA in the 4 to 8 day embryo heart and brain relative to lens, reported by Thomas et al (1990), are lower than those reported for other non-lens tissues by Head et al (1991). Furthermore, of this extralenticular δ-crystallin RNA, Head et al (1991) estimate δ1-crystallin to be approximately one hundred times less abundant than δ2-crystallin RNA in non-lens cells whereas Thomas et al (1990) estimate a factor of no greater than ten times.

The quantitative differences between these two reports may result from genuine tissue- and stage-specific differences in δ-crystallin expression between certain non-lens tissues but this cannot account for the discrepancy in the δ1/δ2 RNA ratio reported for the lens.

Thomas et al (1990) make considerable effort to convince us of the RNA concentration dependence of their PCR based assay but it is not clear from their data whether this assay remains quantitative when samples of cytoplasmic lens RNA are compared with a PCR reaction primed with a $3x10^4$ excess of heart or brain total RNA. On the other hand dot-blot quantitation is a reliable and well documented technique within the limitations set by the dilution factor selected, and the attainment of an appropriate degree of stringency. However it may be that the RNA concentration range over which a linear concentration/signal relationship holds true is reduced when oligonucleotide probes are substituted for cDNA probes.

These potential sources of error not withstanding, it is possible that both studies provide compatible quantitative data for the following reasons. Firstly, δ-crystallin RNA processing intermediates are a common feature of both the very early embryonic lens and embryonic non-lens tissue total cellular RNA but are considerably less abundant or absent from cytoplasmic RNA fractions (Bower et al 1983a,b). The PCR primers employed by Thomas et al (1990) are complementary to sequences in exons 2 and 3 of the δ1- and δ2-crystallin genes and the PCR products quantified could not be transcribed from any δ1- or δ2-crystallin RNAs prior to the splicing out of the second intron. It follows, therefore, that the technical procedure adopted by Thomas et al (1990) would provide consistent under-estimates of the abundance of δ1- or δ2-crystallin RNA in direct proportion to the abundance of partially processed transcripts containing the second intron.

Northern transfers of embryonic lens and selected non-lens tissues show that δ-crystallin RNA processing is both tissue- and, more importantly, transcript-specific (Head et al 1991). Lens δ1-crystallin transcripts are super-abundant they are also proportionally more fully processed than the lower levels of δ2-crystallin RNA found in the lens or the even lower levels still of δ1- and δ2-crystallin RNA found in non-lens tissues. Although δ2-crystallin RNA is more abundant in non-lens tissues it is poorly processed relative to δ1-crystallin RNA. These considerations taken together would have the effect of bringing the results of Thomas et al (1990) and Head et al (1991) into closer agreement.

In any event, whether by translation of the δ1- or δ2-crystallin transcripts, the day-old post-hatch chick retina, cornea, iris, cerebrum, midbrain, optic lobes and kidney all contain an immunodetectible 48kD δ-crystallin polypeptide, at lower levels than that found in lens, but electrophoretically indistinguishable from it (Head et al, in press). The level of δ-crystallin in the kidney is higher than that found in other non-lens tissues and several lines of evidence suggest that it is δ2-crystallin (see Head et al, in press).

Although the deduced amino acid sequences of the two δ-crystallin polypeptides are 91% identical in the chick (Nickerson et al 1986) these differences are significant and probably determine whether the

resultant protein possesses argininosuccinate lyase activity. Both δ1- and δ2-crystallin genes display considerable sequence homology with the mammalian argininosuccinate lyase gene, the homology being greater for δ2-crystallin and the chick genome does not contain any sequences other than the two δ-crystallin genes which hybridise to as mammalian ASL cDNA (Piatigorsky et al 1988). The δ-crystallin band in Western blots of retina accounts for the ASL activity of that tissue (de Pomerai et al 1991). The implication that ASL activity results from the expression of the δ2-crystallin gene is further strengthened by the finding that exogenous δ2-crystallin expressed in mouse cells confers elevated ASL activity but δ1-crystallin does not (Kondoh et al 1991). Although birds are uricotelic and therefore do not require high liver ASL activity for the production and excretion of urea, low level ASL activity in many cell types may be required for arginine and fumerate biosynthesis.

If a complete differentiation has occurred, or is in the process of occurring between the δ1-crystallin gene product (super abundant in, and, specialised for the lens) and the δ2-crystallin gene product (expressed in non-lens cells at low levels as a functional enzyme), two issues must be addressed. Firstly, what role does the δ2-crystallin polypeptide perform in the lens where it possibly occurs, as assessed on the basis of RNA levels, at a level incompatible with an enzymic function and secondly what function might δ1-crystallin perform in non-lens cells if it lacks ASL activity ?

In the absence of any evidence to the contrary it is fair to assume that the sub-unit composition of native tetrameric δ-crystallin protein will be a function of the relative abundance of any δ1- or δ2-crystallin present in a given cell. If mRNA abundance levels are directly translated into polypeptide levels (as described in Thomas et al 1990) the balance of native δ-crystallin sub-unit composition would vary drastically between cell types. The δ-crystallin found in lens fibre cells would therefore be largely δ1 homopolymers, the δ-crystallin found in the lens epithelium, composed of a mixture of hetero- and homopolymers while that found in non-lens tissues would mainly be δ2-crystallin homopolymers. This is clearly the view favoured by Thomas et al (1990) who go on to suggest that the δ1-crystallin polypeptide may contribute to or modulate ASL activity and

that mixed δ1/δ2-crystallin heteropolymers result in both multiple lens δ-crystallin isoelectric variants and in ASL isoforms in birds. This argument may apply to the embryonic lens, where all the available evidence suggests that all cells express both genes to some degree (Thomas et al 1990; Head et al 1991; Head et al, submitted for publication,b) but this cellular homogeneity is not a valid assumption where extralenticular δ-crystallin is concerned. In fact, δ-crystallin expression is heterogeneous in extralenticular tissues, including retina, (Jeanny et al 1985; Ueda and Okada 1986; Linser and Irvin 1987; Clayton et al 1986a,b; Clayton et al submitted for publication). Where specific data does exist the δ2-crystallin RNA appears to be expressed uniformly at low levels in the non-lens tissues of early chick embryos (Head et al submitted for publication,b). This raises the possibility that the δ-crystallin transcripts found concentrated, at a high level, in small, infrequent clusters of cells in non-lens tissues by Jeanny et al (1985) are, in fact, δ1-crystallin transcripts. A comparative developmental study using discriminant probes hybridised to both tissue squashes and sections would be necessary to resolve this point. However, if it transpires that δ1-crystallin is expressed in certain extralenticular cells in excess of, or in the absence of δ2-crystallin expression, an explanation other than ASL activity must be sought.

Two, perhaps related, possibilities may be inferred from the <u>in vivo</u> and <u>in vitro</u> characteristics of lens δ-crystallin. Firstly, soluble δ-crystallin binds Ca^{2+} by virtue of a calmodulin-like amino acid motif (Sharma et al 1989) and secondly, in addition to forming soluble aggregates in the lens fibre cells , δ-crystallin is also found as a Ca^{2+}-dependent extrinsic lens plasma membrane protein (Alcala et al 1977, 1982).

5. Concluding Remarks

Inter-species diversity in lens crystallin composition is thought to have come about by the recruitment of several unrelated pre-existing enzymes, in separate vertebrate lineages, supplementing or partially replacing the α- and ß/γ-crystallins which are expressed in the lenses

of all vertebrate species. In some lineages an apparent evolutionary trends is discernible away from the expression of lens hardening γ-crystallins. This is effected by the dilution of the γ-crystallins in these lenses by newly recruited enzyme/crystallins (see Wistow and Kim 1990) or by inactivation of γ-crystallin expression, as seen in the hominoid lineage (Brakenhoff et al 1990).

There is a general consensus that orderly packing at high protein concentration and thermodynamic stability are necessary pre-requisites for recruitment as a lens crystallin (Wistow and Piatigorsky 1987; Doolittle 1988; de Jong et al 1989; Piatigorsky and Wistow 1989). Furthermore, de Jong et al (1989) point out, the fundamental common denominator and a possible factor in the selection of all lens crystallins may be molecular properties positively beneficial under conditions of cellular stress, a condition, they suggest, in which the lens permanently finds itself. Other interpretations may also be valid and need not be mutually incompatible. Substrate binding by enzyme crystallins has been suggested as lens osmoregulatory mechanism (Wistow and Kim 1991) and the binding of NAD(P) may be presumed to have a number of functions including that of UV filtration (Wistow et al 1987).

The capacity of α-, ß- and δ-crystallins to form association with cytoarchitectural and plasma membrane components may be of considerable importance to lens fibre cell ultrastructure and in the formation of ordered protein aggregates in lens cells and may equally be important in the selection of these proteins as lens crystallins.

The following overview of crystallin evolution may be found in the literature (for example de Jong et al 1989; Wistow and Piatigorsky 1989). A crystallin (lens abundant) function and a extralenticular enzymic or structural role may be encoded by the same gene (ε-, and τ-crystallin). Alternatively, gene duplication may have occurred allowing each duplicated copy to evolve and specialise both in terms of sequence and expression for lenticular or extralenticular roles (αA/αB- and δ1/ δ2-crystallins). Where no sequence homologies with vertebrate non-crystallins are evident, it may be that the relevant ancestral gene no longer exists or that sufficient divergence has occurred to obscure any such relationship (ß-crystallins).

The findings reported here that αA-, δ1- (in addition to δ2-) and ß-crystallins are expressed in embryonic and, in some cases, post-hatch chick tissues, including retina, suggests that the model presented above is in need of revision. Although the current extralenticular functions of αA-, δ1- and ß-crystallins remain a matter for speculation, their restricted localisations in the retina and developmentally regulated quantitatively distinct tissue-specific expression argues in favour of a role for these proteins in non-lens tissues. Both developing lens and retina react adversely to targeted inhibition of αA-crystallin synthesis. The cellular response to inhibition of ß-crystallin synthesis is not known but is currently under investigation. However, the significance of finding ßB2-crystallin in the retina of both chick and mouse should not be overlooked. The fact that orthologous ß-crystallin genes are found in amphibia, birds and mammals demonstrates that the duplication events which gave rise to the current ß-crystallin gene-family occurred prior to the point of divergence leading to these three classes (see Lubsen et al 1988). Unless the expression of ßB2-crystallin in the chick, cat and mouse retina represents an example of convergent evolution we must assume that expression of ß-crystallin in the retina is an equally ancient phenomenon and therefore presumably confers some selective advantage.

These findings emphasise the importance of changes in the regulation of gene expression as an evolutionary mechanism in the generation of novel structures and indicate that protein multifunctionality may be more common than previously thought. They also present new challenges for studies of the factors involved in the regulation of the crystallin genes since a full account of these mechanisms must encompass both lenticular and extralenticular expression.

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Cellular and Molecular Aspects of Eye Research
Post-Congress China Conference of the 7th ICER
Beijing, China
October 6-9, 1986

Non Coordinate Regulation of Crystallin RNAs and Proteins in Lens and in Transdifferentiating Retina

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Tissue distribution of crystallin transcripts

Investigations of the regulation of crystallin gene expression may present problems more interesting and complex even than those of other systems, for three reasons: some at least of the crystallin genes are transcribed at least during embryonic life, in certain non lens tissues, the fiber cells laid down in old and young lenses differ in crystallin gene expression both qualitatively and quantitatively, and lens fiber cells with high levels of crystallins may be derived from lens epithelium, and by transdifferentiation from certain other sources.

Transdifferentiation is the redifferentiation of differentiated cells into another cell type normally of different or even unrelated origin. The best studied examples are from neural retina or tetina pigment epithelium to lens cells with high levels of lens crystallins (recent reviews: Clayton, 1982a, 1982b; Clayton *et al.*, 1986; Okada, 1983, 1986). Other transdifferentiations between these cells are also possible: between pigmented and neuronal cells (see Clayton, 1982b for references) from lens epithelium to pigmented

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0-929031-00-8 (1988) 15$2.00/0

or neurone-like cells (Clayton and Patek, 1982) and from lens epithelium to mesenchyme-like cells (Greenburg and Hay, 1986). However, the potential for transdifferentiation is widespread, and has been reported in several other tissue systems (reviewed by Okada, 1986).

We have suggested (Clayton, 1978, 1982a,b; Clayton *et al.*, 1979) that tissues able to transdifferentiate to lens already express crystallin RNA at moderate levels. A similar type of molecular relationship may also exist in other transdifferentiating systems (Wyllie *et al.*, 1982).

Crystallins were first reported in certain extra-lenticular tissues almost a quarter century ago, and, since crystallins were regarded as particularly clear examples of wholly organ-specific proteins, the early reports were treated with reserve by many investigators, who adduced tissue contamination, tissue leakage or non-specific antisera as explanations of the findings. The earlier literature and the arguments for and against the existence of extra-lenticular crystallins have been reviewed and discussed elsewhere (Clayton *et al.*, 1968; Clayton, 1978). The use of monospecific antisera has shown that δ-crystallin, the major protein of the chick lens during early development, is transiently expressed in 30 % of cells in the embryo adenohypophysis of chick and quail (Barabanov, 1977; Ueda and Okada, 1986), and in certain neurones in defined locations in the embryonic quail brain (Barabanov, 1982). The use of cDNA probes to δ-crystallin RNA has shown that it is transcribed, although not fully processed in a number of tissues of the developing chick embryo which are able to transdifferentiate to lens (Bower *et al.*, 1983a; Agata, *et al.*, 1983) and trace levels were found in some other tissues (Bower *et al.*, 1983a). The chick δ-crystallin gene is transcribed and translated in the lenses of transgenic mice but also in a specific set of neurones, the pyramidal neurones of the anterior piriform cortex of the cerebrum (Kondoh *et al.*, 1987a). All δ-crystallin RNA transcribing tissues in the embryo were heterogeneous, with clusters of cells transcribing δ-crystallin RNA at relatively high levels amongst histologically indistinguishable cells with no δ-crystallin transcripts (Jeanny *et al.*, 1985; Clayton *et al.*, 1986). Clustering may point to a clonal relationship, in which the original derepression of the gene may be a random event, or to a positional effect, in which case the cells may be, in principle, definable by fate or function.

Tissues not shown to have transdifferentiation potential for lens appear to have rare and small groups of transcribing cells with no discernible cytoplasmic labelling. In embyro heart, for example, transcribing cells averaged 27 cells to a cluster and formed about 0.1 % or less of the cell population. Tissues with transdifferentiation potential, however, have more frequent and

larger groups of cells: for example, we have estimated that at least 15 % of cells in neural retina of 3.5-day chick embryo transcribe δ-crystallin RNA, with an average of 44 cells per cluster. There was some evidence of cytoplasmic label in retina and adenohypophysis.

It seems possible that there may be two kinds of extralenticular crystallin expression: in tissues like heart, activation of the δ-crystallin locus may be random, giving rise to a small clone of transcribing cells, while in tissues like epiphysis, adenohypophysis, and retina, transcription may be non-random and characterize a period in normal differentiation of structures which have an evolutionary relationship to one another, are topologically homologous, and between which structural interconversions are possible (Clayton, 1982a, 1982b; Clayton, 1978). The labelling of specific subpopulations of neurons may also be non-random. The implication of such an interpretation is that δ-crystallin protein may have a function in non-lens tissues related to the original ancestral properties of these proteins, antedating the lens.

There have also been several reports of α-crystallin antigenicity in iris and retina (reviewed by Clayton *et al.*, 1968; Clayton, 1978) and an antiserum to a lens protein fraction enriched for α-crystallin localized in Muller glia cells and their precursors in neural retina (Moscona *et al.*, 1985). The possibility that this is actually α-crystallin is strengthened by the detection of αA-crystallin transcripts in freshly isolated neural retina (Errington *et al.*, 1985). However, transgenic mice with an α-crystallin promoter sequence -364 to +65, ligated to a chloramphenicol acetyl transferase (CAT) reporter gene sequence express CAT at low levels in the lens but none was detected in the retina (Overbeek *et al.*, 1985). It is likely that if it were also expressed in glial cells, it could be at levels too low for detection.

Figure 1 shows that low levels of some β-crystallins are also transcribed in freshly isolated neural retina.

Chick δ-crystallin was expressed in transfected mouse lens cells *in vitro*, at lower levels in some other epithelia, and not at all in other mouse cell types (Kondoh *et al.*, 1983). A 5' sequence from -80 to +35, including the initiation site, is required for high level expression and a further 12 bp sequence upstream is required for low level transcription. Two possible binding sites for trans-acting proteins occur in the relevant sequence (Kondoh *et al.*, 1986a,b).

Tissue specific regulatory factors are indicated by other studies. Chepelinsky *et al.*, (1985) transfected chick lens epithelium *in vitro* with mouse α-crystallin upstream sequences and a CAT reporter gene and found a sequence from -364 to +45 of the mouse α-crystallin promotes lens cell specific expression. Lok

et al., (1985) used a mouse γ-crystallin sequence from -392 to +47, ligated to a CAT sequence, to transfect chick lens cells. Deletion of -392 to -171 abolishes the promoter activity. Overbeek, *et al.*, (1985) suggested that although their construct appeared to be co-regulated with the intrinsic α-crystallin, other DNA sequences besides the promoter sequences used might also be needed for high level expression, or that the CAT sequence itself poses unanticipated problems to the mouse cells. Lens cells of both chick and mouse must contain factors which bind to promoter sequences of α- δ- and β-crystallins. They are, therefore, highly conserved in evolution, are tissue specific and neither species specific nor crystallin class specific. Nevertheless, no sequence homologies between these sequences have yet been reported (Kondoh *et al.*, 1986). Such conservative tissue specific factors are however not found in the medaka (a bony fish) carrying the chick δ-crystallin gene (Ozato *et al.*, 1986).

In addition to these tissue-specific factors, there must also be mechanisms for distinguishing between different crystallin classes and between members of a class. There must, therefore, be more than one *cis*-acting sequence per crystallin gene and the number of *trans*-acting factors may be fairly high. However, Murer-Orlando *et al.*, (1987) have presented evidence that the ontogenic changes in levels of the different γ-crystallins may be regulated post transcriptionally, since they are evidently superimposed on simultaneous transcriptional activity.

A range of post transcriptional differences between the crystallins have been reported (reviewed by Clayton, 1979; Piatigorsky, 1987) including mRNA turnover, and translational regulation.

Transcription of δ-crystallin RNA outruns processing in 6-day embryo lens but it is fully processed later (Bower *et al.*, 1982). Neural retina appears to have latent processing capacity for δ-crystallin RNA since precursors are found in freshly excised tissue, but fully processed RNA in tissue allowed to stand for 20 minutes before processing (Clayton *et al.*, 1986). High molecular weight α-crystallin precursors were not seen in lens, but were seen in neural retina (Errington *et al.*, 1985). There are, therefore, tissue and stage specific differences in processing capacity.

Cellular conditions affecting crystallin expression during transdifferentiation

Transdifferentiation is a multistep process (Clayton, 1982a) and requires dissociation of the cells followed by an essential period of growth in monolayers. Reassociation of the cells is then necessary whether by aggregation (Moscona, 1986; Okada *et al.*,

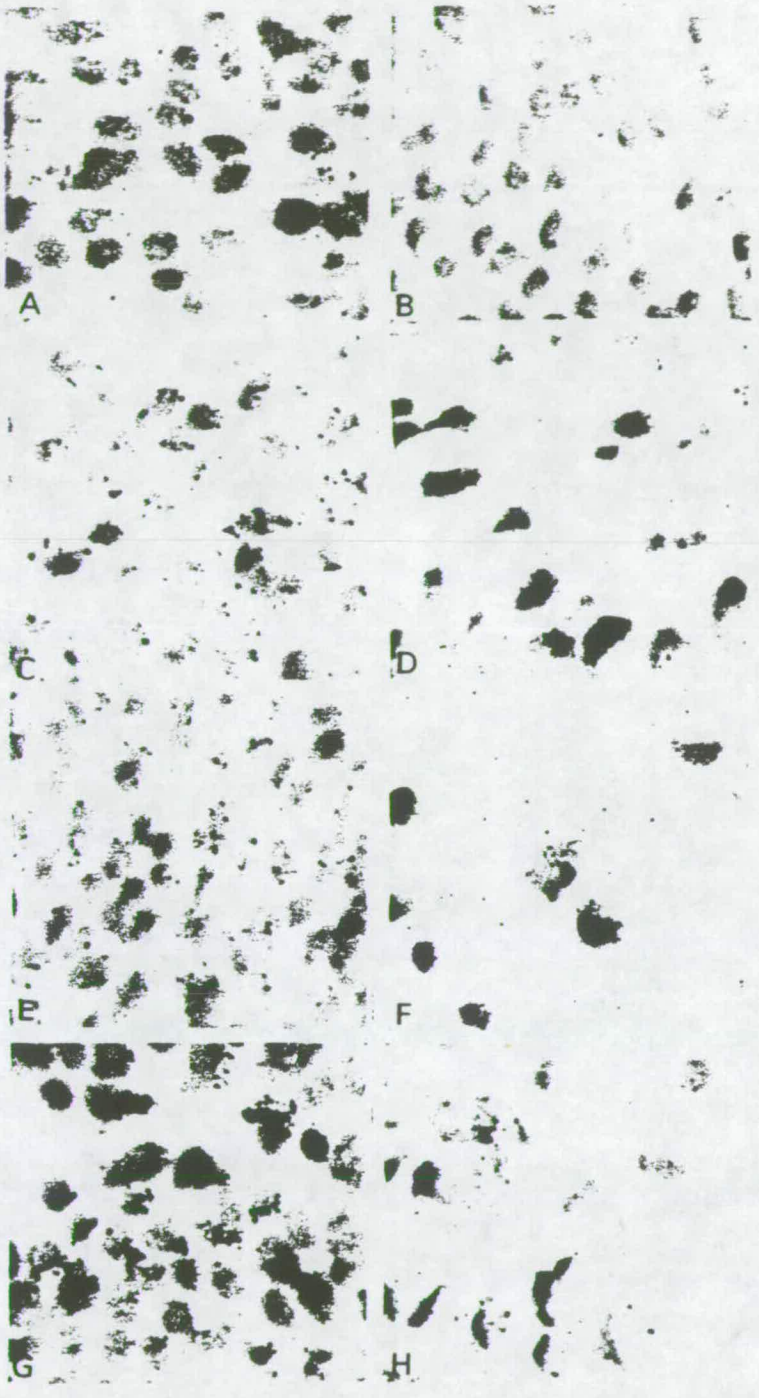
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FIGURE 1 (*facing page*): *In situ* hybridization of cDNA probes to tissue squashes: **A, B, C, E, G,** obtained from freshly isolated 8-day chick embryo neural retina. **D, F, H,** 21-day cultures (These cultures are fully determined for lentoid formation, which begins from four to six days later). The probes used recognize RNAs for the following crystallins: **A** δ-crystallin (pM56: Bower *et al.*, 1982), **B** plasmid vector control: pBR322, **C, D** 19/26 k β-crystallin (Cr 42), **E, F** 23 k β-crystallin (Cr 52), **G, H** 25 k β-crystallin (Cr 61). The β-crystallin probes were a generous gift from Dr. J. Piatigorsky and are described in Hejtmancik and Piatigorsky (1983). *In situ* hybridization was carried out as in Jeanny *et al.* (1985), but using more stringent hybridization and wash conditions (Head, Cuthbert and Clayton, *in preparation*). Each squash was probed with 10 ng of denatured probe DNA nick translated to give 3×10^6 dpm/μg, using the BRL kit.

1983), by folding of the cell sheet (Clayton *et al.*, 1977), or by natural multilayering which occurs in culture. The levels of crystallin RNA begin to rise (Thomson *et al.*, 1979; 1981) at a time roughly coincident with the end of the required monolayer growth period (Okada *et al.*, 1983). The levels of δ-crystallin RNA can be increased about four thousand fold by growing 8-day neural retina cells in spreading culture for 10 days followed by 20 days in aggregate culture, compared to cultures grown without this period of aggregation (Okada *et al.*, 1983).

Although the levels of δ-crystallin RNA transcribed rise steadily, it is at first mainly nuclear, and cytoplasmic label becomes appreciable (Bower *et al.*, 1983b) at a time roughly coincident with the period at which cells become responsive to the effects of insulin on the eventual crystallin proportions of the lentoids, which appear several days later (de Pomerai and Clayton, 1980) and it is after a roughly similar period in cell culture that δ-crystallin DNA in mouse neural retina transfected with δ-crystallin DNA is transcribed at the high level found in lens cells instead of the low level found in non lens cells (Kondoh *et al.*, 1987b).

A metastable population of depigmented pigment epithelial cells was obtained which transcribed but did not process δ-crystallin RNA. Two growth conditions were defined, in one of which transcription continued and the transcripts were processed, while the second totally suppressed transcription of this gene (Eguchi, 1986).

Cellular conditions and crystallin expression in the lens

The regular ontogenic shift in crystallin content in successive cells of the developing lens is probably related to the gradient in refractive index relevant to the functioning of the lens at a given diameter as the animal grows and of the functional requirements of the species (Clayton, 1970) and has presumably been subject to strong selection pressure during evolution. The relationship

postulated (Clayton, 1970) between crystallin composition and lens diameter and retinal curvature (rather than stage of development) was confirmed by Doyle and MacLean (1978).

The cellular signals which bring about the differential temporal, spatial and quantitative regulation of expression of the different crystallin polypeptides in the lens are presumably related to the particular conditions under which an actively transcribing and translating cell finds itself at any given stage of development. However, the ontogenic shift in crystallin composition observed in neural retina-derived lentoids both during lentoid formation (Araki and Okada, 1977) and when lentoids derived from neural retina of different embryonic ages are compared (de Pomerai and Clayton, 1978; Nomura and Okada, 1979) cannot have been subject to such evolutionary selection pressure: on the other hand, the signals and effector systems which operate in the lens to govern the crystallin syntheses within a cell are presumably also operative in transdifferentiating tissue. These may include the following: cell cycle duration, cell position, cell-cell contacts, cell orientation, proximity to iris or retina, growth regulating factors, both systemic and locally synthesized, genetic or ontogenic differences in specific receptors, availability of *trans*-acting cell-specific factors, accessibility of *cis*-acting DNA sequences. Many of these categories overlap with one another. Discussion *in extenso* of such information as is currently available is beyond the scope of this paper: a few illustrated examples must suffice.

1. Cell position and cell contact: cells in the central epithelium differ from more peripheral cells in proximity to the iris, in their mitotic status and in the crystallins synthesized (McAvoy, 1978). Proximity to underlying fiber cells, the composition of which is itself governed by previous cellular history might be relevant. The synthetic patterns of epithelial sheets removed intact from the lens and of dissociated epithelial cells differ from each other and from intact epithelium *in situ* (Clayton *et al.*, 1976).

2. Factors affecting cell growth and differentiation: in general, any agency or condition which increases the mitotic rate and shortens the cycle tends to favor δ-crystallin expression, longer cell cycles and a diminution of mitosis favors β-crystallins (Clayton *et al.*, 1976; Clayton, 1979). This may be, at least in part, due to the optimum period in the cell cycle for synthesis of a particular crystallin (Clayton *et al.*, 1980).

   Insulin affects cell elongation in early embryo epithelium and is mitogenic to epithelium of older stages (Piatigorsky, 1981). It modulates crystallin expression both in lens cells and in transdifferentiating neural retina cells (Clayton, 1979). Re-

tinoic acid prevents loss of δ-crystallin expression in long term cultures of lens epithelium (Patek and Clayton, 1986b, and in preparation).

Other factors affecting epithelial mitosis or cell elongation include lentropin (Beebe *et al.*, 1980) or EDGF (FGF) (Arruti *et al.*, 1985).

3. Cell shape and cell orientation: α-crystallin synthesis takes place but that of other crystallins does not occur in the ak/ak mouse in which cells are randomly oriented (Zwann and Kirkland, 1976).

   Disruption of cell shape by agencies affecting components of ultra-structural architecture also affect crystallin synthesis in a specific way (reviewed by Clayton, 1979).

4. Extra-cellular material: transdifferentiation of pigment epithelium was prevented by growth on bovine collagen (Yasuda, 1979) although transdifferentiation occurs normally in cells permitted to synthesize their own extracellular collagen. Growth in three dimensional collagen gels led to fibroblastic morphology and prevented crystallin synthesis (Greenburg and Hay, 1986).

5. Receptor or effector systems: genetic differences in response to agencies such as insulin (Clayton, 1979) may be due to differences in receptors, such as are known in many other systems.

6. Conditions affecting metabolism can modify the pathways of transdifferentiation. See Pritchard *et al.*, (1978) as well as Galli and de Pomerai (1984).

The sequences of transcriptional events in lens and in transdifferentiating tissues

Long term primary cultures of day-old chick lens epithelium exhibit changes in crystallin composition which have resemblances to post hatch changes *in vivo* (Patek and Clayton, 1986a) but the rate of change is governed by the genotype of the cells, cells of the fastest growing strains differentiate the most rapidly. However, very long term aging changes in serial subcultures, manifested as a sequential loss of crystallin gene expression, are also fairly similar in all strains, but occur more rapidly in cells of slow growing than of fast growing strains (Fig. 2). In all strains, δ-crystallin is lost first: after one passage in the slowest growing and after the third passage in the fastest growing. In all cases, the 26k and 35k β-crystallins are those most persistent, although they are lost after the second passage in the slowest growing strains, and remain the

most abundant of the crystallins in fifth passage cultures of the fastest strains. The remaining β-crystallins and the two α-crystallin polypeptides are lost at intermediate stages.

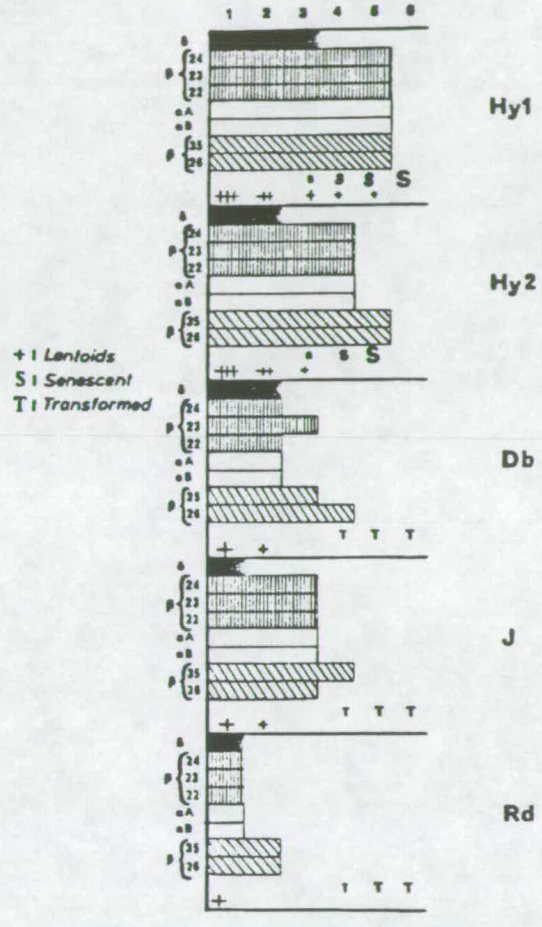

FIGURE 2: Loss of expression of crystallin polypeptides in aging cultures of day-old chick lens epithelium. Cultures were maintained by serial passage for about six months. Passage number is shown at the top of the diagram, and the five genotypes are ranged in descending order of intrinsic growth rate of the cells *in vitro*; strain Hy1 being the fastest growing and strain Rd the slowest (Patek and Clayton, 1986b, 1988). The crystallin polypeptides are identified on the left. The horizontal bars show the passage number in which a given crystallin was no longer detectable.

Table I: Data obtained from the use of cDNA or genomic probes[a].

Days Cultured		0	7	14	21	28	35	42
Neural Retina	δ	+*N*	-*N*tr*D*	tr*N*	++*N*	++*N*	+++*N*	+++*N*
	αA	tr*N*	+*N*	+*N*	+*N*	+*N*	+*N*	+*N*
	β23	+*S*			+*S*	+*S*	*NT*	*NT*
	β25	-*N*tr*DS**	-*N*	-*N*	+*NS*	+*N*	+*N*	+*N*
	β19/26	+*DS**		+*D*	+*SD*	+*SD*	++*D*	*NT*
	β35	tr*D*			tr*D*	tr*D*	tr*D*	*NT*
Pigmented Retina	δ	-*N*	-*N*	-*N*	-*N*	+*N*	+*N*	++*N*
	αA	tr*N*	-*N*	-*N*	++*N*	++*N*	++*N*	++*N*
	β23	tr*S**						
	β25	-*N*tr*S**	-*N*	-*N*	+*N*	+*N*	+*N*	++*N*
	β19/26	tr*S**						

Days Incubated		3.5	6.5-7	15-16	21
Embryo Lens	δ	+*N*	++*N*	+++*N*	+++*D*
	αA	-*N*	+*N*	+*N*	++*D*
	β23				
	β25	-*N*	-*N*+*S*	+*N*	++*D*
	β19/26		+*S*	++*D*	+++*D*
	β35		-*D*		+*D*

[a] Summarizes data from Errington *et al.* (1985; 1986), Bower *et al.* (1983b), and Head, Cuthbert and Clayton (*in preparation*).

The data for αA-crystallin RNA was obtained from Northern transfers.

The data for δ-crystallin and 25 k β-crystallin RNAs from Northern transfers, with *in situ* hybridization giving data in general agreement.

The data for 19/26, 23, 25 and 35 k β-crystallin RNAs comes from *in situ* hybridization and dot blots.

The αA, δ- and 25 k β-crystallin cDNA probes used in Northern transfers were those described in Errington *et al.* (1985), Bower *et al.* (1982), and Errington *et al.* (1986).

The β-crystallin probes used for *in situ* hybridization were a gift from Dr. J. Piatigorsky, as described in the legend to Fig. 2.

We have not yet confirmed that our 25 k β probe used for Northern blots is the same as the 25 k β probe from Dr. Piatigorsky, used for *in situ* hybridization and dot blots.

Key: *D* Dot blot *N* Northern blot. tr trace
* 6.5-day embryo *NT* Not Tested
S *In situ* hybridization, some cells positive
\+ relative intensity of reaction

The time of first appearance of the crystallin classes is modified in the early embryonic lens by the Hyl genotype which affects the cell membrane (McDevitt and Clayton, 1979) but subsequent differences between these genotypes are only quantitative (Clayton, 1979).

Crystallins are not synthesized synchronously in neural retina, pigment epithelium and lens epithelium, although the age-related shift from δ- to β-crystallins occurs both in development and in transdifferentiation (Clayton, 1979). The tendency to maintain a similar sequence of events in lens cells of a range of genotypes (Fig. 2) contrasts with the very different sequence of events in transdifferentiation cultures (Table I), and may indicate that the spectrum of *trans*-acting factors in lens cells is at least qualitatively conserved: however, it is also clear that the sequence of transcriptions does not represent an obligate cascade but depends on the tissue of origin of the lens cells. Thus δ-crystallin is the first transcribed and translated in lens and the last in transdifferentiating 8-day embryo pigment epithelium, and α-crystallin is the first crystallin transcribed and synthesized in transdifferentiating 8-day embryo neural retina (Table I).

Although the regulation of crystallin expression is clearly complex, the crystallins remain an exceptionally suitable system for investigations of the problem of differential gene expression.

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Mutation Research, 00 (1991) MTA00162

ADONIS 092187340

MUTAGI 00162

Ageing in the chick lens: in vitro studies

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(Accepted 17 July 1991)

Keywords: Lens cell; Ageing; Chick; Crystallins, in vitro

Summary

In principle, ageing may be due to the interaction of several factors, including the accumulation of random changes both genomic and non-genomic, secondary changes in a tissue contingent upon the changing function of other tissues, and programmed non-random changes in the tissue-specific expression of various genes.

The use of a single tissue comprising one cell type only, in which the major gene products are well defined, in which there is a well attested series of developmental and age-related changes in cell properties and gene expression and which can be studied and compared in vivo and in vitro, offers advantages for investigation of these questions. The vertebrate eye lens possesses these advantages. The crystallins (proteins expressed at super-abundant levels in the lens) are well characterised. The lens epithelial cells (LEC) grow readily and can differentiate into the lens fibre cells in vitro, and, finally, such terminally differentiated cells may also be derived, by a process of transdifferentiation, from neural retina cells (NRC) in vitro. Thus the effect on ageing changes of the tissue of origin may also be studied. This article reviews our previous studies on long-term changes in growth potential, differentiation capacity and crystallin expression of chick lens cells in ageing cultures, their overall similarity to events in vivo and the effect on ageing changes of genotypes affecting the growth rate. It presents new information on these genetic aspects, and on crystallin expression in long-term ageing cultures of transdifferentiated neural retina, and compares the behaviour of ageing chick lens cells with that reported for mammals.

The lens of the vertebrate eye is composed of concentric layers of lens fibre cells and the epithelial cells from which they are derived. The fibre cells are laid down, albeit at a declining rate, throughout life, and they differentiate successively from cells at the periphery (the equatorial zone) of the sheet of epithelial cells which lies on the anterior face of the lens. The whole is enclosed in the lens capsule.

Lens transparency is achieved by a high concentration of proteins, the crystallins, which are assembled in highly ordered fashion. The majority of the crystallins fall into three gene families, the α-, β- and γ-crystallins, each comprising several related polypeptides, and there are also other

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crystallins restricted to certain vertebrate groups and species (Wistow and Piatigorsky, 1988). The particular assemblage of crystallins synthesised in successively formed lens fibre cells changes throughout life (reviewed in Clayton, 1974; Piatigorsky, 1981), and the representation and relative levels of particular polypeptides within a gene family also change during development. The earliest formed fibres contain high levels of γ-crystallins in mammals and δ-crystallins in birds. Fibres formed later in development contain a higher proportion of α- and β-crystallins, with an increasing ratio of β- to α-crystallins (Clayton, 1974; Piatigorsky, 1981; McDevitt and Brahma, 1982; Harding and Crabbe, 1984). Cells in the older animal express α- and β-crystallins only, and finally, the content of actin, vimentin, and other cytoarchitectural proteins is increased, while that of the crystallins is diminished in the outermost lens fibre cells of very old animals (Bagchi et al., 1983, 1985). The refractive index is highest in the centre (or nucleus) of the lens, which is composed of the fibre cells laid down in the embryo and foetus, and falls towards the cortex, which is composed of later formed cells. The refractive gradient is related to the function of the lens (see Sivak et al., 1989) and to its size (Clayton, 1970; Doyle and MacLean, 1978) and is associated with a gradient in crystallin concentration, a function of the particular crystallin composition, which determines the properties of the assemblages formed (Slingsby et al., 1988; Veretout and Tardieu, 1989).

The older human lens is liable to develop opacities, and this "senile cataract" was once thought to be a reflection of an intrinsic ageing change. However there is evidence that an important effect of increasing age is to increase the time available for exposure to adverse conditions, the likelihood of opacification being related to the number and weighting of risk factors accumulated (Clayton et al., 1984, 1990). This weighting is presumably related to their mode of action, which directly or indirectly may affect lens metabolism, water content, protein solubility or membrane characteristics (see Harding and Crabbe, 1984), and thereby affects the state of the crystallin aggregates. Age-related changes in crystallin composition will modify the proportions of those crystallin polypeptides in the cell which are more susceptible to various chemical modifications by reason of their molecular structures (de Jong et al., 1988). There are also age-related changes in the biochemistry of lens cells (Harding and Crabbe, 1984), and of cell membrane components (Takemoto and Takehara, 1986) which, while not necessarily in themselves causing pathological change, may yet render the cell more susceptible to deleterious change.

Lens epithelial cells (LEC) may be grown and maintained in cell culture and can differentiate to form lentoids, structures composed of lens fibre cells, expressing high levels of crystallins and with membrane components and junctional connections characteristic of lens fibre cells. Mammalian lens cells have, in general, a restricted capacity for lentoid formation and crystallin expression in cell culture (see Patek and Clayton, 1986d) although cell lines can be established, some of which are transformed (Lenstra et al., 1982), but some can still synthesise crystallins (Reddan et al., 1986). However, differentiation in vitro is particularly readily obtained with chick lens cells, and the changes they undergo in vitro closely resemble those occurring in vivo (Okada et al., 1973; Menko et al., 1984; Patek and Clayton, 1985; Patek et al., 1986). They therefore provide an excellent model for studies of the factors involved in ageing, whether intrinsic or extrinsic (Patek and Clayton, 1986a).

Changes in the frequency and duration of mitosis, and the rate of fibre cell differentiation, both of which fall with age (reviewed in Harding et al., 1971) and changes in the specific crystallin content of a cell may be interrelated, and may, in principle, be due to changes in the regulatory signals received as the eye ages, to changes in the number, affinity or availability of cell receptors, or to changes in post-receptor systems (see below). Ageing changes might also be random, associated with the action of free radicals, metabolites or other noxious agencies. There is evidence pointing to the operation of at least some of these latter factors in senile or experimental cataracts, but data which we have obtained from studies of in vitro ageing in the chick point to significant non-random factors in this system.

In the outline which follows, we have de-

scribed the effect, on the rates of differentiation in vitro and the rates of subsequent dedifferentiation, of several genotypes of chick distinguished by fast growth and a rapid rate of mitosis (Fast, or F strains) or slow growth with a lower mitotic rate (Slow or S strains). They are described more fully below.

Changes in cell properties

Growth potential, fibre differentiation and crystallin expression

The growth potential of bovine, human, and rat lens cells in vitro diminishes with increasing donor age, and crystallin content is restricted and falls overall (for example Tassin et al., 1979; Eguchi and Kodama, 1979; Reddan et al., 1981; Rink, 1984; Lipman and Taylor, 1987; Richardson and McAvoy, 1988).

Similarly chick lens epithelial cells (LEC) in long-term culture undergo a series of changes both in growth potential and in crystallin expression which are, overall, very similar to the changes occurring in ageing birds in vivo (Patek and Clayton, 1985, 1986a,b,c, 1988) (Figs. 1 and 2 and Table 1A). Both in vivo and in vitro ageing is associated with a loss of δ-crystallin expression due to a loss of δ-crystallin RNA (Treton et al., 1982; Patek and Clayton, 1986b,c), followed by a fall in α- and β-crystallin expression which is accompanied by the appearance of low-molecular-weight protein, and an increase in actin (Table 2A, Figs. 3 and 4).

This sequence of ageing changes takes place even although cells are grown in a standardised medium. It would appear therefore that there are inherent, yet regular modifications in cell properties which do not depend, whether directly or indirectly, upon any systemic metabolic changes which may occur in vivo and which could affect the eye, although local changes with age in the synthesis of regulatory factors may occur in vivo (for example, Karim and de Pomerai, 1990).

The fall in growth potential is not related to cell death in vivo, but to a reduction in the incidence of mitoses in the cell population which occurs in all lenses in vivo (Harding et al., 1971). The reduction and final loss of differentiation potential of chick cells in vitro still takes place if

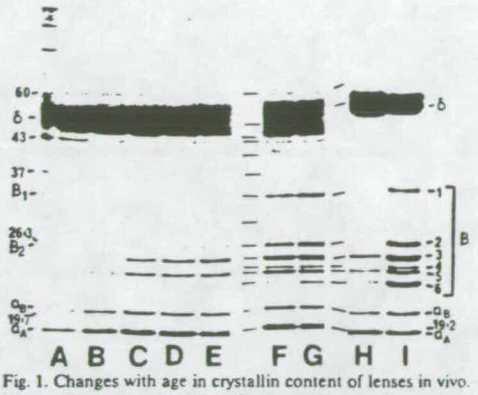


Fig. 1. Changes with age in crystallin content of lenses in vivo. Water-soluble proteins (60 μg) present in lens fibre masses from chicks of different ages. Proteins were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue-R as in Patek and Clayton (1985). (A–E) 7-, 10-, 13-, 16- and 19-day chick embryos respectively, (F) 19-day chick embryos; (G, H) day-old post-hatch chicks (day 21 of incubation), (I) 8-week adult chicks. The nine major crystallin polypeptides resolved are designated δ (48 kDa), β1 (34 kDa), β2 (26 kDa), β3 (24 kDa), β4 (23.5 kDa), β5 (23 kDa), β6 (22 kDa), αB (20 kDa) and αA (19 kDa). All nine major crystallin polypeptides are represented in the 7-day embryonic lens. Development is marked by an increase in crystallin protein and the loss of non-crystallin protein, including actin (43 kDa), and a 19.7-kDa polypeptide. 8-week adult lenses contain less δ-crystallin but substantially more β-crystallin than day-old post-hatch lenses. Quantitative changes in β-crystallin included a shift from an early high abundance of β5 (23 kDa) to relatively high levels of β3 (24 kDa) and β5 (23 kDa), and finally to a high abundance of β3 (24 kDa) and β6 (22 kDa) in the adult lens. β6 (22 kDa) was least abundant in the early embryonic lens but β4 (23.5 kDa) was least abundant in the adult lens. The amount of αB (20 kDa) increased relative to αA (19 kDa) during development but αA (19 kDa) remained the most abundant throughout. Only adult lenses contained detectable amounts of a 19.2-kDa polypeptide.

the cells in successive subcultures are grown at the original cell density (Patek and Clayton, 1986c, 1988), so that the concentration of any autocrine growth factors (such as lens bFGF: Schweigerer et al., 1988) should, in principle, be equivalent.

The rates at which cell growth and cell differentiation fall, and crystallin expression is lost were affected by the genotype, slow-growing (S) strains showing earlier loss than fast-growing (F) strains (see below). The rates were also affected

by the time of passage, which may relate to the number of elapsed mitoses. We found that cells dedifferentiate in an earlier passage if they were replated at 28 days, after many lentoids had formed, but dedifferentiated in a later passage if they were replated at 7 days, before differentiation in vitro began. For example in the slowest growing strain, (NRd), δ-crystallin expression was lost in the second passage of the 28-day series but was still present at a low level in the second passage 7-day series. However even 28-day fourth passage transformed cells still expressed low levels of α- and β-crystallins as detected by fluorography of proteins from labelled cells (Fig. 3). The loss of differentiation capacity in third passage cultures was not complete if cultures were passaged after 7 days, before lentoid differentiation, instead of after 28 days. Small lentoids were still found and α- and β-crystallins were still detectable (Patek and Clayton, 1988).

There is increasing cellular heterogeneity in successive passages (Fig. 5), the epithelial cells being gradually replaced by irregular cells connected by filaments, large polygonal cells, and mutually oriented cells which are epitheloid in early passages but fibroblast-like in later passages of S cultures. In contrast, F cultures contained swollen vacuolated cells with a high rate of cell

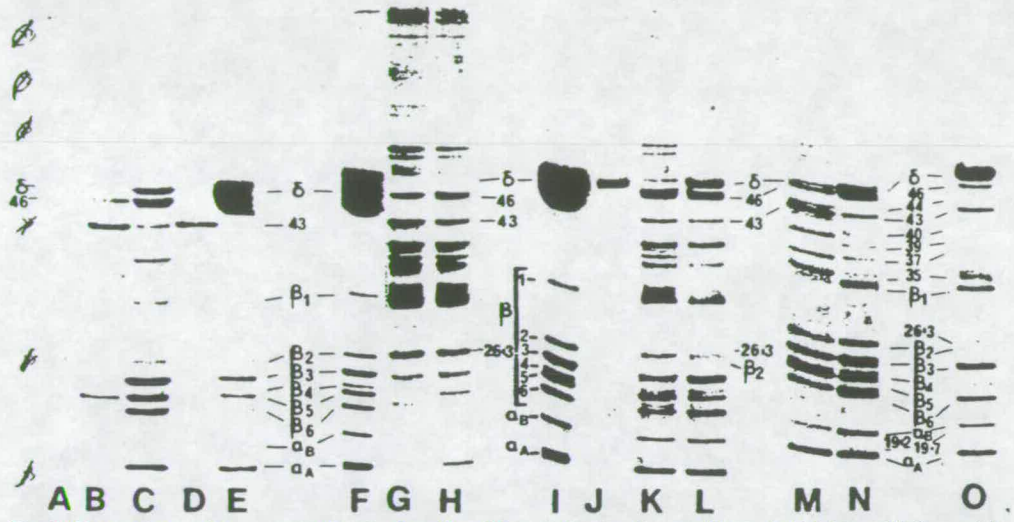


Fig. 2. Changes in proteins accumulated during lens fibre differentiation in primary cultures of chick lens epithelium and in transdifferentiating neural retina. Lens epithelial cell cultures (LEC) and neural retina cell cultures (NRC) were derived from day-old post-hatch chicks and 8-day embryos respectively of the fast-growing Hy-2 strain. Lentoid bodies first appeared by days 12–15 in LEC and at days 32–35 in NRC. Water-soluble proteins (60–90 μg per lane) were analysed as for Fig. 1. Sizes in kilodaltons as for Fig. 1. (A) Molecular weight size markers (from top to bottom); myosin H chain, 200 kDa; phosphorylase b, 92.5 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; α-chymotrypinsogen, 25.7 kDa; β-lactoglobulin, 18.4 kDa; (B, C) 2- and 24-day LEC respectively; (D) chick muscle G-actin, 43 kDa; (E, F, I) lens fibre masses from day-old post-hatch chicks; (G, H) 17- and 30-day NRC respectively; (J) day-old chick lens fibre mass, underloaded (5 μg) to show the position of the most abundant protein, δ-crystallin; (K, L) 40- and 50-day NRC respectively; (M, N) 7- and 40-day LEC respectively; (O) 70-day NRC. There are certain similarities in the changes in crystallin composition between LEC and NRC: for example α- and β-crystallins appear before δ-crystallin, αA is more abundant than αB, β4 is at a low level in both cultures, and both express high levels of β3 and β5 in early stages, shifting to high levels of β3 and β5 in later stages (lanes N and O). These characteristics are similar to those shown by the developing lens in vivo (see Fig. 1). There are also certain differences: for example, there is relatively more δ-crystallin and less β-crystallin in NRC cultures compared to LEC cultures; LEC cultures contain a 19.2-kDa polypeptide, found also in adult lens, but NRC cultures contain a 19.7-kDa polypeptide. β2 is present in undifferentiated LEC from day 2 (lane B) but was not detected in NRC cultures until day 50 (lanes K and L). All nine major crystallin polypeptides are detected in 50-day NRC cultures.

TABLE 1A

SEQUENCE OF APPEARANCE OF THE CRYSTALLIN POLYPEPTIDES IN LONG-TERM PRIMARY CULTURES OF DIFFERENTIATING LENS EPITHELIAL CELLS FROM DAY-OLD CHICKS

Crystallin	Day of culture			
	2	7	24	40
δ	−	+	+	+
β1	+	+	+	+
β2	+	+	+	+
β3	+	+	+	+
β4	+	+	+	+
β5	+	+	+	+
β6	+	+	+	+
αB	−	+	+	+
αA	+	+	+	+

First lentoid bodies appear by days 10–12.

TABLE 1B

SEQUENCE OF APPEARANCE OF CRYSTALLINS IN LONG-TERM PRIMARY CULTURES OF TRANSDIFFERENTIATING NEURAL RETINA FROM 8-DAY EMBRYOS

Crystallin	Day of culture							
	7	14	20	25	30	35	45	50
δ	−	−	−	−	+	+	+	+
β1	?	?	?	?	?	+	+	+
β2	−	−	−	−	−	−	−	+
β3	+	+	+	+	+	+	+	+
β4	+	+	+	+	+	+	+	+
β5	−	−	−	+	+	+	+	+
β6	+	+	+	+	+	+	+	+
αB	−	−	−	+	+	+	+	+
αA	+	+	+	+	+	+	+	+

First lentoid bodies appear by days 32–35.

Cultures were derived from the fast-growing strain Hy-2. 1A, lens epithelial cell cultures, 1B, neural retina cell cultures. Crystallin polypeptides were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue-R (Patek and Clayton 1985). The molecular sizes of the crystallin polepeptides are: δ (48 kDa), β1 (34 kDa), β2 (26 kDa), β3 (24 kDa), β4 (23.5 kDa), β5 (23 kDa), β6 (22 kDa), αB (20 kDa) and αA (19 kDa).

−, crystallin polypeptide not detectable; +, crystallin polypeptide present; ?, detection uncertain.

In both sets of cultures α- and β-crystallins appear before δ-crystallin, αA appears before and is more abundant than αB/β3 and β5 are the most abundant β-crystallins in cultures composed of newly formed lentoids, but β3 and β6 are the most abundant in lentoid-rich cultures.

death. A seventh passage was attempted for cultures of the fastest-growing genotype (Hy-1) but failed because of cell death (Patek and Clayton, 1988).

There is some circumstantial evidence for two of the several possible factors which may lead to the parallelism between in vivo and in vitro changes. These are the possible relationships between mitosis and crystallin expression, and the role of cell-cell contact.

The δ : β ratio is strongly affected by the mitotic rate: a shorter mitotic interval favours δ-crystallin and a longer one β-crystallin. Genotypes, agencies, or culture media which increase the rate of mitosis in vitro lead to a shift towards preponderance of δ-crystallin while those slowing down mitosis in vitro lead to a preponderance of β-crystallin (see de Pomerai et al., 1978; Clayton, 1979, 1982a; Patek and Clayton, 1985, 1986b, 1988).

Mitoses in the lens are synchronised in the day-old chick and remain so for a short period in cell culture (Randall et al., 1979). Brief pulses of labelled mixed amino acids showed that δ-crystallins were more strongly labelled from pulses at the beginning of the cell cycle and β-crystallins from later pulses (Clayton et al., 1980): suggesting the possibility that this temporal sequence might account for the relative importance of δ-crystallin synthesis during short cell cycles and of β-crystallin during longer cycles. However this cannot account for the cessation of transcription of δ-crystallin RNA in young adults in vivo (Treton et al., 1982) and during the ageing of in vitro cultures both of LE and of NR (Patek and Clayton, 1988, 1991). Since δ-crystallin expression is maintained for longer in vitro by retinoic acid or by the constant presence of insulin in the medium (see below), it would seem that certain external signals may be significant, or that receptor synthesis is maintained by the presence of the relevant factor.

Lens cells of all species tested lose crystallin expression in long-term cultures: in rats and mice (Hamada et al., 1979; Rink and Vornhagen, 1980; Rink, 1984), in the bovine lens (van Venrooij et

TABLE 2A

SEQUENCE OF LOSS OF CRYSTALLIN POLYPEPTIDES IN SERIAL SUBCULTURES OF CHICK LENS EPITHELIUM

Crystallin	Passage number					
	1	2	3	4	5	6
δ	+	+	−	−	−	−(−)
β1	+	+	+	+	+tr	−(+)
β2	+	+	+	+	+tr	−(+)
β3	+	+	+	+	−	−(+)
β4	no data					
β5	+	+	+	+	−	−(+)
β6	+	+	+	+	−	−(−)
αB	+	+	+	+	−	−(+)
αA	+	+	+	+	−	−(+)
Cell number	100	70	45	25	15	5

No lentoids were seen after the fifth passage.

TABLE 2B

SEQUENCE OF LOSS OF THE CRYSTALLIN POLYPEPTIDES IN SERIAL SUBCULTURES OF CHICK NEURAL RETINA

Crystallin	Passage number				
	1	2	3	4	5
δ	+	+	−	−	−(−)
β1	+	+	?	−	−(+)
β2	+	+	+	−	−(+)
β3	+	+	+	+tr	−(+)
β4	+	+	?	−	−(+)
β5	+	+	−	−	−(−)
β6	+	+	−	−	−(−)
αB	+	+	+tr	−	−(+)
αA	+	+	+tr	−	−(+)
Cell number	100	70	50	30	10

Sequence of loss of crystallin polypeptides in ageing serial subcultures of: 2A, lens epithelium from day-old chicks of the fast-growing strains Hy-2; 2B, transdifferentiating neural retina from 8-day embryos of the same genotype. LEC cultures were analysed and passaged at 35-day intervals, and NRC cultures were analysed and passaged at 50-day intervals. In both cases all cultures were reseeded at the cell density of the primary culture. The preparation of stained gels was as for Table 1.

+, crystallin polypeptide present; −, crystallin polypeptide absent; ?, detection uncertain; tr, trace.

Molecular sizes as for Table 1.

Cell number is shown as a percentage of the count at first passage. Figures in parentheses indicate that overloaded gels, and fluorographs (based on ^{3}H-mixed amino acid labelling of the cultures) gave evidence for synthesis, but high turnover. Actin (43 kDa) and low-molecular-weight proteins (< 19 kDa) were the major components of late passage cultures, which were composed of large pleiomorphic senescent cells. Both tissues show a progressive loss of crystallins, δ-crystallin being lost first, followed by α- and then β-crystallins. β1 and β2 were the most persistent crystallins detected in LEC and β3 was the most persistent in NRC cultures. Fewer and smaller lentoids were seen in successive passages, and none were seen in sixth passage LEC or in fourth passage NRC.

al., 1974; Courtois et al., 1978; Rink, 1984; Simonneau et al., 1983; Ramaekers et al., 1984), and in humans (Ringens et al., 1982; Reddy et al., 1988; Arita et al., 1990), and we have found loss of crystallin expression in long-term cultures of chick lens (Figs. 3 and 4, Table 2A). Loss of crystallin expression may be reversible in the early stages, by mitotic arrest (Rink, 1984), cold shock (Creighton et al., 1981), or by retinoic acid (Patek and Clayton, 1990).

In general, even aged cultures may still express one or more crystallins at low levels: in mammals it is most often α-crystallin but a β-crystallin (Hamada et al., 1979) and, surprisingly, a γ-crystallin (Arita et al., 1990) have also been reported, and in the chick, we find two β-crystallins are persistent (Tables 2A and 3). However Reddan et al. (1986) have described a rabbit lens cell line with long-term crystallin expression. High levels of actin or vimentin are also reported in aged cultures, both of mammals and chicks (see below).

Cell-cell contact is necessary for lentoid formation and crystallin synthesis, and experimentally increased cell-cell contact, by folding the cell sheet or by aggregating the cells, accelerates the process both with LE and NR derived lentoids (reviewed Clayton, 1982a, 1990). The low growth rate in vitro which characterises LEC from older donors and LEC in ageing cultures will diminish the extent of such cell-cell contact. Arita et al. (1990) reported that forced cell aggregation permitted lentoid differentiation of cells taken from old human lenses, but we have not tested the effect of forced aggregation on ageing chick cultures. It would not appear that cell-cell contacts change during ageing in vivo but there are changes in membrane properties (Takemoto and Take-

hara, 1986; Duncan et al., 1989) which may affect the nature of the contact.

Fibroblastic transformation

Lens epithelial cells have a propensity for fibroblastic-like transformation under a wide range of conditions, both pathological and experimental, and in ageing cultures in vitro. Fibroblastic-like cells have been found in human lens pathology (reviewed Patek and Clayton, 1988) and in certain mutants (Clayton, 1979; Muggleton-Harris and Higbee, 1987). Growth of lens epithelial cells in vitro on abnormal substrates or in collagen gels leads to fibroblast-like morphology with certain mesenchymal characteristics (Greenburg and Hay, 1986; Muggleton-Harris and Higbee, 1987). The change appears to be irreversible (Zuk et al., 1990).

There are several reports of fibroblastic change in ageing mammalian lens cell cultures (for example van Venrooij et al., 1974; Hamada et al., 1979; Miller et al., 1979; Ramaekers et al., 1983, 1984; Creighton et al., 1976; Reddan et al., 1983; Rink et al., 1980; Mungyer and Jap, 1978). We also found fibroblast-like transformation in the second or third passage (Fig. 5C), with an increase in growth rate, when cultures were established on several occasions from three genetically

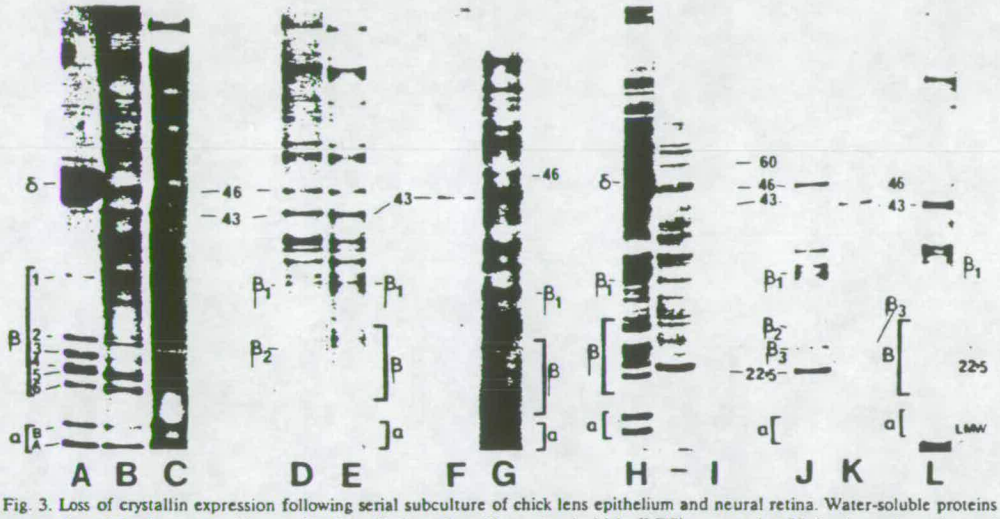


Fig. 3. Loss of crystallin expression following serial subculture of chick lens epithelium and neural retina. Water-soluble proteins present in serial subcultures of lens epithelial cells from day-old post-hatch chicks (LEC), passaged at 28-day intervals, and neural retina cells from 8-day chick embryos (NRC), subcultured at 50-day intervals. Cultures were reseeded at the cell density of the primary culture. Gels were prepared as for Fig. 1. Molecular sizes in kilodaltons as in Fig. 1. (A) Lens fibre masses from day-old post-hatch chicks (NRd chick an S strain); (B, D, F) accumulated proteins from second passage and third passage cultures (NDb chick an S strain) and sixth passage (fast-growing Hy-2 chick strain) LEC; (C, E, G) corresponding fluorograms, showing proteins synthesised from ^{3}H-mixed amino acids. A comparison of stained gels and fluorographs shows that second passage LEC neither contained nor synthesised δ-crystallin, and the third passage LEC still synthesised α-crystallin, although no protein was detectable in the stained gel. Sixth passage LEC synthesised but did not accumulate either α- or β-crystallins, and actin (43 kDa) was the major component expressed. (H–K) Accumulated proteins present in 50-day primary cultures and third, fifth and fourth passage NRC respectively of the fast-growing Hy-2 chick strain. Third passage NRC contained no δ-crystallin, fourth passage NRC contained neither δ- nor α-crystallins, and fifth passage NRC contained no crystallins but contained high levels of actin (43 kDa). (L) Fluorogram showing proteins synthesised by fifth passage NRC from ^{3}H-mixed amino acids; α- and β-crystallins are synthesised but δ-crystallin is not. As in lenses in vivo, the loss of δ-crystallin expression in both LEC and NRC cultures was matched by the loss of δ-mRNA as determined by Northern transfers to total cellular RNA, using a cDNA probe (Patek and Clayton, 1986c; Patek et al., 1991). Both LEC and NRC cultures ~~then lose expression of~~ α-crystallins, ~~followed by~~ β-crystallin, but their synthesis continues, and in both cases actin (43 kDa) is the major constituent of aged dedifferentiated cultures.

distinct strains of slow-growing (S) chick (Fig. 5D) (Patek and Clayton, 1988),but this did not occur in any cultures established from fast-growing (F) chick strains. This difference may be related to factors associated with growth rate, since human lens cells grown in conditions which limited their growth capacity became spindle shaped but in conditions permitting better growth became senescent (Jacob, 1987).

An increase in vimentin and actin occurs in the ageing rabbit and chicken lens in vitro (Bagchi et al., 1983, 1985). Lens cells in ageing mammalian cultures which become fibroblastic may express high levels of actin or vimentin (Bloemendal et al., 1980; Simonneau et al., 1983; Ramaekers et al., 1984; Ringens et al., 1982) and actin is the major component of aged chick lens cell cultures, whether fibroblastic or pleiomorphic (Patek and Clayton, 1988). Finally, pathological lens cells may contain α-smooth muscle actin, and express other mesenchymal markers (Schmitt-Graff et al., 1990).

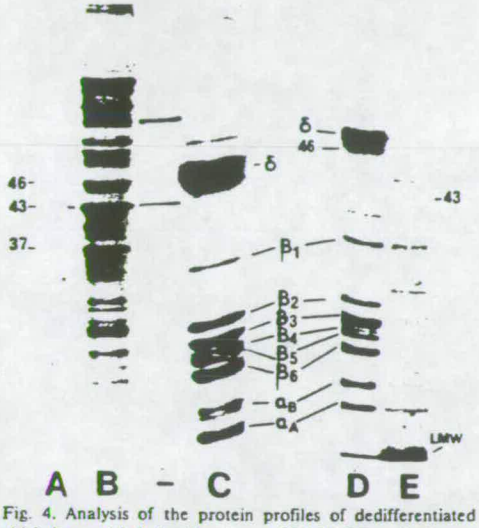


Fig. 4. Analysis of the protein profiles of dedifferentiated chick lens epithelial cell cultures. SDS-PAGE as in Fig. 1, showing water-soluble proteins present in dedifferentiated cultures of lens epithelial cells from the slow-growing NRd strains, replated and reseeded as in Fig. 3. (A) Proteins at 60 μg/lane, (B) proteins at 600 μg/lane, fourth passage LEC. (C) total day-old lens fibre tissue, 60 μg/lane. Only actin is detectable in lane A, but the overloaded lane B shows that a wide range of proteins is present at low levels, including bands in the position of α- and β-crystallins. No δ-crystallin is detectable. (D) 28-Day primary culture of lens epithelial cells; (E) 28-day third passage culture, showing that cells at this stage contain a high proportion of low-molecular-weight (LMW) protein (< 19 kDa). The presence of LMW and the absence of α- and β-crystallins, although they are still being synthesised, implies that the loss of soluble protein in aged LEC is due to rapid degradation.

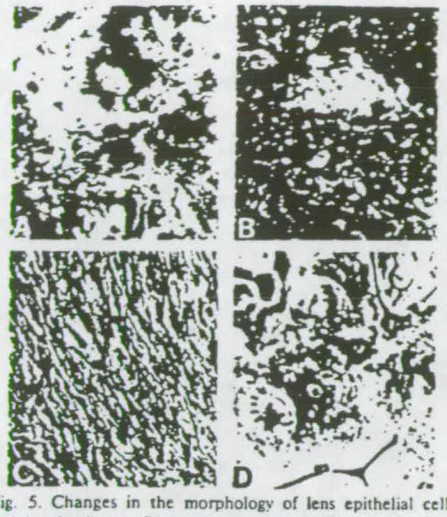


Fig. 5. Changes in the morphology of lens epithelial cells serial subcultures. Photomicrography (×320) showing lens epithelial cell cultures from day-old post-hatch chicks. Cultures were photographed and subcultured at 28-day intervals and replated at the original cell density. (A) Primary cultures composed of numerous large lentoid bodies with characteristic bottle cells; (B) passage cultures composed of rare and small lentoid bodies; (C) late passage cultures with no lentoid bodies, and composed of rapidly growing fibroblast-like cells; (D) late passage cultures with no lentoid bodies, but composed of pleiomorphic senescent cell types. (C) and (D) are derived from slow- and fast-growing chick strains respectively.

A number of cytoskeletal proteins have been identified in non-aged lens epithelial cells (Ramaekers et al., 1980; Alcala and Maisel, 1985) and an actin-myosin contractile system is found both in lens epithelium and in cortical fibres (Rafferty et al., 1990). Thus cytoskeletal proteins found at high levels in mesenchymal cells are also found in normal lens cells.

Transdifferentiation is defined as a change of one cell type into another cell type which occurs

when increased expression of a minority component of a cell achieves the higher level which characterises the cell type towards which the change occurs, while the major constituents of the original cell type are no longer expressed at high levels (Clayton, 1982b, 1990). A high level of expression of vimentin in transgenic mice is apparently incompatible with lens fibre differentiation and crystallin synthesis (Capetaniki et al., 1989) while crystallins are barely detectable when actin has become the major component in aged cultures of LEC (Patek and Clayton, 1988). The apparent incompatibility between high levels of actin or vimentin and high levels of crystallins suggests that the epithelial-mesenchymal type of change represents transdifferentiation. The difference between a late formed cell in an old lens and a fibroblastic lens cell may be a matter of quantitative balance.

Genetic variables

We examined the cell behaviour in vitro of several genetically unrelated chick strains, distinguished by their growth rate in vivo and the rate of cell replication in vitro (Patek and Clayton, 1986b, 1988) using two fast-growing (F) strains, Hy-1 and Hy-2 (Hy referring to the in vivo hyperplasia of the lens epithelium) and three slow-growing (S) strains, NJ, NDb and NRd (N referring to the normal morphology of the lens epitheium in vivo.

Compared to cells from S strains, lens epithelial cells of both F strains have reduced plating efficiency in vivo, a higher growth rate, and show precocious lentoid formation in cell culture (Eguchi et al., 1975). They also form more microvilli, have fewer gap junctions and increased binding of several lectins (reviewed in Clayton, 1979). At all stages of primary culture, F genotypes express more δ-crystallin than S genotypes both in vivo and in lentoids formed in vitro, whether derived from LEC or NRC (reviewed Clayton, 1982a).

Both F strains differentiate earlier in primary culture than S strains; for example large lentoids appear by 8–9 days in the Hy-2 primary cultures and smaller lentoids by days 14–15 in NRd. At 28 days, 70% of the NRd culture is undifferentiated compared to 40% of the Hy-2 culture (Patek and Clayton, 1990). During the ageing process in vitro, both F strains continue to express a higher level of δ-crystallin than the three S strains and both F strains retain crystallin expression longer than S strains. Replicate cultures of the three different S strains set up on several occasions all exhibited transformed behaviour and become fibroblastic in an early passage, while replicate cultures of both

TABLE 3

LOSS OF DETECTABLE CRYSTALLIN POLYPEPTIDES IN SUCCESSIVE PASSAGES OF DAY-OLD CHICK LENS EPITHELIAL CELLS OF FIVE UNRELATED CHICK GENOTYPES, CHARACTERISED BY GROWTH RATES OF CELLS IN VITRO

~~Crystallins~~		~~Passage number~~							Loss of lentoid-forming capacity
		δ	αA, B	β3	β5	β6	β2	β1	
Hy-1	F strains	3	5	5	5	5	5	5	> 5
Hy-2		2	4	4	4	4	5	5	4
NDb	S strains	2	2	3	3	3	3	4	3
NJ		1	3	3	3	3	3	4	3
NRd		1	1	1	1	1	2	2	2

The genotypes are ranked, top to bottom, in order of their growth rate in vitro (Patek and Clayton, 1986a, 1988); F, fast-growing; S, slow-growing. Crystallin polypeptides were resolved as in Table 1. Cells were analysed and passaged at 28-day intervals and reseeded each time at the cell density of the primary culture. Numbers refer to the last passage number in which the protein is still detectable in stained gels. In general, crystallin loss occurred earlier in cultures of the slow-growing strains, and δ-crystallin is lost first, then α-crystallin, and β1 and β2 are the most persistent. In a few cases, crystallins are expressed in an earlier or later passage than would be expected if relative growth rate were the sole determining factor (for example α-crystallin expression in NDb and NJ). Lentoids were proportionally smaller and more sparse in succeeding passages. NRd formed no lentoids in the second passage but there were rare small lentoids in fifth passage Hy-1.

F strains all showed eventual senescence in a late passage (Patek and Clayton, 1988).

Genotype-related differences were superimposed upon the generalised pattern of loss of crystallin expression, which remained similar in all cases (Table 1). The expression of δ-crystallin was lost first in all cultures, as it is in vivo, but it is lost at the first passage in cultures of the slowest growing NRd cells and in the third passage of the fastest growing Hy-1 cells (Table 3). In subsequent passages, α- and most β-crystallin polypeptides became undetectable in sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) except at very high protein loadings, while much of the protein in the cells was of low molecular weight. Again, this occurred at a rate inversely proportional to the growth rate in vitro. Since these crystallins were still being synthesised, as judged by fluorography of SDS-PAGE separations of radiolabelled proteins (Figs. 3 and 4) we concluded that they were being rapidly turned over by proteolysis. An increase of proteolysis and of low-molecular-weight proteins also occurs in vivo (Srivastava, 1988). Finally, two of the β-crystallins, β1 (35 kDa) and β2 (26 kDa) were still detectable in all genotypes, albeit at very low levels, in the senescent pleiomorphic cultures of both F strains, and in the fibroblastic rapidly growing cultures of all three S strains (Patek and Clayton, 1988). In all genotypes, actin became the predominant protein in late passages (Table 3).

Within these consistent trends, there were minor variations: the slow-growing NJ retained α-crystallin expression longer than NDb which had a somewhat higher growth rate, and NRd, the slowest growing, expressed β2-crystallin longer in relation to β1-crystallin than the other strains (Table 3). To conclude, F strains differentiate more rapidly and dedifferentiate more slowly than S strains, suggesting that they retain more juvenile characteristics.

The role of growth factors

The differentiation of lens epithelial cells to primary fibre cells is brought about in vivo by proximity to the retina (Coulombre and Coulombre, 1963; Genis-Galvez and Castro, 1971; Yamamoto, 1976), and later fibre differentiation still involves retina (McAvoy and Fernan, 1984). Growth, differentiation, and crystallin expression are affected by a variety of hormones and growth factors, some of which have been isolated from the retina, other ocular structures, and the vitreous (see Beebe, 1985). To date, these include insulin and insulin-like factors (Piatigorsky, 1973; Beebe et al., 1987, 1990), FGF (Mascarelli et al., 1987, 1989; Chamberlain and McAvoy, 1987), PDGF (Brewitt and Clark, 1988; Campochiaro et al., 1989), and retinoic acid (Patek and Clayton, 1986d, 1990). A fall in growth potential and rate of differentiation may therefore reflect either a fall in growth factors with age or an age-related change in cellular response.

Growth factor peptides exhibit multi-functionality, the range of their effects depending on the tissue and the prior state of the target cell (Sporn and Roberts, 1988). For example FGFa and b may be mitogenic or inhibitory, act as embryonic inducers, or regulate the function and affect cell survival of many different tissues (Gospodarowicz et al., 1986; Mascarelli et al., 1989), and their effect on lens cells depends on the concentration (McAvoy and Chamberlain, 1989), and the age of the lens donor: bFGF has a preferential effect on γ-crystallins of rat lens cells in vitro up to the third day post partum, but thereafter on α- and β-crystallins, while cells from 6-month-old rats failed either to differentiate or to synthesise crystallins (Richardson and McAvoy, 1990).

Similarly, insulin stimulates δ-crystallin synthesis in 6-day chick embryo lens epithelium (Millstone and Piatigorsky, 1977) but not in the 19-day embryo (Beebe and Piatigorsky, 1977), and has a marked but differential effect on β-crystallins of the day-old post-hatch lens epithelium (Clayton and Zehir, 1982). However, δ-crystallin synthesis in day-old chick lens epithelium can still be strongly promoted by retinoic acid (Patek and Clayton, 1986d, 1990), an effect which may be, at least in part, a function of the mitotic rate, since insulin stimulates mitosis in day-old chick lens by 30% but retinoic acid by 400% (Patek and Clayton, 1990). This would be in line with findings that a variety of factors which increase mitosis are also associated with an increase δ-crystal-

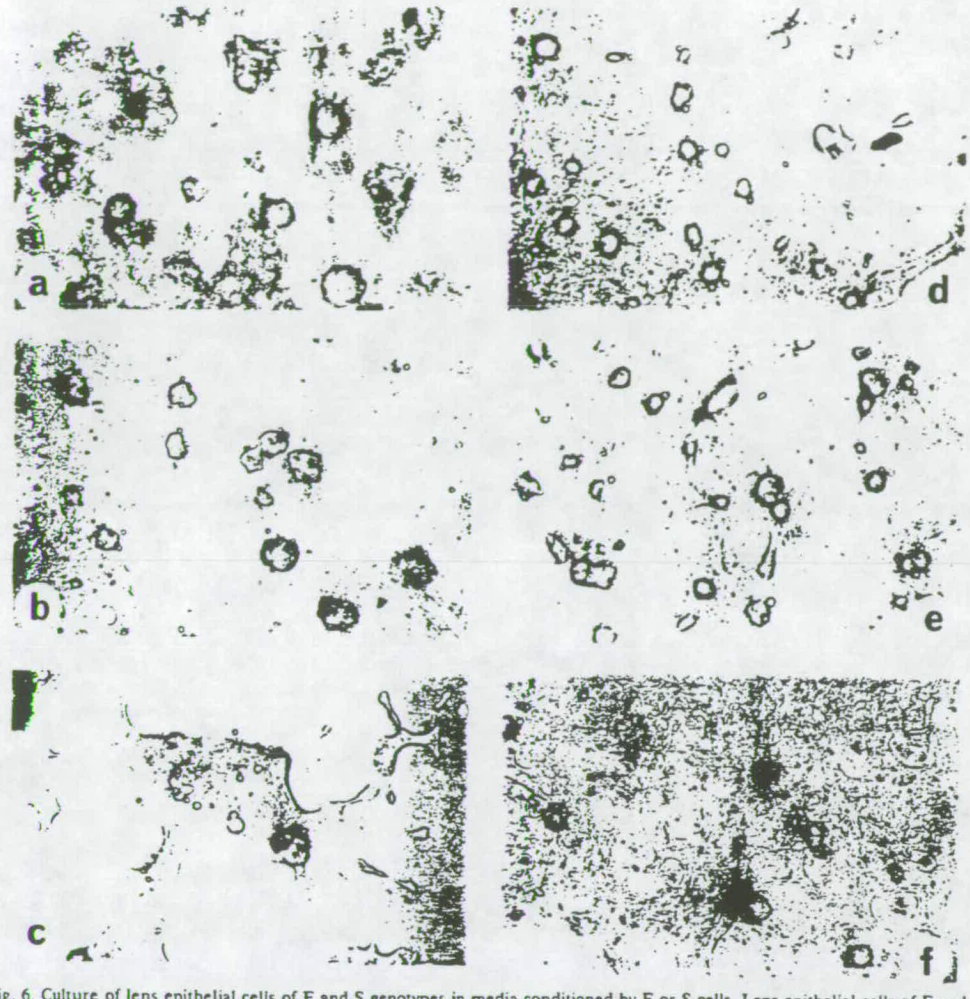


Fig. 6. Culture of lens epithelial cells of F and S genotypes in media conditioned by F or S cells. Lens epithelial cells of F and S genotypes (NRd and Hy-1) grown in culture medium conditioned by cells of either genotype. (A, B) Hy-1 and NRd cells in control medium, showing large lentoids in Hy-1 and small ones in NRd; (C, D) Hy-1 and NRd cells grown in the presence of Hy-1 conditioned medium. The effect seen is small but the number of lentoids is increased, although the size of the lentoids is similar to those of each genotype in control medium. (E, F) Hy-1 and NRd cells grown in NRd conditioned medium. Both cultures are inhibited, showing that NRd cells produce a genetically determined inhibitor of differentiation, but the effect is more severe on Hy-1 cells, pointing to a genetic difference in response. Similar effects were found for retina and chondrocyte cultures (Cuthbert et al., 1991).

lin levels both in lens and transdifferentiating neural retina (reviewed in Clayton, 1982a).

A progressive loss of differentiation capacity with age may reflect a diminution in the levels of the appropriate growth factor. For example, the effectiveness of retina extract in promoting lentoid

formation from neural retina in vitro falls with increasing retinal age (Karim and de Pomerai, 1990). It may also reflect a loss of cellular response. We examined the response of cells in successive subcultures of chick lens epithelium of the slow-growing NRd strain (see above) to retinoic acid (RA), insulin, cAMP, and a bovine retina extract (BRE), which presumably contains FGF (Patek and Clayton, 1990). RA, insulin and BRE all stimulated growth in second passage cultures. RA and BRE had a preferential effect on three of the β-crystallins, β3 (24 kDa), β5 (23 kDa) and β6 (22 kDa), but insulin had a preferential effect on β1 (34 kDa) expression. α-Crystallin expression was promoted by RA and BRE but BRE had a stronger effect on αB- than on αA-crystallin; finally, RA had a strong preferential effect on δ-crystallin. The only detectable effect of cAMP on second passage cultures was to cause a loss of αB and a diminution of levels of actin (43 kDa) and a 46-kDa polypeptide. Insulin, BRE, and RA increased cell number by 30%, 30% and 400% respectively, and RA and BRE also increased the number and size of lentoids differentiated, RA being the most effective.

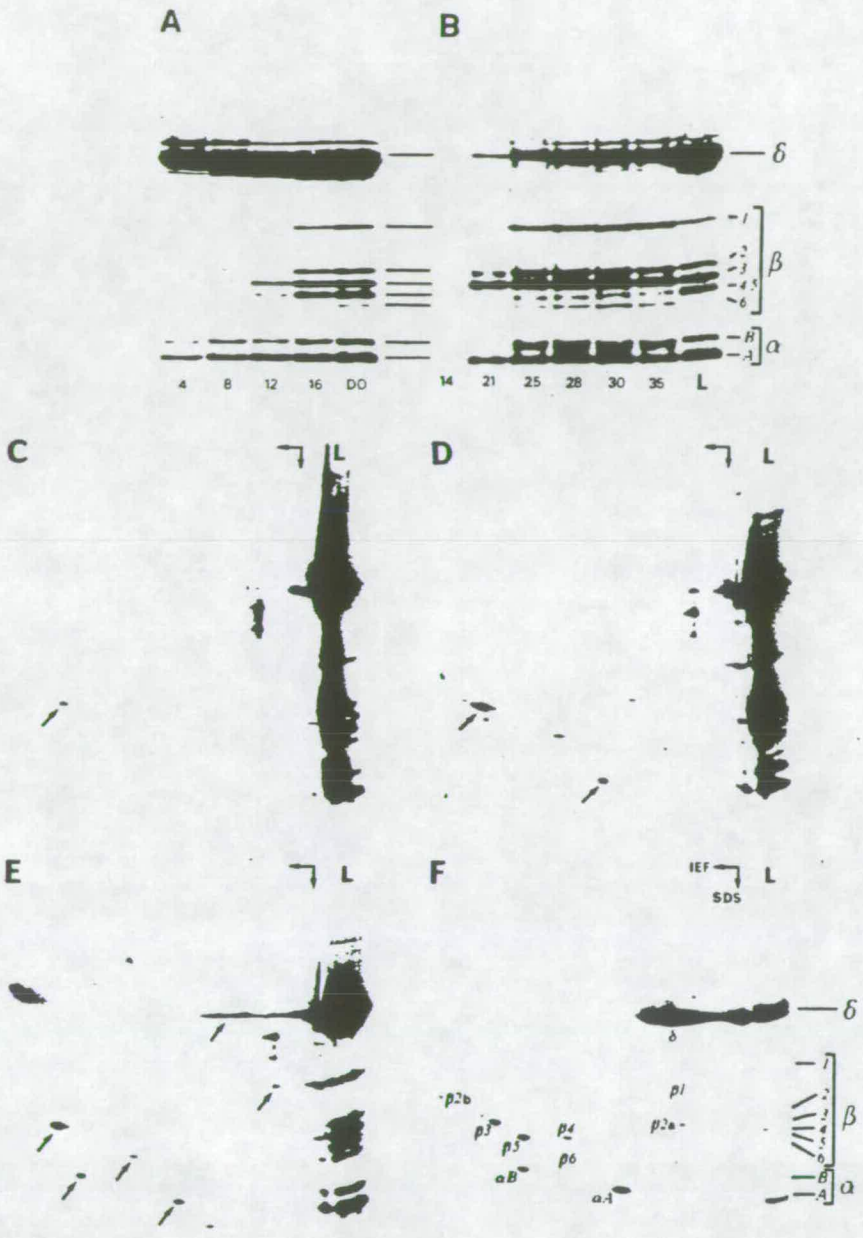
Although δ-crystallin and δ-crystallin mRNA were lost in control, insulin- and BRE-treated secondary cultures, irrespective of cell density or whether cells were passaged before or after the onset of lentoid formation, secondary cultures treated with RA still maintained δ-crystallin expression. Continuous rather than short-term acute insulin treatment also preserved the capacity for δ-crystallin synthesis in secondary cultures. These data suggest that in the initial stages, loss of expression of δ-crystallin is reversible.

None of the agencies affected crystallin synthesis in third passage cultures, but each agency had a specific effect on non-crystallin proteins. Only RA still promoted mitosis and maintained normal cell morphology in third passage cultures. The only effect found in fourth passage cultures (which still expressed very low levels of α- and β-crystallins) was that of RA, which preserved epitheloid morphology. The failure to affect differentiation in late passages could not be due to an absence of crystallins since α- and β-crystallins were still synthesised at very low levels, nor to a failure of response since non-crystallins were affected. Changes occur during early development in insulin and IGF-1 receptors in the chick ocular tissues (Bassas et al., 1987) and it is therefore possible that changes in receptors continue during ageing.

These data indicate that cellular response to a growth factor changes during ageing in vitro, that each growth factor has a characteristic spectrum of effect on the crystallins, that the synthesis of each crystallin can be affected by more than one growth factor, suggesting that some of the effects may be indirect, and also that each growth factor also affects other cell parameters, each with its own timetable of loss of response.

The persistent differences in growth rate be-

Fig. 7. Western blotting of crystallins in developing chick lens and transdifferentiatin neural retina. Comparison of the pattern of crystallin accumulation, (A) during embryonic lens development and (B) during transdifferentiation of 8-day embryonic chick neural retina cells (NRC) of the NJ genotype. Days of embryonic development are shown for lens and days in culture for NRC. Lenses were analysed by Western blotting of a SDS-PAGE separation 50 μg water-soluble protein per lane. DO (A) and L (B) indicate day-old post-hatch chick lens protein. (C, D, E) 14-, 21- and 28-day samples (200 μg/gel) of transdifferentiating NRC analysed by Western blotting of 2D gel electrophoretic separations using isoelectric focussing in the first dimension (IEF), followed by SDS-PAGE (SDS). The horizontal arrow indicates the direction of the acidic to basic pH gradient of the IEF separation. All Western blotting employed a polyvalent rabbit anti-chick lens protein antiserum, a horseradish peroxidase (HRP) conjugated donkey anti-rabbit antibody and 4-chloro-1-naphthol as the HRP substrate. The faint immunostaining on the left-hand margin of E is an artefact due to transitory contact of the 2D gel with the blotting membrane in the reversed orientation during the assembly of the blotting apparatus. (F) Day-old post-hatch chick lens proteins separated on a 2D gel and stained with Coomassie Brilliant Blue-R. Each 2D gel includes a reference lane of 50 μg DO lens protein separated by SDS-PAGE only. Comparison of these components in Fig. 7A with those in Figs. 1, 2, 3, and 4 confirms the identity of the crystallin polypeptides. Comparison of 7A and F with 7B, C, D and E confirms that transdifferentiating neural retina expresses crystallins. Diagonal arrows (C, D, E) indicate polypeptides accumulated by NRCs, identified as crystallins by their immunoreactivity and electrophoretic mobility. β3 is both the first crystallin detected by Western blotting during transdifferentiation of NRCs to lens (B and C) and the most abundant crystallin during the early stages of transdifferentiation (D and E).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tween lens cells of F and S strains and similar differences in retina cells and chondrocytes (Cuthbert et al., 1991) suggest that there may be genetically determined changes in the characteristics or the levels of autocrine growth factors, or in the cellular response to these factors. These three cell types, taken from two F and three S strains, were therefore each grown in conditioned media or on extracellular matrix, prepared from either F or S genotypes. The effects of these extrinsic factors on any one cell type depended on the genotype of the cells which provided the conditioned medium or the substrate, but the cellular responses to any one conditioned medium or substrate depended on the genotype of the responding cell. Fig. 6 shows that medium conditioned by F cells has a small effect on cell growth and differentiation: lentoid number is slightly increased, but lentoid size is not affected. Medium conditioned by S cells is inhibitory, but F cells are far more severely affected than S cells. These findings point to genetic differences both in autocrine factors and in receptors or post-receptor pathways.

A preliminary examination of possible differences in factors affecting growth and differentiation has been made by separating heparin-binding factors (HBF), which include FGFa and b, from F and S birds by reverse phase chromatography. The HBFs were found to differ from each other in the number, amplitude, and charge of the component peaks, and in the number of these components which were mitogenic for bovine lens cells in vitro (Mascarelli et al., 1989). Age-related changes were also found. These data may account for some at least of the genetic differences in growth rate, cell behaviour and rate of ageing.

Ageing in transdifferentiated neural retina cultures

Lentoids composed of lens fibre cells expressing crystallins may be obtained in vitro from lens epithelial cells (LEC), or from embryo neural retina cells (NRC) by the process of transdifferentiation (reviewed in Clayton, 1990). There are some differences between differentiation of LEC and transdifferentiation of NRC in the order of appearance of crystallin transcripts and detectable crystallins (Table 1A, B and Figs. 2 and 7) (Clayton et al., 1986a,b) but the overall similarities in the age-related changes in long-term culture of LEC and NRC are considerable (Table 2A, B, Fig. 3). Although δ-crystallin appears before α- and β-crystallins in lens development, in both sets of cultures, α- and some β-crystallins appear before δ-crystallin (Patek et al., 1991). δ-Crystallin transcripts and protein are lost first in ageing NR cultures and the expression of both α-crystallin polypeptides and of most of the β-crystallins is lost in later passages (Table 2A, B, Fig. 6). Aged cultures of both LE and NR express high levels of actin, and contain a high proportion of low-molecular-weight protein (Figs. 3 and 4) and both LEC and NRC cultures also retain some residual β-crystallin expression. This, however, is tissue-specific: in aged LE cultures β1 and β2 (34 kDa and 26 kDa) are still detectable but in aged NR cultures it is β3 (25 kDa) (Patek et al., 1991), which is also the major β-crystallin expressed in fresh retina (Head et al., 1991) (Figs. 3 and 7, Tables 2 and 3). There are also quantitative differences between some of the crystallin polypeptides between LE and NR lentoids, for example β1- is more abundant than β5- in NR lentoids but β5- more abundant than β1- in LE derived ~~tissue~~ irrespective of age difference in the tissues of origin, lentoid size or any other variable.

There are marked similarities between lentoid differentiation and crystallin expression from NR and from LEC in vivo and in vitro. With increasing donor age, the time required for transdifferentiation of NR to lens cells increases, and the ratio of β- to δ-crystallin rises, as it does in LEC lentoids, until the lentoids which form from embryonic NR in the last prehatch week express little or no δ-crystallin (de Pomerai and Clayton, 1978; Nomura and Okada, 1979). The high ratio of δ- to β-crystallin in the NR cultures and of β- to δ-crystallin in the LE cultures is probably due, at least in part, to the respective donor ages of these two tissues in the reported experiment.

Thus there are differences in the ontogeny of crystallin expression between LEC and NRC cultures (Table 1A, B) but once lentoid-rich cultures have been achieved the similarities of subsequent ageing changes (Table 2A, B) suggest a feed-back

mechanism, the dedifferentiation patterns of each generation of cells being affected by the state of their progenitor cells.

Conclusions

The data on ageing mammalian lenses suggest a greater randomness in the pattern of crystallin loss than found in the chick, but this may reflect differences between laboratories in the species, donor age and the culture methods used.

We may summarise our findings on chick lens cells in vitro as follows. Cultures set up in vitro from five different genotypes, each on more than one occasion, and cultures treated with growth factors (Patek and Clayton, 1985, 1986a,b, 1988, 1990) all underwent a similar programme of changes in crystallin expression, although continuous growth factor administration caused some delay. Transdifferentiating neural retina and differentiating lens epithelium show certain initial differences in ontogeny of crystallin expression but ageing long-term NRC cultures follow a similar programme to that shown by ageing LEC, the main exception being the identity of the β-crystallin most resistant to loss (Patek et al., 1991).

Lens function requires a series of orderly changes in the representation and quantitative balance in the molecular composition of successive lens cells. Whatever the mechanism, the overall similarities of the programme of change in vivo, and between NRC, LEC and different genotypes in vitro suggest that the mechanism is conserved, and that it continues to operate in the cells until they become senescent, or until transdifferentiation to fibroblasts occurs.

Acknowledgements

We thank the following bodies who at various times have supported different aspects of the work presented here: Age Concern, the AFRC and the MRC. We are grateful to the following for their expert technical and photographic assistance: A. Peter, M. McDonald, G. Brown and F. Johnstone. We are most grateful to Mary Ramsay for patiently dealing with the manuscript in its various drafts.

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