Effects of age and genetic growth rate on the crystallin composition of the chick lens

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ABSTRACT This two part study continues and extends our examination of the effects of age and of genetically determined intrinsic growth rate on the overall protein composition of chick lens epithelia, lens fiber masses and whole lenses. Water-soluble proteins were analyzed by SDS-polyacrylamide and 2-dimensional gel electrophoresis. First, detailed age-related changes in protein expression between day 4 of embryonic development and the 8-week adult stage are described for one normal chick strain. Secondly, comparisons are made between day-old post-hatch chicks of four different genotypes: two genetically unrelated chick strains with a high growth rate and propensity for hyperplasia of the lens epithelium and two unrelated slow-growing strains, both with normal lens morphology. We find that the B/δ-crystallin ratio in lens epithelia and fiber masses is higher in both the slow- than in both the fast-growing strains. The data emphasize the importance of quantitative and non-coordinate changes in crystallin polypeptide expression during lens growth and development, and implicates growth rate as a modifier of the pattern of crystallin expression.

KEY WORDS: chick lens, crystallin, ageing, genetic growth rate

Introduction

Chick lens development is marked by a series of changes in crystallin expression: these include ontogenic changes (δ-crystallin appearing first followed by β-, α- and finally by γ-crystallins) and quantitative changes including an increase in α-crystallin and notably in β-crystallin relative to δ-crystallin, the loss of δ-crystallin expression in the early adult cortical fibers, and post-translational changes in αA content (Voorter et al., 1987; Wistow and Piatigorsky, 1988; van Rens et al., 1991; Inoue et al., 1992; Piatigorsky, 1992). These changes, which are both spatially and temporally regulated, act to maintain the appropriate refractive property of the lens as it grows by continual recruitment of lens fiber cells from the LE (see Harding and Crabbe, 1984; Sivak et al., 1989).

However, little is known about the overall protein composition of the growing and aging chick lens at the level of individual crystallin polypeptides. Past studies based on SDS-PAGE have examined very restricted periods of development, and have generally failed to resolve simultaneously all the polypeptide members of all three major crystallin classes (α, β and δ), and most previous studies have analyzed proteins in the presence of urea which can generate artefactual heterogeneity among polypeptides through carbamylation (De Pomera et al., 1978; Thomson et al., 1978; Ostrer et al., 1981; Bagchi et al., 1982; Pal and Modak 1984). Furthermore, these studies have largely concentrated on synthetic changes, but the existence of post-transcriptional differentials do not permit extrapolation to the actual levels of the various crystallin polypeptides in functioning fibers, yet these are relevant to the optical properties of the lens and must therefore be the focus of adaptive evolutionary pressure. Using high resolution SDS-PAGE we described changes in the overall protein composition of chick lenses during early post-hatch development (Patek and Clayton, 1985). Here, using both one and 2-D gel electrophoresis, the study is extended to include changes from day 4 of embryonic development through to the 8-week adult stage. This continuous record is intended to provide a baseline for studies in lens aging, and in the response of the lens to conditions affecting growth rate, to genetic variations, and to various pathologies, or for comparison with ontogenic changes in the composition of lens fibers transdifferentiated from different extra-lenticular sources or at different stages of development. To date the analysis of post-hatch chick LFM by SDS-PAGE has revealed striking similarities between the protein composition of lens fibers formed in vivo and in vitro (Patek and Clayton 1985, 1986, 1988; Patek et al., 1986; Clayton et al., 1991).

While the factors responsible for regulating the various developmental changes in crystallin expression are still poorly understood there is evidence, partly based on analysis of mutant chick strains, for...
which implicates the cell cycle. Hy-1 and Hy-2 are genetically unrelated chick strains rigorously selected for high early growth rate. Both exhibit hyperplasia of the LE which is very marked in day-old post-hatch chicks (Clayton, 1975, 1979; McDevitt and Clayton 1979; Randall et al., 1979). Hy-1 and Hy-2 lens cells exhibit a higher rate of mitotic activity in vivo and in vitro than cells from two genetically unrelated slow-growing inbred strains, N-J (previously referred to as strain N) and N-Rd with normal lens morphologies (see Clayton, 1979; Randall et al., 1979; Patek and Clayton 1986, 1988). Reports that the \( \beta/\delta \) ratio is higher in both freshly excised LE and LE cell cultures derived from day-old post-hatch chicks of the N-J than Hy-1 strain (De Pomerai et al., 1977, 1978) suggest that a short mitotic cycle favors \( \delta \)-crystallin expression while a longer cycle favors \( \beta \)-crystallin (Clayton, 1979). However, the evaluation of the data is affected by several factors. Firstly, the in vitro data could be complicated by cell selection since chick LE is heterogeneous for \( \delta \)-crystallin expression in vivo, and Hy-1 cells are very heterogeneous in their capacity for lens fiber differentiation and growth rate, and show poor plating efficiency as compared with N-J cells (see Clayton, 1979; Patek et al., 1986). Secondly, culture age is an important consideration since we now know that after an initial increase, the level of \( \delta \)-crystallin actually falls in differentiated primary cultures while \( \beta \)-crystallins continue to rise (Patek and Clayton, 1985). Thirdly, our original in vivo data was based on hemagglutination inhibition analysis using polyclonal \( \beta \)-crystallin antisera and it is not clear which \( \beta \)-crystallin polypeptides were affected, and since the affinities of the antibodies used differed between the \( \beta \)-crystallin polypeptides some skewing of the data might have occurred if there were a very large difference in the relative proportions of the \( \beta \)-polypeptides between strains. Finally, since only two strains were compared then it was open to argument that the difference was strain-specific and not meaningfully related to the growth rate. Here we address the issue using SDS-PAGE and by examining freshly excised day-old post-hatch LE from four unrelated chick strains, fast- (Hy-1 and Hy-2) and slow-growing (N-J and N-Rd), in order to assess the effects on the overall crystallin polypeptide composition and determine whether there is a general relationship between mitotic rate and the \( \beta/\delta \)-crystallin ratio. For the first time the effect on the crystallin composition of the corresponding lens fibers was also investigated.

### Results

#### Age-related changes

The protein composition of LE and LFM resolved by SDS-PAGE, and of whole lenses resolved by 2-D gel electrophoresis are
shown in Figures 1-3. The resolution of the α-, β- and δ-crystallin polypeptides by SDS-PAGE and their immunological identification in day-old post-hatch chicks has been described (Patek and Clayton, 1985; Head et al., 1991). The 2-D gel profiles are in general agreement with earlier reports (Voorter et al., 1987; Rudner et al., 1990; Head et al., 1991; Inoue et al., 1992). The study does not deal with γ-crystallin since it is not expressed during embryonic development and comprises <1% of the total protein content of the early adult chick lens (see van Rens et al., 1991).

Numerous qualitative and quantitative changes in polypeptide expression occurred during lens development but only the major changes are detailed below. In relative terms, the levels of each of the α- and β-crystallin polypeptides increased throughout development but the amount of δ-crystallin rose initially between days 4 and 10 and then fell between the day-old post-hatch and 8-week adult stages (Figs. 1 and 2). All three polypeptides resolved in the δ-crystallin region (48 KDa, 50 KDa and 52 KDa) showed parallel changes. Thus a significant increase in the β/δ-crystallin ratio occurs in LFM as early as days 10-13 of development, and metabolic labeling studies find that the level of δ-crystallin synthesis falls in whole lenses between days 13-16 of development while α- and β-crystallins continue to rise (Fig. 4). τA and τB were both detected in 7-day embryonic lenses (Fig. 5A). At this stage τ-crystallin was more abundant than αB and δ-crystallin (only 65 detected) but less abundant than αA and δ-crystallin. τ-crystallin became the least abundant class of crystallin in day-old post-hatch lenses (Fig. 5C). β3 was not detected in 4-day embryonic lenses analyzed by SDS-PAGE but all major α-, β- and δ-crystallin polypeptides were present in LFM from at least day 7 (Fig. 1A). In contrast, β2 was the only β-crystallin detected in 7-day embryonic lenses analyzed by 2-D gel electrophoresis (Fig. 5A). This discrepancy between SDS-PAGE and 2-D gels presumably reflects the different protein loadings, 100 μg per lane in SDS-PAGE gels versus 150 μg total protein in 2-D gels.

Changes in the proportions of the α- and β-crystallin polypeptides observed in both SDS-PAGE and 2-D gels included an increase in the amount of αB relative to αA, but αA remained the most abundant throughout, and a shift from a high abundance of β3 to β3.
and βh between days 10-13, to a shift to a high abundance of β3 and βh by the 8-week adult stage (Figs. 1A and 5A and B). The latter changes in post-hatch LFM were more apparent at lower protein loadings (Fig. 1B). βh was the least abundant β-crystallin in the embryo whereas β3 (26.3 kDa) and βh were least abundant in post-hatch LFM and lenses (Figs. 1 and 5C). Development was also marked by an increase in the αA/αB ratio but αA was predominant in both 7-day embryonic and day-old post-hatch lenses (Fig. 5A and C). In contrast, the ratio of the 48 kDa, 50 kDa and 52 kDa polypeptides resolved in the δ-crystallin region remained unchanged at 10:1:0.5 during development (Fig. 2).

The patterns of accumulated protein in both SDS-PAGE and 2-D gels generally reflected those of protein synthesis (Figs. 4 and 5). However this correlation was not absolute; for example in 2-D gels βh was synthesized but not accumulated in 7-day lenses, βh showed high synthesis but low accumulation in day-old post-hatch lenses, and the intense synthesis of αA over αB was not matched by accumulated levels at either of the developmental stages examined. Finally, the appearance of spot e (19.2 kDa, pl 5.6) in post-hatch lenses did not correspond to any component in either the 2-D gel or SDS-PAGE synthetic profiles (Fig. 5C and D, see 2-D gels and SDS-PAGE reference marker lanes). The latter polypeptide, which corresponds to the 19.2 kDa polypeptide detected in adult LFM by SDS-PAGE, is therefore not a primary gene product, and it corresponds to the age-related post-translational product, αA 149-Asp, derived from αA by deamination, (Voorter et al., 1987), and more recently referred to as αA3 (Inoue et al., 1992).

We confirm reports by both these groups that this component first appears in day-old post-hatch chicks, and increases in abundance thereafter (Figs. 1 and 5).

**Effect of genetically determined growth rate**

All four strains (Hy-1, Hy-2, N-Rd and N-J) were very similar in the crystallin polypeptides present and their relative abundance in LE and LFM (Fig. 6). The only qualitative difference was the presence of the 19.7 kDa polypeptide in LE of the slow-growing

**Fig. 3** 2-D gel electrophoresis showing water-soluble proteins present in day-old post-hatch chick lenses from the normal N-J strain. Molecular sizes are indicated in kilodaltons (KDa). Separation was by IEF in the first dimension followed by SDS-PAGE (SDS), 150 μg protein per gel. Orientation at the IEF arrow denotes the acidic to basic pH gradient. DO, reference lane showing proteins present in LFM from day-old post-hatch chicks resolved by SDS-PAGE. δ-crystallin was resolved as a broad band (pl 3.5-6.1) resulting from protein overloading and possibly also reflecting the mixture of oligomeric species, mainly dimers, due to incomplete dissociation of δ-crystallin (see Patigorsky, 1984). Seven β-crystallin polypeptides were resolved: β1 (34 KDa, pl 4.9), β3 (24 KDa, pl 6.8), β4 (23.5 KDa, pl 6.2), β5 (23 KDa, pl 6.6), β6 (22 KDa, pl 6.2) and two in the β2 region designated β2B (9.0 KDa) and β2A (5.0 KDa). On the basis of relative abundance the β2A and β2B polypeptides probably correspond to the 26 KDa and 26.3 KDa polypeptides respectively which could be resolved by SDS-PAGE in the β2 region under conditions of high current density (Fig. 1C). α-crystallin was resolved as αA (19 KDa, pl 5.9) and αB (20 KDa, pl 6.5). Under optimal conditions (see B) e-crystallin (48 KDa) was resolved as two spots αA (pl 5.1) and αB (pl 6.2) as reported by Rudner et al. (1990). Spot αA (19.2 KDa, pl 5.6) corresponds to the 19.2 KDa polypeptide detected in adult LFM (Fig. 1) and is probably αA 149-Asp (Voorter et al., 1987). This component was not detected by SDS-PAGE in day-old post-hatch LFM but was detected at trace amounts in some day-old post-hatch lenses (Fig. 5C). As well as actin (43 KDa, pl 5.5) and vimentin (V, 57 KDa, pl 4.1), four products labelled αA (a, 36 KDa, pl 8.2: b, 26 KDa, pl 6.8: c, 24 KDa, pl 7.1: d, 21 KDa, pl 6.5) were routinely detected. Spots a and b were only evident at high protein loadings (150 μg in Fig 3A versus 100 μg in Fig. 5c). Spot a shares similar size and pl values with duck and swan e-crystallin (37-38 KDa, pl 7-8; see Stapel et al., 1985; Wistow and Patigorsky, 1988). The molecular sizes of the crystallin polypeptides are indicated in Fig. 1.

![Fig. 4 SDS-PAGE showing developmental changes in water-soluble protein synthesis by chick lenses from the normal N-J strain.](image-url)
strains (Fig. 6 lanes A-G). However there were several strain-specific quantitative differences. In general, the β/δ-crystallin ratio was higher in lens tissues from both the slow-growing compared to both fast-growing strains.

These quantitative differences between strains, although small, were confirmed by densitometry and were consistent between all the analytic studies made of numerous separate preparations. LE and LFM from both the fast-growing strains contained relatively more δ-crystallin than both the slow-growing strains, and the minor 50 kDa and 52 kDa polypeptides which could be resolved in the δ-crystallin region, were, like the 48 kDa polypeptide, also more abundant in LFM from Hy-1 than N-Rd (Fig. 6 lanes O and P). The relative contributions of the β-crystallin polypeptides varied both between strains and between LE and LFM of each strain. In LE, β2_a in N-Rd and β3 in N-J were more abundant than in the fast-growing strains. In LFM, β3 and β4 in N-Rd and β3_δ in N-J were more abundant than in the fast-growing strains. In contrast, the levels of α-crystallin and the non-crystallin polypeptides including actin (43 kDa) and vimentin (57 kDa) remained relatively unaffected in both LE and LFM.

Discussion

We report here for the first time on changes in the overall protein composition of chick LFM throughout embryonic development and readdress the issue concerning the relationship between growth rate and crystallin expression.

Brahma and van der Starre (1976) reported that the β/δ-crystallin ratio remains unchanged in the chick lens between days 6 and 16 of development but we found that the β/δ-crystallin ratio increases very significantly from at least day 13 of development, in terms of both accumulated and synthesized protein, and that the increase in α- and β-crystallins relative to τ- and δ-crystallins involves all the polypeptide members differentially. While δ-crystallin was the predominant crystallin class in both embryonic and day-old post-hatch lenses, τ-crystallin was more abundant than β-crystallin in 7-day embryonic lenses. Thus τ-crystallin is an unexpectedly abundant component of the early embryonic chick lens. β3 was not detected in 4-day embryonic lenses but all eleven major crystallin polypeptides [α2, α3, τ1, τ3, δ (48 kDa) and β3_δ] were represented in LFM and whole lenses by at least day 7 of development, at which
time the chick lens becomes uniformly transparent (see Harding and Crabbé, 1984; Stirling and Wakely, 1987). It remains to be determined whether these events are causally related. Secondary lens fiber production commences between days 3½ and 4 of development in the chick (see Stirling and Wakely, 1987). Thus the absence of β₁ in 4-day lenses may not only point to selective regulation within a class of related polypeptides but may indicate that β₁ is predominantly or exclusively expressed in secondary fibers. There is other evidence that β-crystallin polypeptides are selectively expressed in different compartments of the chick lens; for example ββ₁, which is linked with the process of fiber differentiation (Ostrer et al., 1981), is expressed in lens fibers before day 18 of development, and thereafter in LE but only in the annular pad (Brahma, 1988).

Lens development was also marked by an increase in the amount of α relative to β between the 7-day embryonic and day-old post-hatch stage. If this change continues during post-hatch development then this would account for the report that there is only one isoform of t-crystallin in adult chick lens fibers (Rudner et al., 1990). Other changes included an increase in the amount of βB relative to αA and a shift from a high abundance of β₂ in early embryonic LFM and lenses to high levels of β₂ and β₂ in adult tissues. Thus αA, β₂ and tB, like β-crystallin, may be regarded as largely embryonic and possibly nuclear components while αB, β₂, β₂ and tA may be regarded as predominantly adult and possibly cortical products. However it is difficult to speculate on their precise spatial expression since nuclear fibers, in aged mammalian lenses at least, still retain some capacity for protein synthesis (see Lieska et al., 1992). The 19.2 kDa polypeptide (spot p, p15.6) detected in post-hatch LFM and lenses, which here we directly show is not a primary gene product, is probably formed in the lens nucleus since it corresponds to αA 149-Asp which is derived from αA by age-related post-translational modification (Voorter et al., 1987).

The number of δ-crystallin polypeptides in the chick lens is presently unclear since the number observed varies with the electrophoretic technique, as well as the use of urea (Reszelbach et al., 1977; Thomson et al., 1978; Pal and Modak, 1984; see Piatigorsky, 1984). Using SDS-PAGE Reszelbach et al. (1977) resolved two δ-crystallin polypeptides in the 15-day embryonic chick lens, 48 kDa and 50 kDa (in the ratio 3:1), but only in the presence of urea. In the present study three polypeptides, 48 kDa, 50 kDa and 52 kDa, were resolved in the δ-crystallin region by SDS-PAGE in the absence of urea. Although they were the most abundant products present in δ-crystallin-rich 51/2, and 7-day embryonic LFM, the identities of the 50 kDa and 52 kDa polypeptides is uncertain. However their ratio remained fixed throughout development (10:1:0.5) suggesting that their expression might be regulated by a shared mechanism. If all three are δ-crystallin polypeptides then it would appear that the ratio of the δ-crystallin polypeptides, unlike that of the α₁, β₂- and t-crystallin polypeptides, is unaffected by development and aging.

Li and Beebe (1991) reported that crystallin mRNA stability did not change during development, and the strong correlation found here between crystallin polypeptide accumulation and synthesis supports the view that there is little post-transcriptional turnover in the lens (see McDermott et al., 1992). However we found that this correlation was not absolute, indicating that differential turnover of some polypeptides, including α₁, β₃ and β₄, appears to occur over a limited period of development.

Fig. 6 SDS-PAGE showing water-soluble proteins present in LE and LFM from day-old post-hatch chicks of slow-growing (N-J, N-Rd) and selected fast-growing (Hy-1, Hy-2) strains. All lanes contain 60 μg protein except for lanes L-N (120 μg) and O and P (4 μg). Molecular sizes are indicated in kilodaltons (kDa). DO. LFM from day-old post-hatch N-Rd chicks; ac, chick muscle G-actin (43 kDa); MW, molecular weight size markers as Fig. 2. In each gel, the strain(s) containing the higher level of a particular polypeptide is indicated by an arrow. Due to protein overloading in LFM it was only possible to assess quantitative differences in δ-crystallin in lanes O and P. All three 48 kDa, 50 kDa and 52 kDa polypeptides resolved in the δ-crystallin region were more abundant in Hy-1 than N-Rd and their ratio remained at 10:1:0.5.
The genetic study, based on four unrelated chick strains each with different intrinsic growth rates of the LE, found that strain differences only involved δ-crystallin and some of the β-crystallins, and that the β/δ-crystallin ratio was higher overall in LE from the slow- than the fast-growing strains. The results support the view that crystallin expression is affected by growth rate, with a short cell cycle time favoring δ-crystallin expression and a longer interval favoring β-crystallin (Clayton, 1979; see Introduction). This hypothesis is further supported by other independent evidence. Firstly, early embryonic chick LE cells which have a high growth rate, synthesize more δ- than β-crystallin, whereas the converse is true with slower growing cells from later stages of development (see Clayton, 1979; Harding and Crabbe 1984; Clayton et al., 1991). Secondly, agencies which shorten or, conversely, lengthen the mitotic interval of cultured chick lens cells also have such effects on the β/δ-crystallin ratio (reviewed Clayton et al., 1991). Thirdly, the β/δ-crystallin ratio rises more rapidly in long-term LE cultures derived from day-old post-hatch chicks of the two slow-growing strains compared to the two fast-growing strains (Patek and Clayton, 1988). Finally, the report by Sawada et al. (1992) that β-crystallin is only expressed by non-dividing chick lens cells in vivo and in vitro also supports this view. It is significant that the higher β/δ-crystallin ratio in LE of the slow-growing strains was also found in the corresponding LFM which implies that the properties of the post-mitotic fiber cells reflect the epithelial cells from which they are derived. This relationship, which presumably only applies to the recently differentiated cortical fibers, would explain the ontogenetic shift from high levels of δ-crystallin to β-crystallin expression in the chick lens which commences on about day 13 when LE mitotic activity is in decline (reviewed Harding and Crabbe, 1984).

The data emphasizes the importance of non-coordinate and quantitative changes in crystallin polypeptide expression both within and between crystallin classes, throughout the post-inductive stages of chick lens development, and implicates the cell cycle as an important factor in this regulation.

Materials and Methods

For aging studies we used the normal N-J strain and compared the composition of 4-day embryo lenses, LFM from day 51 of incubation to the 8-week adult, and LE, including most of the annular pad, from day-old post-hatch lenses. Growth rate studies were undertaken by using LE and LFM from day-old post-hatch chicks of the two fast-growing (Hy-1 and Hy-2) and two slow-growing (N-Rc and N-J) strains (see Clayton, 1979; Patek and Clayton, 1986, 1988). In all cases tissues were pooled from at least twelve chicks and stored immediately in liquid N2 until analyzed.

To assess protein synthesis freshly excised whole lenses were incubated in culture medium (Patek and Clayton, 1985) supplemented with L-[U-14C] mixed amino acids (0.18 MBq/mmol) (Amersham, UK) then stored in liquid N2 until analyzed. Water-soluble proteins were analyzed within 2 h of extraction to avoid possible artefacts due to degradation. Protein extraction and SDS-PAGE was performed as described previously (Patek and Clayton, 1985) and 2-D gel electrophoresis was by IEF in the first dimension followed by SDS-PAGE (Head et al., 1991). Proteins were stained with Coomassie Brilliant Blue R, and fluorography and densitometry were performed as before (Patek and Clayton, 1985). Molecular weight markers and chicken muscle G-actin were purchased from The Sigma Chemical Company, UK.

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