The optical properties of the transparent cellular eye lens depend on a diverse group of globular proteins called crystallins (1, 2). Almost all birds and reptiles have an additional major crystallin called δ-crystallin (see Ref. 15). In contrast to the α- and β-crystallins, which contain mainly a β-pleated sheet structure, δ-crystallin is highly α-helical, enriched in leucine, and low in aromatic amino acids. This tetrameric protein is encoded by two closely linked genes in chickens. It used to be thought that the presence of δ-crystallin in birds and reptiles was an exception to the normal cast of the α- and β-crystallins in cellular lenses. We now know, however, that it is not uncommon for different species to include a number of different proteins, known as taxon-specific crystallins, among their abundant lens proteins (see Refs. 1, 2, and 16). We have also learned that crystallins were selected in evolution from proteins with entirely different non-lens roles which can be retained in multiple tissues of the same organism (see Refs. 1, 2, 17, and 18). It has not been determined to what extent, if at all, the non-refractive functions of crystallins are required in the lens, or how their non-crystallin functions may have been important for recruitment as major lens proteins.

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**Lens Crystallins**

**INNOVATION ASSOCIATED WITH CHANGES IN GENE REGULATION**

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The properties of the transparent cellular eye lens depend on a diverse group of globular proteins called crystallins (1, 2). These abundant proteins comprise approximately 90% of the total soluble protein of the lens. Most of the crystallins are present in the lens fibers, which lose their cellular nuclei (as well as other organelles), preventing protein turnover and requiring crystallins in this region to be stable throughout life (see Ref. 3). The accumulation of different crystallins is temporally and spatially regulated in the lens during development, making crystallins useful for investigating differential gene expression during cellular differentiation (see Ref. 4).

All vertebrate lenses have representatives of three major families of crystallins, designated as α-, β-, and γ-crystallins. The β- and γ-crystallin polypeptides are members of a related β-γ-crystallin superfamily (5) (see Refs. 6 and 7). Crystallography has shown that each is composed of four structural motifs (called Greek keys) forming two domains per polypeptide (8, 9). Individual Greek key motifs are encoded by separate exons in all known β-crystallin genes; by contrast, each protein domain consisting of two motifs is encoded by a separate exon in the γ-crystallin genes. Both the β- and γ-crystallin genes have duplicated several times. Despite their phylogenetic relationships, in mammals most of the β-crystallin genes are dispersed, while the γ-crystallin genes, except for γS (formerly βS), are closely linked on the chromosome. The βS-crystallins appear to be related to microbial proteins induced by physiological stress, including osmotic shock (10).

The α-crystallins are encoded by two genes, αA and αB, situated on different chromosomes. In rodents (11, 12) and other mammalian species (13), alternative RNA splicing utilizing a separate exon (the αA-exon) ultimately gives rise to a second αA-crystallin polypeptide containing an extra internal peptide. In humans, which do not produce the αA-crystallin, this exon has been inactivated by a number of mutations and consequently has been termed a pseudo-exon (14).

Almost all birds and reptiles have an additional major crystallin called δ-crystallin (see Ref. 15). In contrast to the α and β-crystallins, which contain mainly a β-pleated sheet structure, δ-crystallin is highly α-helical, enriched in leucine, and low in aromatic amino acids. This tetrameric protein is encoded by two closely linked genes in chickens. It used to be thought that the presence of δ-crystallin in birds and reptiles was an exception to the normal cast of the α- and β-crystallins in cellular lenses. We now know, however, that it is not uncommon for different species to include a number of different proteins, known as taxon-specific crystallins, among their abundant lens proteins (see Refs. 1, 2, and 16). We have also learned that crystallins were selected in evolution from proteins with entirely different non-lens roles which can be retained in multiple tissues of the same organism (see Refs. 1, 2, 17, and 18). It has not been determined to what extent, if at all, the non-refractive functions of crystallins are required in the lens, or how their non-crystallin functions may have been important for recruitment as major lens proteins.

**Crystallins as Multifunctional Proteins: The Concept of Gene Sharing**

The first convincing indication that crystallins were recruited from proteins originally evolved for other functions was the report that the α-crystallins are related to the small heat shock proteins of *Drosophila* (19). Next it was found, surprisingly, that duck ε-crystallin (20), chicken δ-crystallin, turtle γ-crystallin, and squid SII-crystallin (21) were recruited from lactate dehydrogenase B4 (LDH), arginosuccinate lyase (ASL), α-enolase, and glutathione S-transferase (GST), respectively. Subsequent studies showed that other taxon-specific crystallins were also recruited from various metabolic enzymes, and thus these major lens proteins have been called enzyme-crystallins (see Refs. 1, 2, 17, and 18) for lists and reviews. This enzyme-crystallin activity in the lens. Other enzyme-crystallins in vertebrate lenses which have not been shown to possess activity include cytosolic aldehyde dehydrogenase/ε-crystallin in elephant shrews (24), hydroxyacyl-CoA dehydrogenase/γ-crystallin in rabbit (25), and NADPH-dependent reductase/β-crystallin in frogs (26). The apparently inactive enzyme-crystallins may require different substrates, cofactors, or assay conditions than those which were experimentally tested. It is also possible that enzymatic activity is lost with aging in the lens. γ-Crystallin, found in the guinea pig (27), is an interesting case. This enzyme-crystallin is structurally related to the alcohol dehydrogenases (28); however, it has quinone oxidoreductase activity (29).

Some enzyme-crystallins which retain significant amounts of activity in the lens are encoded by the same gene as that which produces the catalytic enzyme in non-lens tissues. For example, LDH/α-crystallin (30) and α-enolase/γ-crystallin (22) in the duck lens are each products of single genes. These generate a low amount of mRNA in non-lens tissues when producing an enzyme and a very high concentration of mRNA in the lens when producing a crystallin.

ASL/δ-crystallin is more complicated in that a gene duplication has occurred (see Ref. 6). In the chicken, the δ-crystallin gene is strongly preferentially expressed in the lens (31), while in the duck there appears to be more equal expression of the two genes (32). The ASL/δ2-crystallin gene is preferentially expressed in numerous non-lens tissues of chicken embryos (33, 34), and recent expression studies using chicken (35) and duck (36) clones have established that δ2-crystallin has ASL activity. Despite their enzymatic activity, it should be underscored that the chicken (33, 34) and almost certainly the duck (32) ASL/δ2-crystallin genes are expressed much more highly in lens than non-lens tissues (although non-lens δ-crystallin expression has not been studied in the duck yet). It is interesting to note that δ-crystallin is not uniformly distributed in non-lens cells (for references and further discussion see Ref. 34).

Although not associated with any enzymatic activity, αB-crystallin is expressed in many non-lens tissues (37–41). αB-crystallin appears particularly abundant in heart, where it forms aggregates as in lens (42), and is expressed in skeletal muscles where it responds to stretching (43). αB-crystallin gene expression has been associated with some neurological disorders (39, 44, 45), with NIH 3T3 fibroblasts expressing HA-ras and v-mos (46), and with fibroblasts from patients with Werner's syndrome (47), an inherited disease of premature senescence. An immunohistochemical study has shown that, except for its absence in liver, αB-crystallin is found in cells with high levels of oxidative function in rat (40). Recently, it has now been shown that αB-crystallin is a stress protein capable of being induced by heat (48) and osmotic (49) shock. Immunological experiments in rats have established that αA-crystallin, like αB-crystallin, is also expressed at a low level in many tissues and at higher levels in spleen and thymus (50). The precise non-refractive functions of the α-crystallins remain unknown.

The bifunctional use of a particular protein (such as occurs for the α-crystallins or some of the enzyme-crystallins) has been called "gene sharing" (23) (see Ref. 17). Two entirely different phenotypes for the same protein mean that that protein is under double selection. An example of this can be found in the mole rat (51). Despite the degenerate and presumably nonfunctional eyes...
of this subterranean rodent, its αA-crystallin gene sequence has drifted less than might be expected once released from selective pressure as a lens protein, apparently due to the non-lens expression of αA-crystallin (50). Another possible example of double selection during gene sharing is the amino sequence replacements believed to have controlled the LDH/CAT promoter constructs in the lens (30, 52). Gene sharing indicates that a protein may develop a second function without gene duplication or loss of its original function. In some cases, partial or complete separation of function may follow as a later event by subsequent gene duplication, as occurred in the δ- and α-crystallins (see Ref. 53).

Convergent Evolution: Lenses of Invertebrates

A number of invertebrate species have complex eyes that are remarkably similar in gross appearance to those of vertebrates (see Ref. 54). Resemblances between vertebrate and invertebrate eyes are believed to have resulted from convergent evolution. Lenses of complex eyes among invertebrates are derived from acellular secretory products, specializations of cellular projections, or intact cells (see Ref. 55).

Cephalopod (octopus and squid) lenses are composed of plate-like cellular derivatives (56, 57) and accumulate high concentrations of crystallins (called S-crystallins) (58, 59) related to the metabolic enzyme GST (21, 60). S-crystallins appear to lack expressed function and are encoded by 9-10bp sequences structurally similar to those of mammalian GST (61). Octopus lenses also accumulate considerable amounts of another crystallin, η-crystallin (62), which is related to mammalian aldehyde dehydrogenase (ALDH) 1 and 2 (63). η-Crystallin is, at best, a very minor component of the GST-like crystallins (64) or an ALDH-derived crystallin is also present in vertebrate lenses; ALDH/η-crystallin amounts to 25% of the soluble protein in the lens of elephant akreus (24), a primitive mammal (macroscelidid), making it the first crystallin known to be similar in vertebrates and invertebrates (63).

A recent study has identified major crystallins of 81, 63, and 28 kDa in the acellular lens of the nudibranch, Aplysia californica (64). (Nudibranchs, like cephalopods, are molluscs.) N-terminal sequences showed that the 80- and 63-kDa proteins are related but not similar to any sequences in the database. No sequence data are available yet for the 28-kDa crystallin of A. californica.

Finally, cubomedusan jellyfish have highly developed eyes with cellular lenses situated on structures called rhopalia, each of which contains a large and a small eye at right angles to one another (65). The lens in the large eye of the cubomedusan Tripedalia cystophora has approximate 35-, 20-, and 19-kDa crys- 


tallins appears at least equal to that of vertebrate crystallins. Crys-

tallin diversity within phyla (such as duck and chicken, or squid and vertebrates) suggests that these lens proteins were recruited by favor only the refractive role of LDH/CAT crystallin in the lens (77, 78) (see Refs. 4 and 79).

Several different sequences which bind lens nuclear factors appear essential for high lens expression of their respective crystallin genes (Table 1). Lens enhancers have also been identified at positions −922/−278 and −226/−123 of the mouse Y-γ-crystallin gene (70), at positions −80/−60 of the rat γ-crystallin gene (81), and positions −263/−85 of the hamster αA-crystallin gene (82), but no gel mobility shift assays have been conducted in these experiments. The mouse Y-γ-crystallin enhancers and the hamster αA-crystallin enhancer are interchangeable in their abilities to increase promoting strength and to expand the spatial domain of expression of the basal mouse Y-γ-crystallin promoter containing 171 bp of 5′-flanking sequence, but they do not change the lens specificity of the Y-γ promoter (82).

Transgenes in mice have demonstrated either lens specificity or enormous preference for lens activity of regulatory sequences from the chicken αA-crystallin enhancer (77) (see Ref. 4). Additional control mechanisms, such as silencers or its interaction with other components is essential for the lens-specific activity of the γ-crystallin gene (83). A zinc finger protein binding to this sequences shows that the regulatory elements include numerous motifs or sequences that bind transcription factors belonging to the NF-κB/dorsal/rel family which controls the expression of numerous genes (see Ref. 85). A single copy of the mouse αA-CRYBP1 sequence (Table 1) at position −66 to −57 was sufficient to increase promoter strength (86).

Transfection, mutagenesis, and gel mobility shift experiments have indicated that lens-specific expression of the chicken αA-crystallin gene is mediated by an enhancer between positions −162 and −79, with protein binding associated with a dyad of symmetry between positions −153 and −140 (75, 87) (Table 1). The putative chicken transcription factor appears to have a subunit size of 61 kDa and is present mainly in lens, slightly in brain, heart, liver and spleen (87). It is interesting that the 5′-regulatory sequences of the orthologous αA-crystallin gene differ as much as they do between chicken, mouse, and humans (88).

Lens specificivity of the chicken δ1-crystallin gene has been attributed to an enhancer with a 120-bp core in mtron 3 (89). Multimeric associations of the δ1 core enhancer strongly increased activity of the tk promoter/CAT fusion gene specifically in transfected lens cells (90). Interestingly, 12-mers of the δ1 enhancer core were able to compete with the SV40 enhancer in co-injection experiments despite the absence of common sequence elements (90). Site-directed mutagenesis experiments identified a 10-bp sequence (Table 1) within the core enhancer which is required for its lens-specific activity (91). This sequence forms a complex with one or more nuclear proteins (called δE/F) from both lens and non-lens cells, indicating that modification of δE/F or its interaction with other cellular components is essential for the lens specificivity of the δ1 enhancer (91). The δ2-crystallin gene contains a similar lens enhancer in its third intron which is interchangeable with the δ1 enhancer (33). Since lens expression of the ASL/ 

82-crystallin gene is much lower than the δ1 gene in the chicken (31, 33, 34), additional control mechanisms, such as methylation (93), or post-transcriptional processing (34), must exist.

Lens-specificity of the mouse Y-γ-crystallin gene has been demonstrated in the −121/+45 fragment in transgenic mice (82) and the −74/+45 fragment in transfection experiments using embryonic chicken lens epithelial cells (94). The proximal domain (−67/−25) is conserved in all γ-crystallin genes (see Ref. 80) and,
when duplicated, strongly stimulated basal activity of the mouse γF-crystallin promoter in transfected chicken lens cells but not in chicken fibroblasts (94). An 11-bp sequence has been identified which forms different nuclear complexes with extracts from lens, fibroblasts, liver, or brain (94). The protein(s) contributing to the lens complex has been called γF-1 (Table I).

As with the chicken δ-crystallin genes (93), recent experiments have also implicated methylation in the regulation of the rat γ-crystallin genes. Hypomethylation of the 5′-flanking sequences of γA-, γB-, and γD-crystallin genes has been associated with lens fiber cell differentiation, and hemimethylation of the γD-crystallin promoter inactivated its ability to drive the CAT gene in transfected retinal cells transdifferentiating into lens (95).

### Non-lens Expression of Crystallin Genes

Progress has been made on the basis for non-lens expression of the mouse αB- and chicken δ1-crystallin genes. In contrast to the δ1-crystallin enhancer core, multimers of the entire δ1 enhancer stimulated the tk promoter/CAT gene in numerous types of transfected cells and multimers of a 105-bp fragment (B3) just upstream of the core-activated activity in a subset of the non-lens cells (90). These and additional competition experiments for enhancer activity in co-injected cells indicated that non-lens expression of the δ1-crystallin gene is governed by a number of cooperating cis-regulatory elements in the third intron which are interacting with multiple trans-acting factors (90).

Investigations are also progressing on the non-lens expression of the αB-crystallin gene. Use of at least two transcription initiation sites results in a smaller αB-crystallin mRNA in tissues with more abundant amounts of αB-crystallin (lens, heart, skeletal muscle, kidney) and a larger αB-mRNA in tissues with lower expression of this gene (lung, brain, spleen) (98, 99), indicating two functional promoters. Minor amounts of αB-crystallin mRNA initiated between these two transcription start sites have been detected in y-mos oncogene expressing NIH-ST3 cells treated with dexamethasone (46). Thus, regulation of the αB-crystallin gene in different tissues appears to involve different regulatory sequences at the 5′-end of the gene. Current experiments indicate that the γ-crystallin gene also utilizes different promoters for expression in lens and liver of the guinea pig.

Transgenic mice carrying an homologous αB-crystallin minigene have established the existence of cis-regulatory elements as major control sites for the differential expression of the αB-crystallin gene in various tissues (38). Other transgenic mice carrying an αB promoter fragment/CAT transgene indicated that cis-regulatory elements between positions −661 and +444 direct αB-crystallin gene expression in lens and skeletal muscle (97). Multiple copies of the αB promoter/CAT transgene increased its activity in skeletal muscle and expanded its expression to include heart and, to a lesser extent, spleen and lung. The broader expression pattern of the minigene, which lacks introns but contains both 5′- and 3′-flanking sequences, suggests that non-lens regulatory elements are also present in the 5′-flanking region of the αB-crystallin gene. Transfection experiments using the CAT reporter gene have also suggested that 3′-regulatory sequences may exist in the human αB-crystallin gene (98). A combination of deletion and transfection experiments has provided evidence that determinants for lens expression exist between positions −2 and +44 and have also shown that a non-preferred lens enhancer is present between positions −426 and −257 of the mouse αB-crystallin gene (97). This is reminiscent of the αA- and γF-crystallin genes, where lens expression is attributed to more proximal sequences and enhancers governing quantitative and spatial expression are located more distally (see above). The αB-crystallin muscle enhancer contains a heat shock consensus sequence, an AP-2-like site, and a potential binding site (MRF) for a number of known muscle regulatory factors (97). A fragment (E2) of the αB-enhancer containing the AP-2-like site has been shown to bind a muscle nuclear protein (97). No cDNAs for factors responsible for the non-lens expression of crystallin genes have been cloned yet.

### Crystallin Recruitment and Changes in Gene Regulation

It has been postulated that regulatory mutations may underlie major changes in form or function (99-101). Indeed, large differences in the concentration of specific proteins (particularly enzymes) among species or tissues have been discussed in terms of their adaptive significance and possible role in evolution (102). The recruitment of crystallins from enzymes and stress proteins provides specific examples of the acquisition of a new function associated with a major change in gene regulation (see Ref. 53). It is not known, of course, whether increased expression in lens heralded the recruitment of an enzyme or stress protein as a crystallin or whether changes in gene regulation followed the discovery of its usefulness for refraction, thereby contributing secondarily to its recruitment as a lens crystallin. In either case, evolutionary changes in gene regulation were clearly an intimate component of crystallin recruitment.

Since crystallins are essentially defined as proteins present at high concentration in the lens, it is obviously necessary that their transcription factors function optimally under the specific conditions required for transparency. Perhaps, then, candidate crystallin genes are selected not only by the properties of their encoded proteins but also by the nature of their transcription factors. Redox state is one possible consideration for selective pressure on crystallin transcription factors, since oxidative stress can lead to cataract (103). In any event, changes in gene regulation appear to have been vital for the innovative use of enzymes and α-crystallin proteins and crystallins during the divergent and divergent evolution of the lens and deserve further study as a key ingredient for the selection and adaptation of crystallin genes.

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**REFERENCES**

1. Wistow, G. J., and Piastigorsky, J. (1988) *Annu. Rev. Biochem.* 57, 479-504
2. Bloemendal, H., and de Jong, W. W. (1991) *Prog. Nucleic Acid Res. Mol. Biol.* 41, 259-291
3. Piastigorsky, J. (1981) *Differentiation* 19, 134-153
4. Piastigorsky, J., and Driessen, P. S. (1991) *Adv. Biochem. Eng.* 1, 211-256
5. Driessen, H. P. C., Herbrink, P., Bloemendal, H., and de Jong, W. W. (1981) *Eur. J. Biochem.* 121, 85-91
6. Piastigorsky J. (1984) *Cell* 38, 620-621
7. Lubeen, N. H., Aarts, H. J. M., and Schoenmakers, J. G. (1983) *Prog. Biophys. Mol. Biol.* 5, 47-76
8. Blundell, T., Lindley, P., Miller, L., Mora, D., Slingsby, C., Tickle, I., Turrell, B., and Wistow, G. (1981) *Nature* 289, 771-777
9. Berg, B., Lapatto, R., Neimi, V., Driessen, H., Lindley, P. F., Mahendavan, D., Blundell, T. L., and Slingsby, C. (1986) *Nature* 324, 770-776
10. Wistow, G. (1990) *J. Mol. Evol.* 36, 140-146
11. Power, C. R., and Piastigorsky, J. (1985) *Cell* 22, 707-712
12. van Rossum, K., Hendriks, W., Warc, W., and Bloemendal, H. (1985)

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1 P. Gonzalez, C. Hernandez-Calzadilla, J. S. Zigler, Jr., and T. Borras, unpublished data.
