PII: S1350-9462(97)00004-9

Gene Sharing in Lens and Cornea: Facts and Implications

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CONTENTS

Abstract—The major water-soluble proteins (crystallins) responsible for the optical properties of the cellular lenses of vertebrates and invertebrates are surprisingly diverse and often differ among species (i.e., are taxon-specific). Many crystallins are encoded by the identical gene specifying a stress protein or a metabolic enzyme which has non-refractive functions in numerous tissues. This double use of a distinct protein has been called gene sharing. Abundant expression of various metabolic enzymes also occurs in a taxon-specific manner in corneal epithelial cells, suggesting that gene sharing extends to this transparent tissue. It has been proposed that one of the most abundant corneal enzymes (aldehyde dehydrogenase class 3) may protect the eye by directly absorbing ultraviolet light, as well as by providing an enzymatic function. It also seems possible that the high expression of corneal enzymes (5–40% of the water-soluble proteins) may reduce scattering in the corneal epithelium by minimizing spatial fluctuations in refractive index as they do in the lens. Thus, gene sharing may be a widespread phenomenon encompassing the lens, cornea and probably other systems. Lens-preferred expression of crystallin genes is integrated in a complex developmental program utilizing in many cases Pax-6. The differential ex-

Progress in Retinal and Eye Research Vol. 17, No. 2, pp. 145 to 174, 1998

Printed in Great Britain
1350-9462/98/$19.00 + 0.00

pression of αB-crystallin (a small heat shock protein) in different tissues involves the combinatorial use of both shared and lens-specific *cis*-control elements. Corneal-preferred gene expression appears to depend in part on induction by environmental influences. Among the implications of gene sharing are that gene duplication is not required for the evolution of a new protein phenotype, a change in gene regulation is sufficient, that proteins may be under more than one selective constraint, affecting their evolutionary clock, and that it would be prudent to consider the possibility that any given gene may have important, unrecognized roles when planning to implement gene therapy in the future.

1. INTRODUCTION

Certain rules for the specialization and evolution of proteins have been steadfastly believed. First, it has been generally accepted that genes encode proteins with distinct functions, even if those particular functions affect multiple biological processes, as might be expected for a metabolic enzyme. Second, no one would argue that the evolution of a new function for a protein occurs by a process of molecular tinkering resulting in small adaptive changes over time (Jacob, 1977). Finally, the prevalence of gene families implies that the freedom for a protein to adopt a new role depends on gene duplication (Kimura and Ohta, 1974). It has also been recognized that the expression of a gene is subject to change, and that this can have profound effects on developmental processes (Britten and Davidson, 1971; Zuckerkandl, 1994) as well as on evolution (King and Wilson, 1975; Wilson *et al.*, 1977, 1987). Recent studies on lens crystallins have provided a compelling case that changes in gene regulation, either without or before gene duplication, can also lead to new protein functions without loss of the original function by a process, described below, called "gene sharing" (Piatigorsky *et al.*, 1988). It is ironic that the crystallins, generally considered lens-specific, structural proteins specialized for the transparent and refractive properties of the lens, have become a model of functional diversity associated with evolutionary changes in gene regulation.

The transparent lens and cornea of the vertebrate eye are responsible for transmitting incident light into the eye and casting an image onto the photoreceptors of the retina (Land, 1988; Land and Fernald, 1992). In terrestrial vertebrates, the cornea refracts about two-thirds and the lens one-third of the incident light, while in aquatic animals the refraction is accomplished entirely by the lens owing to the similarity of the refractive index of the cornea and the surrounding water. The refractive power of the cellular lens is due to a gradient of protein concentration from the center to the edge of the tissue. Surrounded by a collagenous capsule, the lens is neither innervated nor vascularized, and has an anterior layer of cuboidal epithelial cells and a posterior array of fiber cells (Kuzak and Brown, 1994). Lens differentiation involves the cessation of cell division, extensive cell elongation, loss of organelles, including the nuclei in the central fiber cells, formation of cell junctions, and extreme accumulation of crystallins, which represent 80–90% of the water-soluble proteins of the lens (Piatigorsky, 1981). The gradient of protein concentration responsible for lens refraction is established by the differential accumulation of crystallins in the concentric layers of the fiber cells. Lens transparency is due to short range order interactions among the crystallins and reduced spatial density fluctuations in the cytoplasm (Benedek, 1971; Benedek, 1983; Bettelheim and Siew, 1983; Delaye and Tardieu, 1983; Clark, 1994).

The abundant lens crystallins have been generally viewed as static proteins serving a strictly structural role. Even their name—crystallins—reflecting their accumulation in the crystal-clear lens, implies that these soluble, globular proteins have a crystalline structure and inert role within the cells. Thus, although crystallins have been of general interest as markers of lens cell differentiation (Piatigorsky, 1981) and as evolutionary paradigms (de Jong, 1981, 1982; Lubsen *et al.*, 1988), their physicochemical properties have been studied largely in terms of their structural roles in transparency and cataract (Harding and Crabbe, 1984; Bettelheim, 1985; Spector, 1991; Clark, 1994).

Recently, comparative and sequence studies have revealed that the lens crystallins are much more diverse than previously recognized and that many

are related or identical to metabolic enzymes and stress proteins found in numerous tissues. These findings have been reviewed extensively (Wistow and Piatigorsky, 1988; Piatigorsky and Wistow, 1989; de Jong *et al.*, 1989; Bloemendal and de Jong, 1991; de Jong *et al.*, 1993, 1994; Groenen *et al.*, 1994; Wistow, 1993, 1995; Slingsby *et al.*, 1997). The fact that crystallins are multifunctional proteins has raised new questions with respect to crystallin evolution, the nature of crystallin gene regulation, and the roles that crystallins may be playing within the lens as well as in other tissues, which are examined in the present article.

A second goal of the present review is to consider the possibility that the use of enzymes and other specialized proteins for multiple purposes by a gene-sharing strategy is not limited to lens crystallins. The cornea, in particular, is examined in greater detail. The current literature indicates that, like the lens, the corneal epithelial cells accumulate different enzymes at concentrations approaching those of crystallins, suggesting that they have structural, as well as metabolic roles. I thus propose that gene sharing is a widespread phenomenon exploiting the different potentials of proteins and is used by the lens, cornea and probably other systems.

2. HISTORICAL DEVELOPMENT OF THE CONCEPT OF GENE SHARING

Unlike most proteins performing highly specialized tasks, such as globin or rhodopsin, the crystallins have presented the challenge of being very diverse and showing numerous species differences. While essentially all vertebrate lenses contain representatives of the α and β/γ crystallins, which are themselves heterogeneous, some species or selected taxonomic groups were found that also use entirely different proteins as lens crystallins (Piatigorsky, 1984a; Wistow and Piatigorsky, 1988). Table 1 provides a list of the different crystallins in vertebrates and invertebrates that are known today.

The first of these so-called taxon-specific crystallins was δ-crystallin, which was confined to bird and reptile lenses, as judged by immunological criteria (see Clayton, 1974). Chicken δ-crystallin was initially called FISC (for First Important Soluble Crystallin) on account of its appearance before the other crystallins during lens development in the chicken (Rabaey, 1962; Zwaan and Ikeda, 1968), and is probably the "typical songbird crystallin" found as an electrophoretic variant in a number of birds (see Clayton, 1974; de Jong, 1981). δ-crystallin has numerous physical properties, including considerable α-helical structure and surprisingly high proportions of leucine and isoleucine (see Piatigorsky, 1984b), clearly separating it from the α and β/γ-crystallins. Another observation of δ-crystallin that did not fit with the notion of lens-specific specialization for crystallins was the small amounts of it that were found in non-lens tissues (see Clayton *et al.*, 1986).

After δ-crystallin, an immunologically distinct, 37.5 kDa abundant protein was observed in frog lenses (Zigler and Sigbury, 1976). This was shown later by cloning to be a novel protein (Tomarev *et al.*, 1984). It was originally called ϵ-crystallin and then changed to ρ-crystallin. The subsequent discoveries of τ-crystallin in turtles (Williams *et al.*, 1982; Williams *et al.*, 1985) and lampreys (Stapel and de Jong, 1983) followed by-ϵ-crystallin in ducks and crocodiles (Stapel *et al.*, 1985; Brahma and Defize, 1985) established the concept of taxon-specific crystallins in vertebrate lenses and foreshadowed the growing list of these lens proteins (see Table 1). Studies on invertebrates added to crystallin diversity. A novel protein family designated as S-crystallins was shown to comprise the major water-soluble proteins of the squid lens (Siezen and Shaw, 1982) and then Ω-crystallin was discovered as a minor crystallin in the octopus lens (Chiou, 1988). It thus appeared that very different proteins could fulfill the refractive needs of the transparent eye lens, but left unanswered why the diverse lens crystallins are so highly conserved in evolution (de Jong, 1982; Lubsen *et al.*, 1988).

The next development came from sequence data showing that the small heat shock proteins of *Drosophila* are homologous to mammalian αA and αB-crystallin polypeptides (Ingolia and Craig, 1982). This was the first indication that lens crystallins are recruited from pre-existing proteins with non-refractive functions and it had a great

Table 1. *Crystallins in Cellular Lenses. The Phylogenetic Distributions and References of these Vertebrate and Invertebrate Crystallins are Given Elsewhere (Piatigorsky and Wistow, 1991; Wistow, 1995; Tomarev and Piatigorsky, 1996). ρB-crystallin was Described in (Roll et al.*, 1995)

Crystallin	Identity/(Homology)	Gene sharing (species)
	Vertebrates	
αA	molecular chaperone	+ (rat)
αB	small heat shock protein	+ (mammals)
βγ	(Protein S; spherulin 3A; EDSP; AIM1)	-
ϵ	lactate dehydrogenase B	+(duck)
δ1	(argininosuccinate lyase)	-
δ2	argininosuccinate lyase	+(duck, chicken*)
τ	α-enolase	+(duck)
ζ	NADPH:quinone reductase	+(guinea pig)
μ	(ornithine cyclodeaminase)	-
η	retinaldehyde dehydrogenase	+(elephant shrew)
ρ	(aldo-keto-reductases)	-
ρB	(aldose reductase)	-
λ	(hydroxyl CoA dehydrogenase)	-
π	(glyceraldehyde-3-phosphate dehydrogenase)	-†
	Invertebrates	
SL11/Lops4	glutathione S-transferase	+(squid, octopus)
S	(glutathione S-transferase)	-
Ω/L	(aldehyde dehydrogenase)	-
J	(novel proteins)	-

*Although there is relatively little chicken ASL/δ2-crystallin in the lens, it is still present at much higher concentration in the lens than any other tissue examined.

†Although π-crystallin appears to have glyceraldehyde-3-phosphate dehydrogenase activity as judged by tests on

impact on the vision science community. The following surprise came when it was shown that a number of the taxon-specific crystallins known at the time were similar to different metabolic enzymes. These computer-assisted matches indicated that previously obtained duck ϵ-crystallin peptides match with lactate dehydrogenase B (LDHB, the heart isozyme of LDH; Wistow *et al.*, 1987), and that the chicken δ1 and δ2-crystallin polypeptides are similar to human argininosuccinate lyase, the few determined turtle τ-crystallin peptides occur in α-enolase and the N-terminal sequence of squid S-crystallin is similar to that of glutathione S-transferase (Wistow and Piatigorsky, 1987). These homologies were reinforced with similarities in the electrophoretic behaviors of the crystallins and the enzymes, and with the demonstration that ϵ-crystallin possesses *in vitro* LDH activity. Together, the data showed that a number of lens crystallins are strikingly related to metabolic enzymes or stress proteins. The critical question that remained was whether the lens crystallins and their related enzyme or stress protein are encoded in different, related genes or whether the crystallin and enzyme are encoded in identical genes.

To answer this question, experiments were performed simultaneously at the gene level for duck ϵ- (Hendriks *et al.*, 1988) and chicken δ- (Piatigorsky *et al.*, 1988) crystallin. Southern blot hybridizations of genomic DNA indicated that the same, single-copy gene encodes ϵ-crystallin and LDH in the duck. The situation with chicken δ-crystallin was more complex. Previous experiments had shown that δ-crystallins are encoded in two tandemly linked genes (Nickerson *et al.*, 1985; Nickerson *et al.*, 1986), with the 5′ δ1-crystallin gene being expressed much more actively in the embryonic lens than the 3′ δ2-crystallin gene (Parker *et al.*, 1988). Southern blot hybridizations

using cDNA and oligonucleotide probes of human ASL and chicken δ-crystallin indicated that the chicken δ2-crystallin gene is orthologous to the human ASL gene (Piatigorsky *et al.*, 1988). Moreover, enzyme tests showing little ASL activity for chicken δ-crystallin was consistent with the known, relatively low expression of the ASL/δ2-crystallin gene in the lens (Piatigorsky *et al.*, 1988). By contrast, duck δ-crystallin, also encoded in two genes (Piatigorsky *et al.*, 1987), contained high ASL activity in lens extracts (Piatigorsky *et al.*, 1988). This suggested that, unlike the situation in the chicken, the ASL/δ2-crystallin gene is expressed highly in the duck lens. Subsequent experiments confirmed that the δ2-crystallin gene encodes the active ASL enzyme, while the δ1-crystallin gene encodes an enzymatically inactive protein in chicken (Kondoh *et al.*, 1991) and duck (Barbosa *et al.*, 1991; Piatigorsky and Horwitz, 1996), and that the ASL/δ2-crystallin gene is expressed as highly as the δ1-crystallin gene in the embryonic duck lens (Wistow and Piatigorsky, 1990; Li *et al.*, 1995b).

These data provided convincing evidence that the lens crystallin and enzyme are encoded in the same gene for LDH/ϵ-crystallin (Hendriks *et al.*, 1988) and ASL/δ2-crystallin (Piatigorsky *et al.*, 1988). We called the use of the same protein for refraction or for enzymatic activity "gene sharing" to describe the phenomenon of having the identical gene responsible for entirely different functions (Piatigorsky *et al.*, 1988; Piatigorsky and Wistow, 1989).

3. GENE DUPLICATION AND GENE SHARING

3.1. Taxon-Specific and Enzyme-Crystallin Genes

LDH/ϵ-crystallin showed that gene duplication need not occur after recruitment of the enzyme for a refractive role in the lens. Southern blot hybridization tests of genomic DNAs have indicated that there are other single-copy genes that produce enzymes when expressed at low concentrations in some tissues and structural crystallins when expressed at high concentrations in the vertebrate lens. These include α-enolase/τ-crystallin in the duck (Wistow *et al.*, 1988; Kim *et al.*, 1991), NADPH:quinone oxidoreductase/ζ-crystallin in the guinea pig (Borras *et al.*, 1990; Gonzalez *et al.*, 1994), and μ-crystallin, which is homologous to bacterial ornithine cyclodeaminase, in the Grey Kangaroo (Kim *et al.*, 1992). These enzyme-crystallins are also found in other selected species (see Wistow, 1995) making it possible that they have undergone gene duplications and separation of functions in some species which have not yet been examined at the gene level.

In some cases, as indicated above by ASL/δ-crystallin, gene duplication followed recruitment of the enzyme for a crystallin role, with subsequent specialization of the duplicated gene (δ1-crystallin) for high expression in the lens. It is unlikely that the ASL gene duplicated before ASL became a lens crystallin because: (1) both duck δ-crystallin genes are expressed equally in the lens (Li *et al.*, 1995a); (2) the chicken ASL/δ2 gene, although not expressed as highly as the δ1-crystallin gene, is still expressed more highly in the embryonic lens than in any other tissue that has been examined (Piatigorsky *et al.*, 1988; Li *et al.*, 1993); and (3) the third intron of both the chicken δ1-crystallin (Hayashi *et al.*, 1987) and ASL/δ2-crystallin genes contains an enhancer that directs equally high expression of reporter genes specifically in transfected lens cells (Thomas *et al.*, 1990) and transgenic mice (Li *et al.*, 1997). Moreover, both duck δ-crystallin polypeptides have undergone adaptive changes increasing their thermal stability, a characteristic of lens crystallins (Voorter *et al.*, 1993; Piatigorsky and Horwitz, 1996). These data fit the idea that an ancestral ASL gene achieved high lens expression in the evolutionary lineage, giving rise to reptiles and birds before the occurrence of gene duplication. However, it is not known when ASL/δ-crystallin gene duplication occurred in the reptilian/avian lineage, whether ASL/δ-crystallin gene duplication took place independently in different species, or whether there are species in which it remains as a single-copy enzyme-crystallin gene. δ-crystallin with high ASL activity has been reported in geese (Yu and Chiou, 1993) as well as ostriches (Chiou *et al.*, 1991), but these have not been investigated with respect to gene copy number. In the chicken

(Nickerson *et al.*, 1986) and duck (Piatigorsky *et al.*, 1987; Li *et al.*, 1995b), the two δ-crystallin genes are tandemly arranged in the same order (5′- δ1 - ASL/δ2 - 3′) and are separated by approximately 4.5 kbp of surprisingly conserved spacer DNA (Li *et al.*, 1995b). While it is not known if there is any benefit to such an arrangement, it is consistent with the possibility that a duplication event took place relatively recently in the common ancestor of these two species. It would be especially interesting to examine chimney swifts for δ-crystallin gene dosage, since these birds are unique in having lost expression of δ-crystallin in their lenses (Wistow *et al.*, 1990). In the rat, which does not use δ-crystallin in the lens, ASL is encoded in a single-copy gene whose structure is extremely similar to that of the chicken (Matsubasa *et al.*, 1989).

Aldehyde dehydrogenase 1 (ALDH1)/η-crystallin is another enzyme-crystallin which has undergone gene duplication and specialization for lens and eye expression in elephant shrews (Graham *et al.*, 1996). Like LDH/ϵ-crystallin and ASL/δ2-crystallin, ALDH1/η-crystallin is catalytically active *in vitro*, where it has retinaldehyde dehydrogenase activity (Graham *et al.*, 1996). ALDH1/η-crystallin comprises up to 25% of the soluble protein of the elephant shrew lens and is distinct from ALDH1-nl (for non-lens), which is the predominant ALDH1 transcript in the liver. There are also at least two ALDH1 genes in the rat, but these do not show a difference in expression pattern in the liver and eye, as do the two genes in elephant shrews, and the rat does not use ALDH1 as a crystallin. Thus, in contrast to the situation with ASL/δ-crystallin, the ALDH1/η-crystallin gene of elephant shrews may have been recruited for its crystallin role after duplication of the original gene (ALDH-nl) encoding the enzyme, although it remains possible that the present ALDH-nl gene selectively lost its high expression in the lens after duplication. The enzyme-crystallin encoded in the duplicated ALDH1/η-crystallin gene of elephant shrews has kept its enzymatic activity, unlike that encoded in the duplicated δ1-crystallin gene of chickens (Kondoh *et al.*, 1991) and ducks (Barbosa *et al.*, 1991; Piatigorsky and Horwitz, 1996). It is interesting that ALDH is the only known enzyme to date which has been selected as a lens crystallin in both vertebrates and invertebrates.

In invertebrates, Ω-crystallin, which is homologous to ALDH1/2, is a minor crystallin in the eye lens of cephalopods, especially octopus (Chiou, 1988; Zinovieva *et al.*, 1993), and a major, if not sole crystallin (L-crystallin) in the light organ lens of certain squid (Montgomery and McFall-Ngai, 1992) and the eye lens of scallops (Piatigorsky and Horwitz, unpublished; see Tomarev and Piatigorsky, 1996 for review of invertebrate crystallins). Enzymatic activity has not been demonstrated for these ALDH-like invertebrate enzyme-crystallins, and it is not known yet for certain whether gene duplication has been a part of their evolutionary history. There are some data suggesting that the Ω-crystallin gene has separated from the ancestral ALDH gene and has specialized for lens expression. Cephalopod Ω-crystallin appears as a single-copy gene by Southern blot hybridization and is lens-specific by Northern blot analysis in octopus and squid (Zinovieva *et al.*, 1993). Moreover, ALDH activity using various substrates is extremely low in lens extracts but relatively high in extracts of the squid digestive gland.

Glutathione S-transferase (GST)/S-crystallin of cephalopods is another enzyme-crystallin which may have been recruited for a refractive role by acquiring high expresssion in the lens after one or more gene duplications (Tomarev *et al.*, 1992; Tomarev and Piatigorsky, 1996). The S-crystallin genes have undergone numerous duplications after recruitment, resulting in at least 24 different polypeptides in the squid *Loligo opalescens* (Tomarev *et al.*, 1995). The deduced amino acid sequences of the S-crystallins range from 46 to 99% identity among themselves. This suggests that the gene duplications have occurred progressively throughout evolution, although it is possible that different S-crystallin polypeptides were subjected to different selective pressures owing to as yet unknown differences in function. Tests with lens extracts initially indicated that, in contrast to the authentic GST in the squid digestive gland, S-crystallins as a group contain negligible glutathione S-transferase activity, using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate (Tomarev *et al.*, 1992). Subsequent cDNA ex-

pression studies demonstrated some enzymatic activity with a CDNB substrate for an orthologous, minor S-crystallin in two different species (SL11 crystallin in *Ommastrephes sloani pacificus* and Lops4-crystallin in *Loligo opalescens*), while the expressed cDNAs for the major SL20-1 and Lops12 S-crystallins were inactive (Tomarev *et al*., 1995). Loss of GST activity of the S-crystallins has taken place by the insertion of a central peptide by exon shuffling and by sequence changes throughout the polypeptides (Tomarev and Zinovieva, 1988; Tomarev *et al*., 1992; Tomarev *et al*., 1993, 1995). Crystallographic studies of squid GST suggest that the variable central peptide of S-crystallins exists as a loop between α-helical regions on the surface of the protein (Ji *et al*., 1995; Tomarev and Piatigorsky, 1996), but this requires confirmation by X-ray analysis.

Northern blot hybridization experiments have shown that the inactive S-crystallins are lens-specific in the squid (Tomarev *et al*., 1992) and octopus (Tomarev *et al*., 1991). By contrast, the active digestive gland GST enzyme is only expressed at low concentrations in the lens (Tomarev *et al*., 1993). The present SL11/Lops4 gene, which lacks the exon coding for the central peptide of the S-crystallins and encodes a protein containing some GST activity, may be the original daughter GST gene which acquired high expression in the lens. However, it remains possible that the GST gene was originally recruited to be a lens crystallin and has secondarily lost its high expression in the lens. It seems reasonable that the genes encoding crystallins recruited earlier in evolution, such as GST/S-crystallins in cephalopods, will have undergone additional duplications, possible reversions, and adaptive modifications in structure and expression. The enzyme-crystallins of vertebrates may ultimately follow the same fate as the S-crystallins of the ancient cephalopods.

The only other taxon-specific crystallins that have been investigated at the gene level are the novel J-crystallins of the cubomedusan jellyfish. Jellyfish crystallins are especially interesting because of their antiquity, although it is not known when these species actually developed eyes and cellular lenses. Of the three crystallins identified (J1, J2 and J3) in *Tripedalia cystophora* (Piatigorsky *et al*., 1989), three closely related J1-crystallin genes (J1A, J1B and J1C) (Piatigorsky *et al*., 1993) and one J3-crystallin gene (Piatigorsky and Norman, unpublished) have been cloned. The deduced amino acid sequences of the three J1-crystallin family members are extremely similar (84–98%). However, the untranslated sequences of their mRNAs and the 5′ flanking sequences of their genes are very different from one another, indicating that they duplicated a very long time ago. It is not known yet whether any of the jellyfish crystallins are multifunctional proteins with non-lens functions. The fact that the three J1-crystallin genes all show high lens expression indicates that their duplication took place after recruitment for a crystallin role.

3.2. "Ubiquitous" Crystallins

Since lens crystallins have been studied much more intensively in vertebrates than in invertebrates, the α and β/γ-crystallins are commonly referred to as ubiquitous. Careful examination of squid crystallins, however, showed that these can be absent from eye lenses (Siezen and Shaw, 1982; Tomarev and Zinovieva, 1988), and all subsequent experiments on the cellular lenses of invertebrates indicate that the α and β/γ-crystallins are confined to vertebtrate lenses. It is surprising that no members of the α or the β/γ-crystallins have been found in an invertebrate eye lens, since these ubiquitous vertebrate crystallins belong to superfamilies of proteins that are expressed in invertebrate and microbial organisms.

3.3. The α-Crystallins

The α-crystallins, which played such a pivotal role in the discovery of crystallins as borrowed proteins (Ingolia and Craig, 1982), have continued to be instructive in delineating mechanisms used for gene sharing. There are two α-crystallin genes (αA and αB), which have similar, three exon structures and encode proteins that are approximately 55% identical in amino acid sequence (see Wistow and Piatigorsky, 1988; Wistow, 1995 for reviews). In

humans, the αA-crystallin gene maps to chromosome 21 and the αB-crystallin gene maps to chromosome 11, indicative of their ancient duplication. Expression studies in non-lens cells showed that the αB-crystallin polypeptide is not only homologous to small heat shock proteins but is a functional small heat shock protein (see de Jong *et al.*, 1993; Sax and Piatigorsky, 1994 for reviews). αB-crystallin is induced by heat and other physiological stresses in cultured cells (Klemenz *et al.*, 1991; Dasgupta *et al.*, 1992; Lin *et al.*, 1993), and can protect the cells from elevated temperatures (Aoyama *et al.*, 1993; Iwaki *et al.*, 1994) and hypertonic stress (Kegel *et al.*, 1996). αB-crystallin is also overexpressed in many pathologies, including neurodegenerative diseases, fibroblasts of patients with Werner's disease showing premature senescence, and growth abnormalities (see above reviews for references). In addition to being overexpressed under abnormal conditions, αB-crystallin is constitutively expressed in heart, skeletal muscle, kidney, lung and many other tissues (Bhat and Nagineni, 1989; Dubin *et al.*, 1989; Iwaki *et al.*, 1990b). Indeed, αB-crystallin is found in the embryonic mouse heart even before the eye has formed and its expression pattern follows a complex, transcriptionally regulated developmental program (Haynes *et al.*, 1996; Benjamin *et al.*, 1997). In contrast to αB-crystallin, except for low-level expression in the thymus, spleen and retina (Kato *et al.*, 1991; Srinivasan *et al.*, 1992; Deretic *et al.*, 1994), αA-crystallin is highly specialized for expression in the lens (Dubin *et al.*, 1989) and is not stress-inducible. Despite its specialization for a refractive role, αA-crystallin, like αB-crystallin, is a molecular chaperone and can protect proteins (Horwitz *et al.*, 1992; Horwitz, 1993; Jakob *et al.*, 1993) and cells (van den IJssel *et al.*, 1994) against thermal stress. Together, these observations suggest that the α-crystallin genes in mammals were recruited for a lens function by first having an ancestral small heat shock protein becoming highly expressed in the lens, duplicating, and having one of the daughter genes (αA) specialize for refraction, while the other daughter gene (αB) maintained its original, non-lens functions, as well as taking on its additional refractive role in the lens. Surprisingly, few studies have been conducted on non-mammalian α-crystallin genes. In one case, the duck αB-crystallin gene appears to have lost its constitutive expression and stress-inducibility in cultured embryonic cells (Wistow and Graham, 1995). Like the taxon-specific enzyme-crystallins, the gene-sharing strategy used by the small heat shock protein/α-crystallins differs in detail among species.

In general, the recruitment, gene duplication and subsequent specialization for lens expression of the α-crystallins from small heat shock proteins in mammals are similar to the ASL/δ-crystallin story in chickens and ducks, but there is at least one important difference in the two cases. The specialized δ1-crystallin polypeptide lost its pre-duplicated enzymatic activity, which suggests that its role in the lens is solely as a structural, refractive protein. By contrast, although the αA-crystallin polypeptide has lost its heat shock response, it has retained its ability to act as a chaperone despite the specialized lens expression of its gene, allowing it to serve a refractive as well as a protective, chaperone role in the lens.

A recent transgenic mouse with a targeted disruption of the mouse αA-crystallin gene has provided the first evidence for what might have driven α-crystallin gene duplication and lens specialization of αA-crystallin gene expression (Brady *et al.*, 1997). The αA-crystallin null mice developed cataracts and inclusion bodies containing αB-crystallin in the lens fiber cells. This finding suggests that the accumulation of αA-crystallin in the lens is necessary to keep αB-crystallin in solution at the concentrations required for it to play a refractive role and may be the reason that the 20 kDa αA and αB-crystallin polypeptides are present as aggregates in the normal lens (Spector *et al.*, 1971; Zigler, 1994). The specialization of αA-crystallin expression in the lens after gene duplication may serve less to separate the functions of the two α-crystallin genes, as predicted by adaptive conflict (Piatigorsky and Wistow, 1991; Wistow, 1993), than to aid the solubilization of the small heat shock protein/αB-crystallin in the lens so that it can act as a refractive protein. It remains to be tested whether a similar need has driven gene duplication and lens specialization in other crystallins.

3.4. The β/γ-Crystallins

The β and γ-crystallins were considered to be separate classes of proteins until sequence and crystallographic studies revealed that they belong to the same superfamily characterized by having "Greek key" structural motifs (see Piatigorsky, 1984a; Wistow and Piatigorsky, 1988; Lubsen *et al.*, 1988; van Rens *et al.*, 1992; Slingsby *et al.*, 1997 for reviews). The β/γ-crystallins differ from the α-crystallins in that they are a relatively large, multigene family. Several other proteins have been found that contain Greek key motifs, placing them in a β/γ-crystallin superfamily. These include a bacterial spore coat (Protein S; Wistow *et al.*, 1985; Bagby *et al.*, 1994a; Bagby *et al.*, 1994b), a slime mold cyst protein (spherulin 3A; Wistow, 1990), an epidermis differentiation-specific protein (EP37; Ogawa *et al.*, 1997; EDSP; Wistow *et al.*, 1995) and, most recently, a protein associated with human melanoma (AIM1; Ray *et al.*, 1997). These relationships raise the possibility that the β/γ-crystallins have been recruited from an ancestral protein by a gene-sharing mechanism, as have the other crystallins, followed by a number of gene duplications, but the nature of this putative ancestral protein is not known. No non-refractive function has been found yet for any of the β/γ-crystallins, although non-lenticular expression of βB2-crystallin in chicken (Head *et al.*, 1991a) and mice (Head *et al.*, 1995) and of γ-crystallin RNAs in *Xenopus* embryos (Smolich *et al.*, 1994) have been reported.

4. GENE SHARING IN THE CORNEA: CORNEAL "CRYSTALLINS"?

4.1. Vertebrates

The cornea is a transparent, avascular tissue derived from the embryonic head ectoderm responsible, at least in terrestrial vertebrates, for focusing light onto the retina (Land, 1988; Land and Fernald, 1992). In contrast to the lens, which relies on a smooth gradient of crystallin concentration for its refractive power, the cornea depends on the air:cell interface and its curvature for refraction. The vertebrate cornea is well described in a linked series of three articles (Gipson and Sugrue, 1994; Joyce, 1994; Olsen and McCarthy, 1994). It has an anterior stratified squamous epithelium and a relatively thick extracellular layer composed of collagen fibrils, numerous proteoglycans, glycosaminoglycans and keratocytes. The posterior border of the stroma is lined with Descemet's membrane followed by a single layer of endothelial cells which are responsible for keeping the collagenous stroma dehydrated. Investigations on lens transparency have necessarily focused on the interactions among the crystallins and have pointed to the importance of short-range interactions to minimize refractive index fluctuations within the cells (Bettelheim and Siew, 1983; Delaye and Tardieu, 1983; Clark, 1994). Studies on corneal transparency have concentrated on the extracellular stroma since it comprises the bulk of the tissue. The cornea is transparent because it is thin, because the stromal collagen fibrils are smaller than the wavelength of light, and because of negative interference of scattered light owing to an ordering of the collagen fibers over relatively short distances (Benedek, 1971; Farrell, 1994; Freund *et al.*, 1995). Owing to the differences in the anatomy and mechanisms of refraction used by the cornea and lens, sparse attention has been given to the idea that the cornea may also contain crystallin-like proteins, especially in the epithelial layer, where a gene-sharing interpretation may be appropriate. The evidence suggests that corneal epithelial cells do indeed accumulate enzymes at crystallin concentrations.

Early immunological studies established that a specific antigen called BCP 54 (for bovine corneal protein, 54 kDa) comprises 20–40% of the total soluble protein of the bovine cornea (Holt and Kinoshita, 1973; Alexander *et al.*, 1981). BCP 54 was found in the bovine corneal epithelium, stroma and endothelium, in the lens epithelium and, at lower levels, in the conjunctiva, but not in non-ocular tissues (Silverman *et al.*, 1981). Subsequent studies on the bovine cornea indicated that BCP 54 is ALDH3 (Abedinia *et al.*, 1990; Verhagen *et al.*, 1991). ALDH3 is also a major component of human (Holmes, 1988; Cuthbertson *et al.*, 1992; King and Holmes, 1993), baboon (Holmes and Vandeberg, 1986;

Algar *et al*., 1991), rat (Messiha and Price, 1983; Evces and Lindahl, 1989; Cooper *et al*., 1991), mouse and kangaroo (Cuthbertson *et al*., 1992), pig, sheep and cattle (Holmes *et al*., 1989) and gray short-tailed opossum (Holmes *et al*., 1991) corneas. ALDH3 is a tumor and xenobiotic-inducible, cytosolic enzyme. It differs from ALDH1, which is the main constitutive and inducible liver ALDH, and from ALDH2, which is a constitutive mitochondrial ALDH (Hempel and Lindahl, 1989; Lindahl, 1992; Yoshida, 1992). While ALDH1 and ALDH2 are tetramers utilizing NAD^+ as a cofactor, ALDH3 functions as a dimer and can use NAD^+ and $NADP^+$ as cofactors (see Lindahl, 1992 for review). In contrast to ALDH1 and ALDH2, ALDH3 has a preference for medium-chain (C6–C9) aliphatic aldehydes derived from lipid peroxidation (Lindahl and Petersen, 1991).

It is important to underline that corneal ALDH3 differs from ALDH1 (retinaldehye dehydrogenase)/η-crystallin of the elephant shrew lens (Graham *et al*., 1996) and from the tetrameric ALDH/Ω-crystallin of cephalopod lenses; the latter is 50–60% identical to ALDH1/ALDH2 and only about 25% identical to ALDH3 in amino acid sequence (Zinovieva *et al*., 1993). ALDH3 appears to have been recruited for corneal expression by a gene-sharing mechanism. In addition to being expressed at high concentration in the cornea, it is inducible and constitutively expressed at lower concentrations outside of the cornea, including especially the stomach as well as other ocular and non-ocular tissues (Holmes *et al*., 1988, 1991; Algar and Holmes, 1989; Hsu *et al*., 1992; Lindahl, 1992; Yoshida, 1992; Sladek *et al*., 1995), where it certainly has an enzymatic function. ALDH3 is a single-copy gene in the mouse (Holmes, 1988; Vasiliou *et al*., 1993; Vasiliou *et al*., 1995b), rat (Jones *et al*., 1988; Hempel and Lindahl, 1989), gray short-tailed opossum (Holmes *et al*., 1991) and human (Hsu *et al*., 1992). The marked accumulation of ALDH3 in the transparent cornea suggests that it has more than a strictly enzymatic function in the cornea. The prevalence of BCP 54/ALDH3 in the cornea led Silverman *et al*. (1981) to suggest that it may have major role in the structure or transparency of the tissue and Rabaey and Segers (1981) to name it transparentin.

Another striking similarity to the lens enzyme-crystallins is that BCP 54/ALDH3 is taxon-specific. This was discovered initially when BCP 54 was found at high concentration in the corneas of human, swine and pigs, but not in those of chicken, toad and fish (Alexander *et al*., 1981). More recent studies have also documented the absence of ALDH3 in the cornea of chicken, the garter snake, *Xenopus*, and a number of bony fish (Cuthbertson *et al*., 1992; Cooper *et al*., 1993). Interestingly, two fish (chain pickeral and redhorse) have exceedingly high enzymatic activity for substrates specific for ALDH1 and ALDH2 (Cooper *et al*., 1993), indicating that these species have selectively increased a different member of the ALDH family in the cornea. Another parallel with enzyme-crystallins of the lens is that ALDH3 may be replaced by entirely different proteins in different species and, in some cases, these are the same as the lens crystallins in that species. For example, chickens accumulate δ1-crystallin in the cornea (Cuthbertson *et al*., 1992; Li *et al*., 1993). Moreover, in chicken but not mice, up to 10% of the soluble protein of the corneal epithelial cells is type A peptidyl prolyl *cis–trans* isomerase, also known as cyclophilin (Cuthbertson *et al*., 1992). An example of taxon-specificity of abundant corneal proteins is shown in Fig. 1.

A list of abundant proteins in the cornea is shown in Table 2. A major unidentified bovine corneal protein called BCP 11/24 has a tendency to co-purify with BCP 54 and has a similar spatial distribution within the eye as BCP 54, but it does not immunologically cross-react with BCP 54 (Bakker *et al*., 1992). α-enolase is found at relatively high concentrations (Cuthbertson *et al*., 1992), especially in the basal and limbal cells (Zieske *et al*., 1992; Zieske, 1994). Recently, transketolase (TKT), which was first identified as a 65 kDa abundant protein in the human and mouse cornea (Cuthbertson *et al*., 1992), has been cloned from the mouse and shown to comprise up to 10% of the total soluble protein of the mouse cornea (Sax *et al*., 1996). The highly preferred expression of TKT in the corneal epithelium of the mouse is shown in Fig. 2. TKT is

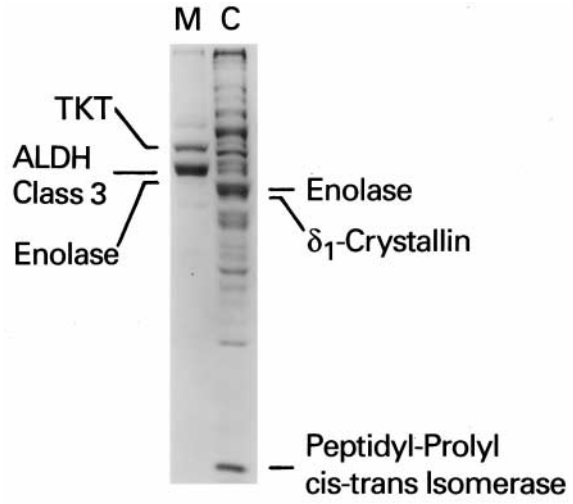

Fig. 1. Coomassie blue-stained sodium dodecylsulfate/10% polyacrylamide gel electrophoresis of proteins from epithelia of cornea from 18-day-old embryonic chicken (C) and 21-day-old mice (M). Note that none of the major bands of corneal proteins co-migrate from these two species

encoded in a single-copy gene in the mouse (Salamon *et al.*, 1997). It is both less enriched in the cornea and more widely distributed in ocular and non-ocular tissues than is ALDH3 (Guo *et al.*, 1997).

4.2. Invertebrates

Invertebrates with complex eyes containing cellular lenses also have cellular corneas with various degrees of complexity. Cephalopod corneas have epithelial cells and a stromal matrix and are derived from a different ectodermal tissue than the lens (Arnold, 1984). Immunoblotting experiments revealed unexpectedly that the major water-soluble proteins of adult squid cornea co-migrate with the S-crystallins in the lens (Cuthbertson *et al.*, 1992). Since the S-crystallins are a very large family (Tomarev *et al.*, 1995), it is not known whether the corneal and lens S-crystallins are encoded by the same genes, or whether one of the abundant S-crystallin-like proteins is GST. We are presently investigating the possibility that the epithelial cell layer comprising the corneas of jellyfish (Laska and Hundgen, 1982; Piatigorsky *et al.*, 1989) and scallops (Barber *et al.*, 1967) contain crystallins that have been found in their respective lenses.

The ommatidia within the compound eyes of insects have corneas composed of secreted proteins (see Tomarev and Piatigorsky, 1996). In *Drosophila melanogaster*, the secreted corneas contain three calcium binding proteins (52, 47 and 45 kDa; Komori *et al.*, 1992). The glycosylated, serine-rich 52 kDa protein is the most abundant of these three proteins. It is synthesized in the pupa, apparently specific for the cornea in the adult, and called drosocrystallin. Immunogold electron microscopy indicated that drosocrystallin is secreted from vesicles of the primary pigment cells. Drosocrystallin appears to be absent from some species of *Drosophila* and two other species of dipteran flies, indicating that it is taxon-specific.

In some invertebrates containing complex rather than compound eyes it is the lens rather than

Table 2. *Abundant Proteins in Corneal Cells. See Text for References*

Vertebrates
Aldehyde dehydrogenase class 3 (mammals)
Aldehyde dehydrogenase class 1/2 (some fish)
BCP 11/24 (bovine)
Transketolase (mammals)
α-enolase (mammals, chickens)
Peptidyl-prolyl *cis–trans* isomerase (also known as cyclophilin; chicken)
δ1-crystallin (chicken)
Invertebrates
S-crystallins (squids)
Drosocrystallin (secreted from *Drosophila melanogaster*)

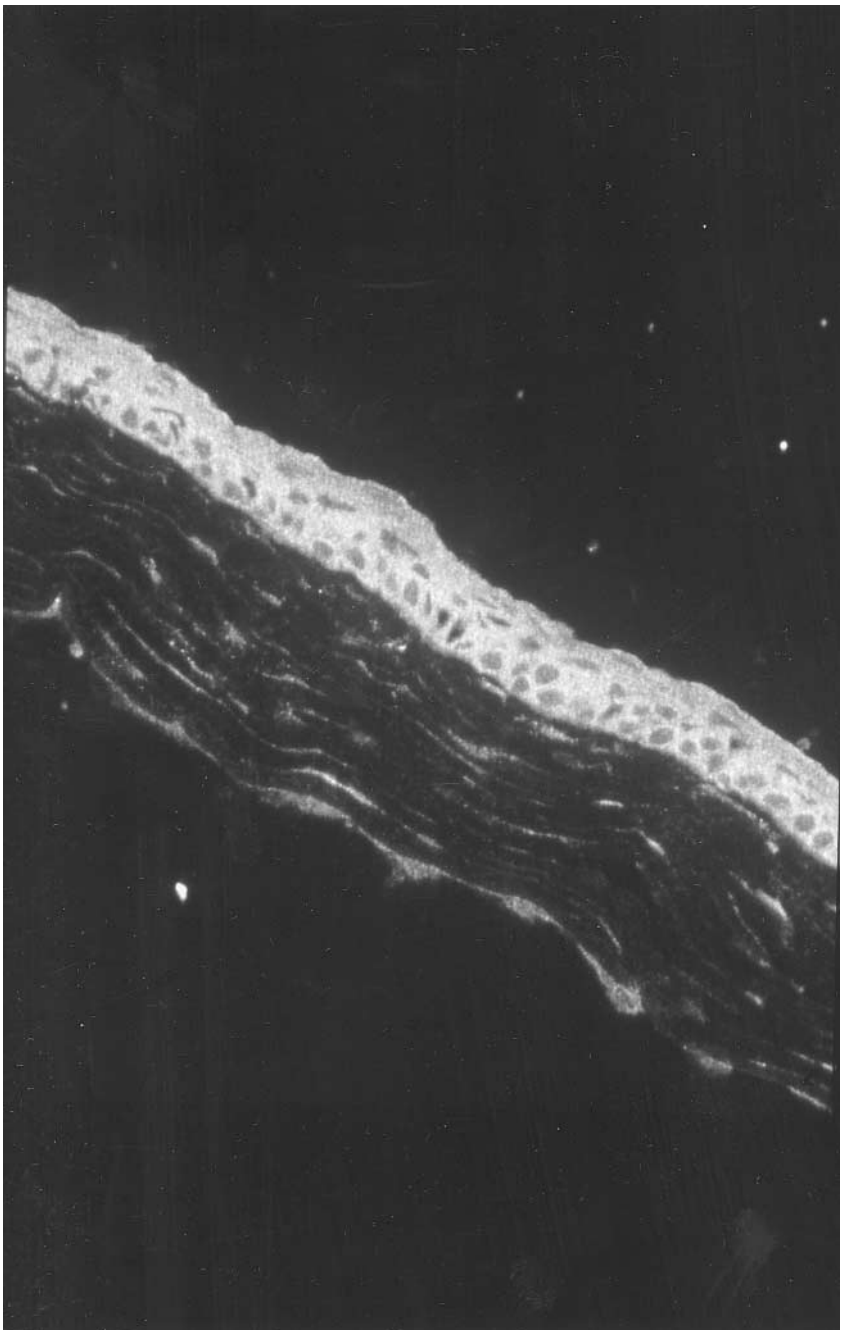

Fig. 2. Immunofluorescence analysis of TKT expression in the mouse cornea. A section of the 17-day-old cornea was subjected to immunofluorescence analysis as described (Gipson *et al*., 1993) using a 1:500 dilution of an anti-TKT antiserum (Sax *et al*., 1996) and a 1:50 dilution of an anti-rabbit IgG-FITC conjugated secondary antibody, and the section was subsequently viewed via confocal microscopy (Sax, Tisdale, Gipson and Piatigorsky, unpublished)

the cornea that is secreted. Electron microscopic observations have provided evidence that acellular lenses are secreted from the corneas of a pulmonate snail (Eakin and Brandenburger, 1967), a nudibranch (essentially a snail without a shell) (Eakin *et al.*, 1967), two species of marine snails (Gipson, 1984; Blumer, 1996), and three species of peripatus (Phylum Onychophora)(Eakin and Westfall, 1965). The corneal cells believed to secrete the lens in these species are extensions of the retina, which may also contribute to the lens material, as do may other ocular tissues (see Blumer, 1996). The secreted lens of the marine opisthobranch, *Aplysia californica* (sea hare), has been shown to contain three crystallin polypeptides of 80, 63 and 28 kDa (Cox *et al.*, 1991). The corneal secretion of proteins comprising the acellular lenses of invertebrates is another indication that corneas may be specialized for crystallin synthesis. Indeed, primitive corneas may have synthesized crystallins even before the evolution of cellular lenses. In this connection, it would be very interesting to explore the composition of soluble proteins in the transparent corneas of invertebrate eyes that lack lenses altogether, such as the ocellus of the sea star, *Henricia leviuscula* (Eakin and Westfall, 1964). It has yet to be established whether the secreted crystallins of invertebrates have non-refractive functions.

5. POSSIBLE NON-REFRACTIVE ROLES FOR CRYSTALLINS AND ENZYMES IN THE LENS AND CORNEA

5.1. Lens

While gene sharing has resulted in many of the lens crystallins being multifunctional proteins with non-refractive roles outside of the lens, it is still uncertain to what extent the enzyme-crystallins use their non-refractive properties in the lens or were selected on the basis of their non-refractive functions. The diversity, taxon-specificity and apparent overabundance of enzyme activity support the possibility that neutral evolutionary events independent of non-refractive abilities contributed to the recruitment of enzymes and stress proteins as lens crystallins (Wistow *et al.*, 1990; Wistow and Kim, 1991). However, a comparative analysis of the lens promoters of NADPH quinone oxidoreductase/ζ-crystallin in the guinea pig and llama led to the suggestion that selection of this enzyme-crystallin was based on its function (Gonzalez *et al.*, 1995). The crux of the argument was that the multiple adaptive changes in gene regulation, which differed in the two species, could only have occurred if the recruited enzyme-crystallin provides a specific benefit to the lens. This idea is consistent with the *in vitro* activity found in many of the enzyme-crystallins. Guinea pig NADPH: quinone oxidoreductase/ζ-crystallin appears to be active in the intact lens (Rao and Zigler, 1992) as well as *in vitro* (Rao *et al.*, 1991).

A possible non-refractive role of enzyme-crystallins includes the generation of or ability to interact with metabolites that stabilize the crystallins or that affect other functions within the lens. NADPH is an example of a small molecular weight compound that heat stabilizes ζ-crystallin (Rao *et al.*, 1991) and makes it susceptible to the chaperone activity of α-crystallin (Rao *et al.*, 1994). Pantethine and glutathione are additional examples of metabolites which affect the chaperone activity of α-crystallin (Clark and Huang, 1996). Another possibility is that retinoic acid generated from retinaldehyde dehydrogenase/η-crystallin plays a role in cellular differentiation or gene expression in the elephant shrew lens (Graham *et al.*, 1996). Further discussions of possible non-refractive roles of enzyme-crystallins in the lens that include the creation of light filters or the accumulation of energy stores via binding of NADH/NADPH, detoxification, osmoregulation, and cytoskeletal remodeling during lens cell elongation can be found elsewhere (Wistow and Piatigorsky, 1988; de Jong *et al.*, 1989; Zigler and Rao, 1991; Wistow and Kim, 1991; Wistow, 1993, 1995).

The α-crystallins provide a convincing case for a lens crystallin using its non-refractive ability within the lens to prevent protein aggregation under a variety of environmental stresses (Horwitz, 1992; Wang and Spector, 1994, 1995; Borkman *et al.*, 1996) and to protect against enzyme inactivation by post-translational modifications such as glycation (Ganea and Harding,

1995; Blakytny and Harding, 1996) and carbamylation (Ganea and Harding, 1996). Without α-crystallin's protective role, these insults, especially those involving oxidative stress, could lead to protein aggregation and cataract. Indeed, α-crystallins undergo numerous age-related post-translational modifications (Horwitz, 1993; Groenen *et al.*, 1994) and have reduced chaperone activity (Horwitz, 1992; Cherian and Abraham, 1995), which probably contribute to the prevalence of cataract in older individuals. The α-crystallins may also play a functional role in the stability and remodeling of the cytoskeleton during fiber cell differentiation in the lens (see Haynes *et al.*, 1996; Wang and Spector, 1996 for discussion and references). In *in vitro* tests, α-crystallin has an ATP-dependent inhibitory effect on the polymerization of vimentin and glial fibrillary acidic protein (Nicholl and Quinlan, 1994) and a stabilizing effect on actin fibrils, especially after treatment with cytochalasin D (Wang and Spector, 1996). The cAMP-dependent phosphorylations (Spector *et al.*, 1985; Voorter *et al.*, 1986) and autokinase activity (Kantorow and Piatigorsky, 1994) of the α-crystallin polypeptides, both of which occur on serine residues, may modulate functions of the α-crystallin polypeptides (see Wang and Spector, 1996; Takemoto, 1996 for further discussion). Different serines are phosphorylated in the cAMP-dependent and autokinase reactions of αA-crystallin, suggesting that these phosphorylation mechanisms may influence different biological processes (Kantorow *et al.*, 1995). The autokinase activity of αA but not αB-crystallin is stimulated approximately 10-fold by conversion into tetramers by treatment with deoxycholate. By contrast, the αB-crystallin polypeptides appear to be better substrates than the αA-crystallin polypeptides for phosphorylation by cyclic AMP-dependent kinase (Kantorow *et al.*, 1995). These findings raise the possibility that the αA and αB polypeptides have different functions and that one or both may participate in a yet to be discovered signal transduction pathway, which would bestow these structural proteins with a metabolic role. DNA-binding studies have even suggested that αA-crystallin is directly involved in the transcriptional regulation of γ-crystallin expression (Pietrowski *et al.*, 1994).

These data strongly suggest that at least some of the non-refractive properties of crystallins are used in the lens as they are in non-lens tissues. Another issue concerns the possible use of a lens crystallin that has specialized for refraction in the lens but is also expressed in other tissues, such as δ1-crystallin. Despite the fact that δ1-crystallin has lost its ASL activity by site-specific mutations in highly conserved regions of the protein (see Piatigorsky and Horwitz, 1996), it is expressed in different tissues of the embryonic chicken (Thomas *et al.*, 1990; Head *et al.*, 1991b; Li *et al.*, 1993) and duck (Li *et al.*, 1995b). The duck δ1 and ASL/δ2-crystallin polypeptides interact without preference to form all combinations of tetramers resulting in ASL isoenzymes (Williams and Piatigorsky, 1979; Piatigorsky and Horwitz, 1996). These isoenzymes derive no known benefit from the presence of the δ1-crystallin polypeptide. They are equally heat-stable and their ASL activity is directly related to the amount of δ2-crystallin polypeptide within the tetramer (Piatigorsky and Horwitz, 1996). Additional studies are required to determine whether δ1-crystallin has a non-refractive function, or whether its expression outside of the lens is an evolutionary quirk.

5.2. Cornea

In contrast to the situation for the lens crystallins, the challenge in the cornea is to find a structural rather than a non-refractive or enzymatic role for the abundant proteins. The cornea is in direct contact with the environment and is the first line of defense against incident light. Moreover, early studies have indicated that most of the UV irradiation, especially below 290 nm, is absorbed by the epithelial layer of the cornea (see Mitchell and Cenedella, 1995 for references). UV-B (290–320 nm) is especially damaging to eye tissues by initiating free radicals which generate toxic lipid peroxidative aldehyes and other reactive oxygen species, such as hydrogen peroxide. Superoxide dismutase, glutathione peroxidase and catalase are among the enzymes that are well represented in ocular tissues, including the cornea, to remove these reactive compounds (see Green, 1995).

Owing to the preference of ALDH3 for medium chain length aldehydes generated by lipid peroxidation (Lindahl and Petersen, 1991), it has been proposed that a primary function of ALDH3 in the cornea is for detoxifying peroxidic aldehydes (Messiha and Price, 1983; Evces and Lindahl, 1989; Uma *et al*., 1996; Lindahl, 1992). In view of the abundance of ALDH3 in the cornea (5–40% of the water-soluble protein, depending upon the species), it has also been suggested that a second, structural role of ALDH3 in the cornea is to directly absorb UV-B light (Abedinia *et al*., 1990; Algar *et al*., 1991). Direct measurements showed that the water-soluble proteins of bovine corneal extracts comprise only about 17% of the total corneal proteins, yet account for almost half of the 290–300 nm absorption of the total protein extracts of the cornea (Mitchell and Cenedella, 1995). These authors have thus proposed that the corneal water-soluble proteins be collectively called absorbins.

The idea that absorbins protect against UV light is supported by experiments demonstrating increased corneal clouding 4 days after brief exposures to UV irradiation (peak at 302 nm) in a mouse strain (SWR/J) that has decreased levels of ALDH3 and alcohol dehydrogenase (ADH) (Downes *et al*., 1994). Corneal clouding and severe reductions (85%) in ALDH3 and ADH activities in the cornea, as well as the retina and ocular fluids were also correlated in irradiated mice (Downes *et al*., 1992). Other detoxifying enzymes were not correspondingly lowered after irradiation, and the specific inactivation of ALDH and ADH has been called a "suicide" response. ALDH1 and ALDH2 are also inactivated by UV light by suicide adduct formation (see Boesch *et al*., 1996 for references). The UV-B absorptions for ALDH3 and ADH are due to tryptophan content and complexing with NAD cofactors. Associations of enzyme-crystallins with these cofactors may have a beneficial effect on light absorbance (Wistow *et al*., 1987) or redox cycling (Zigler and Rao, 1991). The UV inactivation of ALDH3 and ADH may be a reason why these specific detoxifying enzymes have not been recruited as crystallins in the lens (although ALDH1 is η-crystallin), where the cells remain throughout life and loss of fiber cell nuclei prevents protein turnover (see Piatigorsky, 1981). By contrast, the corneal epithelial cells are nucleated and continually renewed (Cenedella and Fleschner, 1990; Beebe and Masters, 1996).

Specific structural roles have not been suggested for the other abundant corneal proteins listed in Table 2. TKT may have an important role in maintaining the reducing environment of the cornea and providing a defense mechanism against free radicals under oxidizing influences, since it is a key enzyme in the pentose-phosphate (PP) pathway producing pentoses and NADPH (see Sax *et al*., 1996; Guo *et al*., 1997). The abundance of cyclophilin in the chicken cornea may be critical for folding collagens or other macromolecules that are secreted into the stroma (Schonbrunner *et al*., 1991; Freskgard *et al*., 1992). The possibility that the abundant corneal enzymes have a structural as well as an enzymatic role is limited at the present time to the fact that they are much more prevalent (about 10% of the water-soluble protein) in the corneal epithelial cells than seems reasonable for strictly enzymatic roles. Even in the lens, individual crystallin polypeptides seldom exceed 5–10% of the total crystallin content, which collectively comprises 80–90% of the total water-soluble protein. In the case of the bovine cornea, 54 kDa (ALDH3), 62 kDa (possibly TKT) and 45 kDa (probably α-enolase) proteins have been estimated to account for 60–70% of the water-soluble proteins (Mitchell and Cenedella, 1995). In addition to light absorption, as discussed above, it seems reasonable to propose that these abundant water-soluble proteins play crystallin roles in corneal epithelial cells, as they do in the lens, by minimizing the spatial refractive index fluctuations that would scatter light (Benedek, 1971; Bettelheim and Siew, 1983; Delaye and Tardieu, 1983).

6. GENE REGULATION AND GENE SHARING

6.1. Lens

Two central problems raised by gene sharing are the mechanism(s) by which crystallin genes are expressed at such high concentrations in the lens

and the molecular bases for their lower expression outside of the lens. Since the rapidly progressing area of crystallin gene expression is beyond the scope of this article and reviews can be found elsewhere (Sax and Piatigorsky, 1994; Cvekl and Piatigorsky, 1996), only some general comments will be made here.

Many transgenic mouse studies have established that lens-specific expression of crystallin genes is controlled largely, if not entirely, at the transcriptional level and functions across species (see Piatigorsky and Zelenka, 1992). The chicken δ1-crystallin gene microinjected into mouse lens cells (Kondoh *et al.*, 1983) or integrated as a transgene in transgenic mice (Kondoh *et al.*, 1987, 1991) are convincing examples of species-independent, lens-preferred expression of a taxon-specific crystallin. Each crystallin gene has its characteristic mix of *cis*-control elements. In some cases it is difficult to believe that orthologous and homologous genes dedicated to high expresssion in the lens have such differences in the nature and/or arrangement of their regulatory elements. An extreme example is the three jellyfish J1-crystallin genes, which have highly conserved coding sequences, while their putative regulatory sequences in their 5′ flanking regions are entirely different (Piatigorsky *et al.*, 1993). Nonetheless, recent studies have begun to unify our understanding of crystallin gene expression in the lens.

A major step forward has been the discovery that Pax-6, a transcription factor with highly conserved paired and homeodomains, appears to be fundamental for eye development in invertebrates and vertebrates (Li *et al.*, 1994; Halder *et al.*, 1995a,b; Tomarev *et al.*, 1997; Callerts *et al.*, 1997; Cvekl and Piatigorsky, 1996; Harris, 1997; Glardon *et al.*, 1997). Pax-6 is able to activate at least five crystallin genes in vertebrates, including those for chicken (Cvekl, 1994) and mouse (Cvekl *et al.*, 1995b) αA-crystallin, chicken δ1-crystallin (Cvekl *et al.*, 1995a), mouse αB-crystallin (Gopal-Srivastava *et al.*, 1996) and guinea pig ζ-crystallin (Richardson *et al.*, 1995). Interestingly, Pax-6 can also repress the chicken βB1- and βA3/A1-crystallin genes (Duncan, Haynes, Cvekl and Piatigorsky, unpublished). It remains unclear if Pax-6 contributes to the, expression of crystallin genes in invertebrates. In squid, Pax-6 is expressed mainly in the surface ectoderm and cornea of the developing eye (Tomarev *et al.*, 1997). A partial Pax homolog related to Pax-2, 5 and 8 has been cloned in the jellyfish and is expressed in the rhopalia containing the eyes, as judged by reverse transcription polymerase chain reaction tests (Piatigorsky and Norman, unpublished).

Retinoic acid receptors are another family of transcription factors that appear to regulate different crystallin genes in the lens. The mouse γF-crystallin enhancer was first shown to contain a novel retinoic acid receptor (RAR) response element (Tini *et al.*, 1993) that can be activated or suppressed by various combinations of the RAR, RXR, RORα and thyroid hormone receptors (Tini *et al.*, 1994, 1995). We have also found that chicken δ1 (Li *et al.*, 1997) and mouse αB-crystallin (Gopal-Srivastava and Piatigorsky, unpublished) promoters are activated in cells co-transfected with retinoic acid receptors and treated with retinoic acid. DNase I footprinting suggests a direct interaction between the crystallin promoters with Pax-6 and retinoic acid receptors, however it remains possible that these transcription factors activate the crystallin regulatory elements indirectly by stimulating other genes. As for invertebrates, an RXR-related gene has been cloned from the jellyfish and can bind the promoters of J-crystallin genes (Kostrouch, Kostrouchova, Lowe, Jannini, Piatigorsky and Rall, in Preparation).

SOX-2 has an activating effect on the mouse γ2-crystallin and chicken δ1-crystallin genes and may be another transcription factor that influences the lens-expression of several crystallin genes (Kamachi *et al.*, 1995). A number of other transcription factors (including AP-1, USF, δEF1, L-maf and HSF2) have been implicated in crystallin gene expression (see Cvekl and Piatigorsky, 1996). Moreover, there is a growing list of transcription factors responsible for eye development in *Drosophila* whose homologs are expressed in the embryonic eyes of vertebrates (for example, Oliver *et al.*, 1993, 1995, 1996; Tomarev *et al.*, 1996; Xu *et al.*, 1997; Shen and Mardon, 1997; Duncan *et al.*, 1997). Like Pax-6, these must be tested for their effect on crystallin gene expression. Current experiments indicate that *prospero*/Prox 1, which is expressed in developing

mouse (Oliver *et al.*, 1993) and chicken (Tomarev *et al.*, 1996) lenses, is able to activate a number of crystallin promoters, including that for chicken βB1 (Duncan, Tomarev and Piatigorsky, unpublished). Thus, it is possible that at least some of the diverse crystallin genes were initially recruited for a refractive function by their responsiveness to transcription factors that are involved in a conserved regulatory cascade directing eye and lens development. It has also been proposed, on the basis of the large array of proteins that function as crystallins and their abundance in the lens, that selective pressures (such as redox state) for lens transparency that also optimize the function of certain transcription factors have contributed to the recruitment of crystallins (Piatigorsky, 1992, 1993).

We have studied the constitutive non-lens expression of the mouse αB-crystallin gene. Transgenic mouse and site-specific mutagenesis experiments have shown that the complex expression pattern of the αB-crystallin gene in different tissues (Haynes *et al.*, 1996; Benjamin *et al.*, 1997) is achieved by using different combinations of at least five control elements in its muscle-preferred enhancer (Dubin *et al.*, 1991; Gopal-Srivastava and Piatigorsky, 1993; Gopal-Srivastava *et al.*, 1995; Haynes *et al.*, 1995) and downstream promoter, which contains two lens-specific regions (Gopal-Srivastava and Piatigorsky, 1994; Gopal-Srivastava *et al.*, 1995; Gopal-Srivastava *et al.*, 1996). An upstream transcription initiation site is used preferentially for expression of the αB-crystallin gene in the lung and brain (Iwaki *et al.*, 1990a; Frederikse *et al.*, 1994), showing additional complexity of regulatory controls. Two transcription initiation sites have also been noted in the duck LDH/ε-promoter (Kraft *et al.*, 1993).

While the αB- and LDH/ε-crystallin promoters employ two initiation sites to direct transcription, the guinea pig NADPH quinone oxidoreductase/ζ-crystallin gene has evolved two separate promoters preceding distinct exons, one for utilization in the liver (and presumably other non-lens tissues) and another within intron 1 for utilization in the lens (Gonzalez *et al.*, 1994). The lens-specific promoter, called ZPE, works in transgenic mice (Lee *et al.*, 1994). In general, however, most crystallin genes use the same transcription initiation site, and consequently promoter, in the lens and other tissues. The modular arrangements of control elements and multiple promoters in crystallin genes reflect the evolutionary playground that gene sharing exploits, leading to new functions for old genes.

6.2. Cornea

Studies on the tissue-specific regulation of the genes encoding the abundant water-soluble proteins of the cornea are just beginning. In contrast to the developmentally regulated high expression of crystallin genes in the lens, inductive effects contribute significantly to the corneal-preferred expression of the abundant water-soluble proteins. Developmental studies in mice have shown that ocular ALDH3 activity in mice is very low at birth and increases markedly at eye opening 2 weeks later (Rout and Holmes, 1988, 1991). Moreover, mice raised on a 12 h light:dark cycle showed higher ALDH3 activities than mice raised in the dark, suggesting that light modulates ALDH3 gene expression in the cornea (Downes and Holmes, 1992). ALDH3 expression is also modulated by light in primary cultures of rat corneal epithelial cells (Feimer *et al.*, 1995; Boesch *et al.*, 1996). Rat corneal epithelial cells cultured in the presence of continuous visible fluorescent light had more ALDH3 activity than cells cultured in the dark or than hepatoma cells cultured under the identical lighting conditions. The expression of TKT also increases substantially in the corneal epithelium of mice after eye opening (Sax *et al.*, 1996). *In situ* hybridization, Western immunoblotting and enzymatic assays all revealed developmental increases for TKT, suggesting transcriptional regulation, although post-transcriptional regulation cannot be excluded. Furthermore, TKT mRNA levels are elevated in cultured αTN4-1 lens cells exposed to hydrogen peroxide or diamide (a glutathione-specific oxidizing agent), or in cultured newborn mouse eyes exposed to a combination of filtered sunlight and fluorescent light (Salamon *et al.*, 1998). The increase in ALDH3 and TKT at eye opening and in response to light fits with the idea that these

enzymes are required to protect against light-induced oxidative stress of the cornea.

The constitutive and inducible patterns of ALDH3 expression indicate that the regulation of its gene is complex. Consensus sequences for stress-inducible control elements are abundant in the 5′ flanking sequence of the rat (Takimoto *et al*., 1992; Asman *et al*., 1993), mouse (Kays and Piatigorsky, unpublished) and human (Hsu *et al*., 1996) ALDH3 gene. Of special significance is the multiple xenobiotic response elements (XRE) implicating the inducible aromatic hydrocarbon (Ah) pathway (Vasiliou *et al*., 1992, 1993, 1995a; Nebert *et al*., 1993; Pappas *et al*., 1994; Korkalainen *et al*., 1995). Positive and negative regulatory elements have been identified by transfection experiments in non-corneal cells in the 5′ flanking region of the rat (Takimoto *et al*., 1994; Xiao *et al*., 1997) and human (Hsu *et al*., 1996) ALDH3 gene. DNase I footprinting experiments have indicated that Sp1-like proteins interact with two positive proximal elements and that NF1-like (and possibly other) proteins interact with two distal negative elements within the first kb of the 5′ flanking sequence of the rat ALDH3 gene (Xie *et al*., 1996). Sequence comparisons have shown that the 5′ flanking sequence, non-coding exon 1 and 5′ region of the first intron are well conserved between the rat and human ALDH3 genes, raising the possibility that these sequences harbor control elements that are important for constitutive expression (Hsu *et al*., 1996). In this connection, we have generated transgenic mice which show that a transgene containing the first kb of 5′ flanking sequence, exon 1 and intron 1 fused to the CAT reporter gene is preferentially expressed in the cornea (Kays and Piatigorsky, 1997). High expression of the rat ALDH3 gene in the cornea does not depend upon utilization of a separate promoter since the same transcription initiation site is used in different tissues (Boesch *et al*., 1996).

The single-copy mouse TKT gene has been cloned and its expression investigated (Salamon *et al*., 1998). Like the ALDH3 gene, it lacks a TATA box and has a series of potential stress-inducible elements in its 5′ flanking sequence, including consensus sequences for binding Sp1, AP1, NF-κB, and four UV-responsive elements, as well as other transcription factors, consistent with the inducibility of this gene. Transfection studies indicate that the first kb of 5′ flanking sequence of the TKT gene has promoter activity. In contrast to ALDH3 (Boesch *et al*., 1996), the TKT gene has a minor upstream transcription initiation site preceding an untranslated exon that is used in the liver but not the cornea; the major liver and sole corneal promoter is present in intron 2 (Salamon *et al*., 1998).

Potential Pax-6 binding sites can be found in both the ALDH3 and TKT genes. Pax-6 is expressed in the corneal epithelial cells of the embryonic chicken and the mature mouse and monkey (Koroma *et al*., 1997; Kays and Piatigorsky, unpublished). It is not known whether Pax-6 has a role in the high expression of these or other abundant corneal genes.

Taken together, the existing data suggest that the corneal-preferred expression of ALDH3 and TKT depends more on inductive processes promoted by the environment than do the lens crystallins. It is likely that there is also a corneal-preferred constitutive component to the regulation of the abundant corneal enzymes, as with the lens crystallins. Since the cornea probably preceded the lens evolutionarily, one may speculate that inductive processes were more important for the high expression of the ancestral stress-related lens crystallin genes than is the case for the modern crystallins, where gene expression has become integrated into a lens developmental program.

7. UNIVERSALITY AND CONSEQUENCES OF GENE SHARING

7.1. How Widespread is Gene Sharing?

There are many reports of multifunctional proteins suggesting that gene sharing is a widespread phenomenon. Thioredoxin (Holmgren, 1985; Huber *et al*., 1986; Russel and Model, 1986) and Qβ-replicase (Blumenthal, 1979) are microbial examples of borrowing a protein with one function to play another role in a different context. Thioredoxin is a 12 kDa, ubiquitously expressed protein that is used as a reductant, as a subunit

of DNA polymerase in *Escherichia coli* infected with bacteriophage T7, and as a critical cofactor for the assembly of filamentous phage. Mutagenesis experiments have shown that different features of thioredoxin are used to accomplish these diverse tasks. Qβ-replicase is an RNA-replicating enzyme found in *E. coli* infected with bacteriophage Qβ. This replicase is composed of four subunits, one derived from the bacteriophage (subunit II) and three borrowed from the bacterium (30S ribosomal protein S1 and elongation factors EF-Tu and EF-Ts). While the 30S ribosomal protein is probably used in similar biochemical ways when part of the ribosome or of the replicase, this may not be true for the protein synthesis elongation factors (see Blumenthal, 1979).

Human nuclear uracil DNA glycosylase is a monomer which has glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity when tetramerized, providing an example of a eukaryotic protein which has different enzymatic activities depending on its oligomeric state. GAPDH has been reported to have many different functions involving microtuble structure, membrane interactions and single-stranded DNA-binding (see Meyer-Siegler *et al.*, 1991) as well as being π-crystallin in diurnal geckos (Jimenez-Asensio *et al.*, 1995). There are a large number of eukaryotic proteins reported that appear to have multifunctional roles by using different properties inherent within their structure. Some examples include neuronal survival factor/γ-enolase (Takei *et al.*, 1991), hyaluronidase/hemopexin (a serum heme-binding protein) (Zhu *et al.*, 1994), 49 kDa *Tetrahymena* 14 nm filament-forming cytoskeletal protein/citrate synthase (Numata *et al.*, 1991), neuroleukin/phosphohexose isomerase (Chaput *et al.*, 1988; Faik *et al.*, 1988), plasminogen receptor/α-enolase (Miles *et al.*, 1991), cation-independent mannose-6-phosphate receptor/insulin-like growth factor II receptor (Morgan *et al.*, 1987; Tong *et al.*, 1988; Haig and Graham, 1991), RNA binding/aconitase (Kaptain *et al.*, 1991), and *Caenorhabditis elegans* isocitrate lyase/malate synthase (Liu *et al.*, 1995). A recent review has considered the possibility that many proteins (i.e., cytoskeletal components, growth factors, glycolytic enzymes, kinases, transcription factors, chaperones, transmembrane proteins and extracellular matrix proteins) serve distinct roles in different cellular compartments (Smalheiser, 1996). Mutations resulting in protein misrouting within or outside of the cell fostering new interactions in different environments was the favored hypothesis for the innovation of new functions for proteins or their isoforms.

Rather than misrouting, changes in gene regulation are responsible for recruitment of enzymes and stress proteins for new structural roles in the lens or cornea. This differs from the more classical separation of function of sibling genes by evolutionary adaptations, resulting in one gene being used exclusively for one purpose and its duplicate used solely for a newly acquired function. Thus, to establish gene sharing for the cases listed above, or any other situation, it is necessary to determine whether the different roles are being fulfilled by the same protein encoded in the identical gene. The multifunctional roles of human GAPDH appear to fall in the category of gene sharing since there is only one functional gene for this enzyme (Ercolani *et al.*, 1988). In other cases, such as the RNA binding protein with aconitase activity (Kaptain *et al.*, 1991), proteins with specialized functions may simply retain ancestral properties which are no longer used in their present role. It is also possible that a protein is multifunctional by a gene-sharing mechanism in one species, while complete separation of functions has occurred for this protein by gene duplication in another species. Finally, gene sharing also requires that multifunctional proteins use different properties of the protein for each role. In the case of crystallins, affecting lens refraction by its abundance is entirely different from catalysing an enzymatic reaction. On the other hand, it is possible that multiple biological roles evolve by using similar chemical or physical properties of a protein, which may be better considered as exploiting the full potential of a selected trait than as gene sharing *per se*.

7.2. Consequences of Gene Sharing

There are many interesting consequences of gene sharing which have been considered elsewhere (de Jong *et al.*, 1989; Piatigorsky and Wistow, 1989,

1991,; Piatigorsky, 1992; Wistow, 1993, 1995). One of the striking, although not surprising, implications of gene sharing is that evolution is pragmatic. Clearly, small heat shock proteins and metabolic enzymes were not originally designed or selected for refraction in eye lenses, yet some of these proteins perform this specialized function today. Gene sharing implies that a protein may expand or redirect its functions either when placed in a different cell, microenvironment or cellular compartment, or even by changing its relative abundance. Thus, a change in gene regulation, without modification of the coding sequences, may be sufficient for the innovation of a new function for a protein.

Gene sharing among lens crystallins establishes that gene duplication is not necessary for the generation of a new protein phenotype. This makes evolutionary tinkering at the level of gene regulation as important for the development of new functions as tinkering with the structural gene. Since mutations in either the *cis*-regulatory elements of the gene or their cognate *trans* factors could have widespread and unexpected consequences on the developmental expression, tissue distribution, microenvironment or abundance of its encoded protein, gene sharing can lead to sudden, completely unpredictable new uses for an old protein by pragmatic processes (see Wang *et al.*, 1996). Moreover, since gene regulatory sequences are able to function by combinatorial and modular mechanisms, all proteins must be constantly subject to experimentation for new functions by changes in their *cis*-elements, *trans*-factors and adaptor molecules. It also follows that changes in gene regulation may add new patterns of expression without necessarily eliminating the old patterns of expression, as has occurred with lens crystallins. Acquisition of a new role without relinquishing the old, one of the hallmarks of gene sharing, would probably be a rare event when a new protein function evolves strictly by the more conventional mechanism of mutations in the coding sequences of its structural gene.

A major consequence of gene sharing is that the resulting multifunctional protein will be under two or more selective pressures, slowing its evolutionary clock, differentially affecting specific regions of its encoded protein, and possibly giving misleading notions concerning divergence time. Stringent conservation could also give the impression that a protein is indispensable for a particular function, while in fact its conservation may be more influenced by its role for an entirely different function elsewhere. This is probably the case for some of the crystallins which are highly conserved, yet are performing a refractive function in the lens that can clearly be accomplished by diverse proteins. Indeed, crystallins have been conserved to a greater extent in the blind mole rat than would be predicted if they lacked a non-refractive function elsewhere (Quax-Jeuken *et al.*, 1985; Hendriks *et al.*, 1987).

Finally, gene sharing has both theoretical and practical consequences with respect to gene therapy. On a positive note, multifunctional proteins that have evolved by a gene-sharing strategy are under complex, tissue-specific and developmentally regulated control mechanisms, making their genes a source of promoter elements and enhancers that could be exploited for directing foreign genes for therapeutic purposes. On a cautionary note, however, the existence of gene sharing may blur the full significance of a protein, creating potential pitfalls for gene therapy. Tampering with a gene that encodes a protein which has distinct, nonoverlapping functions may have unpredictable and deleterious side-effects. The growing number of reports of multifunctional proteins discussed briefly above shows that the danger of influencing unexpected biological pathways by gene therapy is real. It is also conceivable, although perhaps unlikely, that placing a protein within a tissue at a different developmental time or at a different concentration than occurs under natural conditions, could result in the creation of a new function for that protein, which may be harmful.

8. FUTURE DIRECTIONS

It is very important to continue investigating the molecular bases for the pattern of gene expression of the lens crystallins and abundant corneal enzymes. The mechanisms used for the enormously high expression of these genes are only beginning to be understood in the lens and

remain even more mysterious in the cornea. Moreover, information on the quantitative and spatial control of crystallin gene expression in the lens lags behind that of tissue-preferred control. Comparisons of the mechanisms for high, tissue-preferred gene expression in the lens and cornea will be very informative and ultimately important clinically. Although identification of tissue-specific *cis*-elements has lost glitter in recent years, one must remember that those few that have been established for lens have already had a great impact on basic eye research. No *cis*-element or trans-factor has been found yet for crystallin gene expression specifically in the lens epithelium or in the cornea. The identification of these control elements sow the seeds for future clinical application, particularly in the cornea (see Piatigorsky, 1988).

Although many questions remain concerning the lens crystallins, the desert is even more arid for the corneal-enriched proteins. The fragmentary data available indicate that inductive events by the environment are more important for cornea than lens-preferred gene expression, but this remains to be established and the mechanisms need to be delineated. Virtually nothing is known concerning mechanisms that would account for the spatial expression of abundant enzymes in the cornea. These questions have great relevance for the basic understanding of how gene sharing comes about, how it is maintained, and how it can be utilized medically.

Invertebrates represent another area of opportunity for understanding the recruitment of crystallins and the similarities/differences in gene expression between the cornea and lens. Compared to our knowledge of vertebrates, invertebrates are still in the beginning stages of analysis (see Tomarev and Piatigorsky, 1996). It will be extremely interesting to discover if the regulatory cascades resulting in the recruitment and expression of crystallin genes in the lens have been conserved, at least in part, between vertebrates and invertebrates.

A central, albeit difficult, area of gene sharing that should benefit from future exploration concerns the multiple uses of the proteins in the lens, cornea and other tissues. The reasons for the diversity of crystallins among different species remain conjectural (i.e., neutral or fulfilling specific requirements). No non-refractive function is known yet for the β or γ-crystallins, although there is beginning to be evidence that at least some of these are expressed outside of the lens, or for δ1-crystallin, which is clearly expressed in numerous tissues. The situation is reversed in the cornea: the challenge is to provide convincing evidence for a structural role for the abundant enzymes. It will also be of great interest to learn whether crystallin-like concentrations of enzymes or other proteins exist in the non-epithelial cells of the cornea. The different roles played by lens crystallins and abundant corneal enzymes is at the heart of gene sharing and one might anticipate that new insights into the eye, as well as into evolutionary and developmental processes, will be forthcoming by investigating the full potentials and biological uses of these fascinating proteins.

Acknowledgements—I am grateful to Drs Frederick Bettelheim, J. Samuel Zigler, Jr, Joseph Horwitz, Stanislav Tomarev, Rashmi Gopal-Srivastava, Melinda Duncan, Ales Cvekl, Ernst Tamm, Marc Kantorow, Wm. Todd Kays, and Christina Sax for critically reading this manuscript. I am also indebted to Dr Kays for invaluable help in providing cornea references and to Ms Linda Willett for secretarial assistance.

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