INTEGR. COMP. BIOL., 43:492–499 (2003)

Gene Sharing, Lens Crystallins and Speculations on an Eye/Ear Evolutionary Relationship[1]

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SYNOPSIS. The crystallins comprise 80–90% of the water-soluble proteins of the transparent, cellular, refractive eye lens and are responsible for its optical properties. Comparative studies have established that the crystallins are surprisingly diverse and often differ among species in a taxon-specific fashion. In general, the crystallins are derived from or identical to metabolic enzymes or stress (small heat shock) proteins that are expressed to a lesser extent in other tissues where they have non-refractive roles. We call the phenomenon of having the small heat shock protein or enzyme and lens crystallin encoded in the identical gene "gene sharing"; examples include small heat shock protein/αB-crystallin, α-enolase/τ-crystallin and argininosuccinate lyase/δ2-crystallin. Lens crystallins have evolved by gene sharing in vertebrates (all) and invertebrates (cephalopods, scallops, jellyfish). Similar *cis*-elements and transcription factors (including Pax6 among others) appear to unify lens expression of crystallin genes in vertebrates and mollusks (especially scallops). Instead of *Pax6*, cnidarians have a *PaxB* gene encoding a Pax2 DNA-binding paired domain and octapeptide, and a Pax6 homeodomain; PaxB appears important for ocellus (eye) development and crystallin gene expression in the cubomedusan jellyfish, *Tridpedalia cystophora*. Finally, we speculate on the basis of our current studies on *Tripedalia* that eyes and statocysts (associated with mechanoreceptors in many cnidarians and thus possibly ears in vertebrates) are evolutionarily related. Numerous examples indicate that gene sharing is widely used, consistent with changes in gene regulation being an evolutionary driving force for innovation of protein function.

Structural and functional specializations of tissues are typically associated with correspondingly specialized proteins; for example, hemoglobin is inseparable from erythrocytes and oxygen transport, and immunoglobulins are tethered to macrophages and immunity. In the eye, rhodopsin is synonomous with retina and vision, and crystallins signify lens, transparency and refraction. Historically, this led to the belief that proteins are highly specialized and that new functions are acquired by evolutionary changes in their structure by mutation. It was also generally assumed that gene duplication is the foundation for protein sub and neospecializations, since it allows one copy of the gene to continue its original function and its sibling gene to adopt a new role after structural modifications. Our studies on lens crystallins, however, have shown that one mechanism by which proteins can evolve new functions is by changes in gene regulation in the absence of or before gene duplication, without a change in protein structure. We call this evolutionary strategy ''gene sharing'' (Piatigorsky *et al.*, 1988; Piatigorsky and Wistow, 1989). Gene sharing allows the same protein to acquire a new role, without losing its old one, by a change in expression pattern: indeed, since gene sharing can occur without gene duplication, the original protein function must be maintained along with its novel function.

CRYSTALLIN DIVERSITY AND GENE SHARING IN VERTEBRATES

Crystallins comprise 80–90% of the water-soluble proteins of the transparent lens and are responsible for its optical properties. One of the roles of crystallins is to pack closely enough to minimize concentration fluctuations in the cytoplasm in order to reduce scatter of transmitted light (Delaye and Tardieu, 1983; Bettelheim and Siew, 1983). There is also a smooth concentration gradient of crystallins that is highest in the center and lowest in the periphery which bends the light as it traverses the lens; thus the focusing power of the lens is achieved both at the surface and internally (see Land and Nilsson, 2002). The concentration gradient is achieved by regulating crystallin gene expression in the individual fiber cells that compose the vertebrate lens. The situation is complicated by the fact that there is a spatial and temporal regulation of the different crystallin genes throughout lens development, so that the light is refracted by a different combination of the various crystallins in different regions of the mature lens (Piatigorsky, 1981).

It used to be thought that all crystallins were members of the α-, β- or γ-crystallin families. Each of these families is heterogeneous and distinct; however, the β- and γ-crystallins form a structurally related β/γ-crystallin superfamily (see Di Maro *et al.*, 2002 for references). In addition, Rabaey (1962) found that the embryonic chick lens had an entirely different abundant protein, which he called ''first important soluble crystallin'' since it was the first to appear during lens development. Subsequent studies revealed that this protein was a major crystallin in birds and reptiles despite its absence as a crystallin in other classes; it is now called δ-crystallin (see Piatigorsky, 1984).

[1] From the Symposium *Comparative and Integrative Vision Research* presented at the Annual Meeting of the Society for Integrative and Comparative Biology, 4–8 January 2003, at Toronto, Canada.

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TABLE 1. *Some crystallins and their protein counterparts.*

αA-Crystallin	(vertebrates) small heat shock protein homologue
αB-Crystallin	small heat shock protein
ϵ-Crystallin	(ducks, crocodiles) lactate dehydrogenase B_4
τ-Crystallin	(turtles, *Mola mola* fish) α-enolase
δ1-Crystallin	(birds, reptiles) argininosuccinate lyase homologue
δ2-Crystallin	argininosuccinate lyase
ζ-Crystallin	(guinea pig, camel, llama) quinine oxidoreductase
π-Crystallin	(gecko) glyceraldehyde-3-phosphate dehydrogenase
η-Crystallin	(elephant shrew) retinaldehyde dehydrogenase
Ω/L-Crystallin	(cephalopods, scallop) aldehyde dehydrogenase homologue
S-Crystallins	(cephalopods) glutathione S-transferase homologues
J3-Crystallin	(cubomedusan jellyfish) saposin homologue

The discovery of δ-crystallin in birds and reptiles was just the beginning of the surprising diversity of these abundant lens proteins. Moreover, crystallins also vary among species to an extent that would have never been imagined earlier (Wistow and Piatigorsky, 1988; Piatigorsky and Wistow, 1989; de Jong *et al.*, 1989). For example, while all vertebrates have α- and β/γ-crystallins in various proportions (depending upon species) in their lenses, there is a growing list of crystallins that differ among species and are taxon-specific (Table 1). Unexpectedly, many of these abundant water-soluble proteins are derived from or identical to proteins that are used for other roles outside of the lens. This was first recognized for the α-crystallins that are homologous to small heat shock proteins (Ingolia and Craig, 1982). The αA- and αB-crystallins arose by gene duplication from a small heat shock protein; αA-crystallin subsequently specialized for lens expression, while αB-crystallin remains, in addition to being a lens crystallin, a stress-inducible, ubiquitously expressed small heat shock protein (de Jong *et al.*, 1993; Klemenz *et al.*, 1991). The β/γ-crystallins are related to microbial stress proteins and are also expressed in non-lens tissues, where their functions are not known (Wistow and Piatigorsky, 1988; Rosinke *et al.*, 1997). Another surprising discovery was that many of the taxon-specific crystallins are metabolic enzymes. The earliest examples of enzyme-crystallins were duck lactate dehydrogenaseB_4/ϵ-crystallin, chicken argininosuccinate lyase/δ2-crystallin and turtle α-enolase/τ-crystallin (Wistow *et al.*, 1987; Wistow and Piatigorsky, 1987). Many other examples followed (see Table 1), clearly indicating that crystallin function is not restricted to a small set of proteins specialized for the lens.

A pivotal finding was that argininosuccinate lyase/δ2-crystallin (Piatigorsky *et al.*, 1988) and lactate dehydrogenaseB_4/ϵ-crystallin (Hendriks *et al.*, 1988) are encoded in the same gene as their respective, active metabolic enzymes. This meant that there were cases where the abundant crystallin in the lens serving a structural/optical role is encoded by the identical gene as the enzyme expressed to a lesser extent outside of the lens and serving a metabolic role. In this case the different functions of the protein can be attributed to the difference in the expression pattern of the same gene. This does not mean, of course, that the crystallin cannot or does not also carry out a metabolic role in the lens; indeed, the small heat shock protein/αB-crystallin is almost certainly acting both as a crystallin and molecular chaperone in the lens (Horwitz, 1992). This dual role for a given gene governed by gene regulation is called 'gene sharing' (Piatigorsky *et al.*, 1988; Piatigorsky and Wistow, 1989).

GENE SHARING AND INVERTEBRATE LENS CRYSTALLLINS

The use of gene sharing as an evolutionary strategy to recruit lens crystallins is not limited to the vertebrate eye (see Tomarev and Piatigorsky, 1996 for review) (see Table 1). S-crystallins of squid (and other cephalopod) eyes are related to glutathione S-transferase (Wistow and Piatigorsky, 1987; Tomarev and Zinovieva, 1988). S-crystallin genes have undergone multiple duplications and in general encode proteins that lack glutathione S-transferase activity due to exon shuffling and base pair changes. There is one S-crystallin (S11-crystallin), however, that retains limited enzymatic activity and may represent the original sibling of the authentic glutathione S-transferase gene that is still present in the cephalopod genome (Tomarev *et al.*, 1995). Ω-Crystallin is another mollusc enzyme-crystallin; it is related equally to aldehyde dehydrogenase 1 and 2 and lacks enzyme activity with the usual substrates (Piatigorsky *et al.*, 2000). Ω-crystallin is the only crystallin in scallops, where it appears to be encoded in a single-copy gene that is also expressed at lower levels outside of the eye, consistent with it having a non-refractive role as well as being a lens crystallin (Carosa *et al.*, 2001).

Ocelli are another variety of well developed, lens-containing eyes that are present in invertebrates, although it appears as if these do not cast focused images onto the retina (Land and Nilsson, 2002). Perhaps the most ancient species to possess ocelli are jellyfish, especially cubomedusan jellyfish (Conant, 1898; Laska and Hundgen, 1982; Yamasu and Yoshida, 1976; Pearse and Pearse, 1978; Piatigorsky *et al.*, 1989). We have been studying the cubomedusan jellyfish, *Tripedalia cystophora* (Fig. 1), since it has highly developed ocelli on four specialized sensory structures called rhopalia (Piatigorsky *et al.*, 1989). Each of the four rhopalia has a large and a small complex ocellus (situated at right angles to one another) containing a cellular

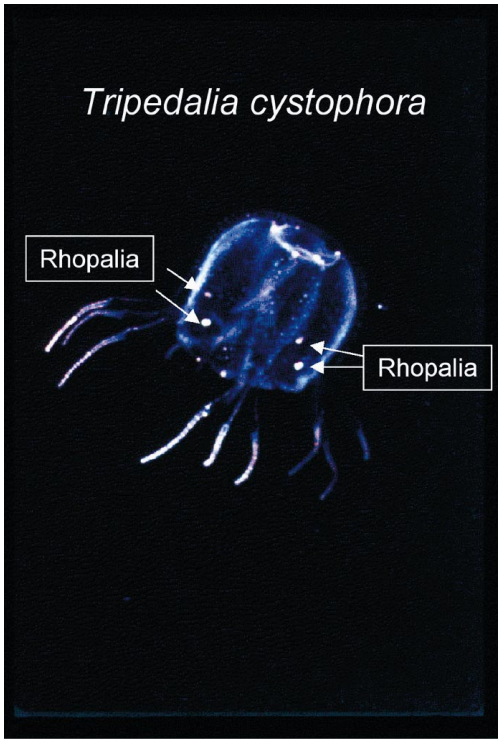

FIG. 1. Photograph of a living *Tripedalia cystophora*, a cubmedusan jellyfish found in the mangroves of Puerto Rico. The four refractive spots indicated by the arrows are the rhopalia, sensory structures that contain the ocelli (eyes) and statocyst (see Fig. 2). This photograph of a live jellyfish was taken by the late Dr. Charles E. Cutress (Marine Station, Department of Marine Sciences, University of Puerto Rico, Mayaguez, PR).

lens, cornea and retina; each rhopalium also has four simple ocelli comprising essentially photoreceptors, one statocyst (see below) and a rhopalial ganglion attached to a nerve net surrounding the jellyfish (Fig. 2). Of particular interest for the present discussion is that *Tripedalia* has three crystallin (J1A-C, J2 and J3) families in the cellular lens (Piatigorsky *et al.*, 1989). While the J1-crystallins comprise three closely related novel proteins (Piatigorsky *et al.*, 1993), J3-crystallin is a single polypeptide that is structurally similar to the saposin protein family (Piatigorsky *et al.*, 2001). J2-crystallin has not been cloned yet. Saposins are present throughout vertebrates, bridge membrane lipids to lysosomal hydrolases and activate enzyme activity and contain peptides that can act as a neuronal survival factor and myelinotrophic protein (see Piatigorsky *et al.*, 2001 for discussion and references). The J3-crystallin mRNA suggests strongly that mature J3-crystallin is derived by cleavage of a precursor protein, although this remains to be proved. The jellyfish saposin-like protein/J3-crystallin gene is expressed highly in the lens, in streaks extending from the ocellus, in cells surrounding the lumen of the statocyst, and at the tips of the tentacles; the novel J1A-crystallin gene is also expressed in the lens and statocyst (Piatigorsky *et al.*, 2001). Together, these data suggest strongly that the *Tripedalia* crystallins have been recruited from multifunctional, ubiquitously expressed proteins by a gene sharing strategy as have the crystallins of mollusks and vertebrates.

CRYSTALLIN GENE REGULATION: HOW CONSERVED IS IT?

Regardless of which protein is utilized as a crystallin, high lens expression is a critical and defining feature of all crystallin genes, since the refractive function of crystallins is directly related to their concentration in the lens. One of several transcription factors involved in lens expression of crystallin genes is *Pax6* (Cvekl *et al.*, 1994; see Cvekl and Piatigorsky, 1996 for review). Interest in *Pax6* and eyes was greatly stimulated when *Drosophila eyeless* (the orthologue to vertebrate *Pax6*) was shown to be required for eye development (Quiring *et al.*, 1994) and able to induce ectopic eyes (Halder *et al.*, 1995). It was subsequently found that *Pax6* from squid (Tomarev *et al.*, 1997) and many other species can direct the formation of ectopic eyes. Consequently, *Pax6* has been considered a ''master gene'' for eye development (Gerhring and Ikeo, 1999; Gehring, 2002). While it remains clear that Pax6 is of fundamental importance for eye development in both vertebrates and invertebrates, it is part of a network of developmental transcription factors. Indeed, *twin of eyeless, eyeless, sine oculis, optix, eyes absent, dachshund, eye gone* and *teashirt* are all able to induce ectopic eyes in *Drosophila* (see Kumar and Moses, 2001; Pichaud and Desplan, 2002). Homologues of these and other factors comprise an eye-field transcription factor self-regulating feedback network specifying the vertebrate eye field that are used for eye development (Zuber *et al.*, 2003). Thus, tissue-specific gene expression in the eye is complex and involves many factors and signalling pathways.

The fact that most if not all of the vertebrate crystallin genes that have been studied are regulated by a common group of transcription factors (CREB, AP-1, Pax6, maf, retinoic acid receptors, Sox, among others) (see Duncan *et al.*, 2004) supports the idea that these diverse proteins are linked by the mode of gene expression in the lens (Piatigorsky, 1992). Much less is known about crystallin gene expression in invertebrates, but any similarity in the mechanism of their high lens expression with that of vertebrate crystallin genes would be of great interest. An early study suggested that the non-homologous chicken βB1 and squid SL20-1 and SL11 crystallin genes have convergently evolved an AP-1/antioxidant responsive element used for high expression in the lens (Tomarev *et al.*, 1994). It is noteworthy that in vertebrates maf, a basic leucine zipper transcription factor important for crystallin gene expression, binds AP-1 sites (Kerppola and Curran, 1994); moreover, L-Maf, which is essential for chick lens development and δ-crystallin gene expression, is downstream of Pax6, consistent with common mechanisms for crystallin gene expression in vertebrates and invertebrates (Reza *et al.*, 2002). This possibility gains additional support from the finding

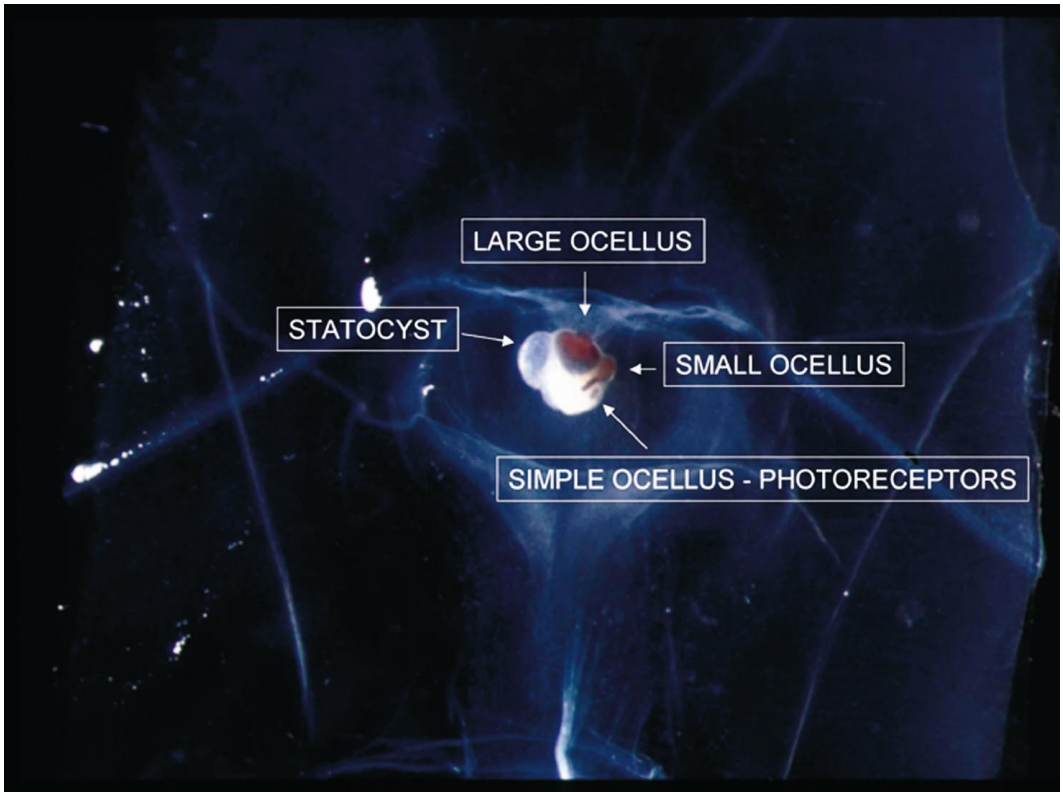

FIG. 2. Photograph of a rhopalium of a living *Tripedalia cystophora*. The rhopalia dangle from stalks and are set within a notch on the surface of the umbrella. Each of the four rhopalia contains a large ocellus and a small ocellus, set at right angles from each other, and a statocyst. This photograph of a single rhopalium was taken by the late Dr. Toichiro Kuwabara (National Eye Institute, National Institutes of Health, Bethesda, MD).

that the highly lens-preferred scallop aldehyde dehydrogenase/Ω-crystallin promoter has putative binding sites for many of the same transcription factors that are used to regulate vertebrate crystallin genes (Carosa *et al.*, 2002). Direct comparison of the alignment of putative *cis*-regulatory elements and their cognate transcription factors of the promoters of scallop Ω-crystallin, and mouse and chicken αA-crystallin showed a striking similarity. The *cis*-elements for cyclic AMP responsive element-binding protein (CREB) and Pax6 appeared functional in the scallop Ω-crystallin promoter as judged by site-specific mutagenesis and transient transfection tests. It needs to be underlined that scallop aldehyde dehydrogenase/Ω-crystallin shows no homology with the αA-crystallins derived from small heat shock proteins; nonetheless, the similarity in the putative *cis*-regulatory elements in their gene promoters suggests evolutionary convergence for lens expression. We speculate that this resulted in the utilization of these very different proteins as lens crystallins.

Current studies on jellyfish support the idea that invertebrate and vertebrate crystallins are related by their mechanism of gene expression in the lens. First, while a classical *Pax6* gene has not been found in Cnidaria, an eyeless jellyfish (the sea nettle) and hydra (Sun *et al.*, 1997), an eyeless hydromedusan jellyfish (*Podocoryne carnea*) (Gröger *et al.*, 2000), a coral (*Acropora millepora*) (Catmull *et al.*, 1998), and a hydrozoan jellyfish with a lens-containing ocellus (*Cladonema californicum*) (Sun *et al.*, 2001) all have a *PaxB* gene that encodes a Pax-6-like octapeptide and homeodomain and a Pax2/5/8-like DNA binding paired domain. We have cloned *PaxB* from *Tridpedalia*, confirm that its structure is similar to that described for other *PaxB* genes and provide evidence that it contributes to the regulation of the jellyfish crystallin genes (Kozmik *et al.*, 2003), as *Pax6* does to vertebrate crystallin genes (Cvekl and Piatigorsky, 1996). The possibility that PaxB regulates jellyfish crystallin genes fits with the fact that the J1A- and J3-crystallin genes are expressed in the lens and statocyst (Piatigorsky *et al.*, 2001), as *PaxB* appears to be (Kozmik *et al.*, 2003).

Although it is not possible to eliminate the idea that cnidarian rhopalia are a later, divergent evolutionary adaptation, our experiments on the *Tridpedalia* ocelli suggest that *Pax* genes have had a primordial and critical role in eye evolution (Gehring and Ikeo, 1999; Gehring, 2002). However, since a *PaxB*-like gene (a *Pax2/5/8* homologue) is present in sponges (Hoshiyama *et al.*, 1998), *PaxB* appears to precede the invention of *Pax6*, suggesting that a Pax2 paired domain was used before the Pax6 paired domain in eye evo-

lution and crystallin gene expression. However eyes evolved, either divergently or convergently, the contribution of PaxB to crystallin gene expression shifted later to the more modern Pax6. *Pax* gene proliferation is seen already in cnidarians thus allowing such a shift to occur early in evolution (Miller *et al.*, 2000).

SPECULATIONS ON THE POSSIBLE EVOLUTIONARY RELATIONSHIPS BETWEEN OCELLI, STATOCYSTS AND VERTEBRATE EARS

That jellyfish ocelli are concerned with vision, in the sense that they are light responsive structures with striking similarities to vertebrate eyes, coupled with the remarkable conservation of key regulatory genes between the diploblastic cnidarians and chordates (Galliot and Schmid, 2002; Hayward *et al.*, 2002), justify their consideration in the context of eye evolution. Our recent experiments raise a new question about the evolutionary relationship of ocelli to other sensory structures. Rhopalial statocysts in hydromedusae (Hyman, 1940; Horridge, 1969; Singla, 1975) and in developing ephyrae of the scyphomedusa, *Aurelia aurita* (Spangenberg, 1991), are known to be used for orientation or a ''righting'' response. These statocysts are associated with cilia and stereocilia that have resemblances to mechanoreceptors and can be considered as evolutionary ancestors to the vertebrate ears (see Singla, 1975). Statocysts contain statoliths, concretions of $MgCaP0_4$ in hydromedusans and $CaP0_4$ in scyphomedusans and cubomedusans (Chapman, 1985), that can act as gravity sensors. Jellyfish statocysts may be open or closed, and Horridge (1969) has proposed that these represent evolutionary stages for sensing water vibrations first and then gravity. To the best of my knowledge, little is known about the closed statocysts of *Tridpedalia*, or even if they have the same orientation functions as the statocysts of other cnidarians. Since the rhopalia of other jellyfish do not have the same elaborate ocelli that cubomedusans have, it is even plausible that the main function of the statocysts of *Tridpedalia* has specialized to keep the rhopalia weighted so that the ocelli are correctly oriented. Thus, *Tridpedalia* statocysts may play an indirect role in the ''righting'' response through its influence on vision. In this connection, *Tripedalia* shows a positive phototaxis and positive responses to an optokinetic drum, implying the use of vision for orientation (Stewart, 1997). By analogy with the statocysts of other jellyfish, however, it seems reasonable to propose that the statocysts of *Tridpedalia* may also have a separate orientation function even though the mechanism remains obscure at the present time. If *Tridpedalia* statocysts have lost or modified direct orientation functions because of their association with highly developed ocelli in this species, one would think that they must still be related to other cnidarian statocysts that have been reported to be directly connected with mechanoreceptors.

That *Tridpedalia* statocysts and ocelli are nested together on rhopalia which are suspended within a notch of the umbrellar periphery of the jellyfish, and that *PaxB* (Kozmik *et al.*, 2003) and the crystallins (Piatigorsky *et al.*, 2001) are both expressed in the statocyst and lens raise the possibility that ocelli and statocysts are evolutionarily related. In addition, the association of statocysts with mechanoreceptors further suggests that these ancient sense organs are distantly related to the vertebrate ear, linking sight and sound. A discussion on ears is beyond the scope of the present communication; however, like eyes, ears appear to be evolutionarily linked in vertebrates and invertebrates (Fu *et al.*, 1998; Eberl, 1999; Fritzsch *et al.*, 2000; Gillespie and Walker, 2001; Baker and Bronner-Fraser, 2001). Numerous homologous regulatory genes are used for development of the chordotonal organs of *Drosophila* and of vertebrate ears (Fritzsch and Beisel, 2001), supporting the idea that invertebrate and vertebrate ears have a common origin. *Drosophila atonal* and its vertebrate orthologue, *Ath5*, are particularly relevant. In the fly, *atonal* specifies stretch receptor neurons and the R8 photoreceptor neuron, while vertebrate *Ath5* specifies retinal ganglion cells (see Kumar and Moses, 2001; Sun *et al.*, 2003). A striking observation consistent with eyes and ears sharing developmental pathways is the appearance of an eye lens in the area of the otic vesicle as a result of ectopic expression of *Six3* in the killifish medaka (Oliver *et al.*, 1996).

Finally, the occasional linkage between genetic disorders of the eye and ear is consistent with the possible evolutionary connection between these sensory modalities. Usher syndrome resulting in deafness and retinal degeneration is well established (see Steel and Kros, 2001; Petit, 2001; Walsh *et al.*, 2002). Connections between the visual and auditory system also exist in the brain, consistent with the concerted evolution of hearing and seeing. A brain region has been identified where auditory and visual signals converge (Regan *et al.*, 1995), and neurons can evoke multisensory interactions at the single cell level in response to visual and auditory cues (King and Calvert, 2001). In barn owls the visual system teaches the auditory system to translate cues for pinpointing prey (Knudsen, 2002). Our experiments thus suggest that we may profit by considering the relationships among the senses as we interpret the evolutionary fragments at our disposal and the complex developmental circuitries that are used repeatedly throughout evolution.

HOW GENERAL IS GENE SHARING?

The striking use of metabolic enzymes and stress proteins for refraction and transparency in the lens, as occurs with crystallins, without relinquishing the other functions of these ubiquitous proteins, is an evolutionary strategy that increases the utility of the genome in a highly streamlined fashion. The crystallin story implies that changes in gene regulation can be instrumental in the innovation of protein function, bypassing the need for gene duplication and/or changes in protein structure by mutation (Piatigorsky and Wistow, 1991). In short, changing the cellular concentration or location of a protein can lead to new functions (Smalheiser,

1996), and one way of effecting these changes is by gene regulation.

Gene sharing has certainly occurred many times in evolution and represents a fundamental (not *only*) mechanism for generating functional diversity of proteins. A number of cases have been uncovered and crystallins represent the tip of the iceberg. The cornea accumulates taxon-specific, water-soluble, ubiquitously expressed proteins as does the lens, however their corneal function is not known yet (see Piatigorsky, 1998; Piatigorsky, 2001*a* for further discussion and references). The F-actin-severing protein, gelsolin, is particularly interesting in that it amounts to 50% of the water-soluble protein of the zebrafish cornea where it is highly expressed (Xu *et al.*, 2000) and modulates embryonic dorsal/ventral pattern formation when expressed in low amounts during early development (Kanungo *et al.*, 2003). *Drosophila* rhodopsin represents a case of gene sharing that developed in the same tissue where its role in phototransduction is complemented by an ability to organize the actin cytoskeleton for photoreceptor morphogenesis (Chang and Ready, 2000). Another recent case of gene sharing is *xanthine oxidoreductase* (Vorbach *et al.*, 2002). This gene encodes the rate-limiting enzyme in purine catabolism; in addition, it is highly expressed in the lactating mammary epithelium of mice, where it is used for enveloping milk droplets with the apical plasma membrane prior to secretion. Multifunctional proteins that represent potential examples of gene sharing can be found elsewhere (Jeffery, 1999; Piatigorsky, 1998, 2001*b*). A caveat in interpreting multiple protein functions as ongoing gene sharing is the necessity of knowing that the same gene is serving both roles. The gene may have multiplied, but the same sibling gene must be serving both functions, otherwise neo- or subspecialization has occurred. Such subdivision of labor would be a later evolutionary event.

In conclusion, studies on lens crystallins have taught us that the same gene may be used for unexpectedly different functions depending upon its expression pattern, and our investigations on the cubomedusan jellyfish, *Tridpedalia cystophora*, have raised the possibility that vision and hearing may have an evolutionary relationship. Clearly, nature is pragmatic, resourceful, and full of surprises.

ACKNOWLEDGMENTS

I am indebted to Dr. Zbynek Kozmik (Institute of Molecular Genetics, Prague, Czech Republic) for numerous discussions, and to Drs. Kozmik, Stanislav Tomarev (National Eye Institute, Bethesda, MD) and Joseph Horwitz (Jules Stein Eye Institute, UCLA School of Medicine, Los Angeles, CA) for critically reading the manuscript.

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