CHAPTER 4

THE GENE RECRUITMENT OF ENZYMES AS CRYSTALLINS

Taxon-specific enzyme crystallins are found in all reptiles and birds which have been examined, in amphibians of the genus *Rana* and in several species of mammal (Fig. 4.1). One atypical enzyme crystallin is also found in certain fish, lamprey, turtles and some other reptiles and birds. With this last exception, all the taxon-specific crystallins can be localized to specific lineages and their recruitment can be attributed to a single event in the ancestry of each lineage. Taxon-specific crystallins may modify the properties of the lens either through "diluting" the effects of γ-crystallins which contribute to hard, high-refractive index lenses, or through secondary protective effects such as UV filtration or contributions to protecting against oxidative or other stresses.

ε-CRYSTALLIN

Our understanding of crystallin gene recruitment and the occurrence of enzymes as taxon-specific crystallins began with ε-crystallin and in many ways this protein remains an archetype. It was discovered as a major component of the lenses in many birds and in all the crocodilians examined.[1] ε-Crystallin can be extremely abundant. In the lens of a hummingbird (*Calypte ana*) it was found to make up more than 40% of total soluble protein[2] (Fig. 4.2). In many other species, particularly seabirds, water fowl and others which hunt their food in bright light, ε-crystallin is 10-25% of total soluble protein.[3] The biggest surprise about ε-crystallin came when the protein from duck (*Anas platyrhynchos*) lens was partially sequenced and was found to be very closely related to lactate dehydrogenase B (LDHB), the heart muscle isoform of the glycolytic enzyme.[1,3] Peptide sequences of purified duck heart LDHB and duck lens ε-crystallins were identical except for age-related deamidation of two asparagine residues in the much older lens protein and ε-crystallin was found to have LDH activity.[3] Subsequently the identity of LDHB and ε-crystallin was confirmed by cDNA and genomic cloning which showed that the heart and lens proteins are indeed the products of the same single gene.[4,5]

Ubiquitous Stress-Protein Crystallins	*Related* or **Identical**	Activity	Cofactor	Species
αA, αB	**small heat shock protein** (mammalian αB)	Solubilize heat-stressed protein	none	all vertebrates
βA1-4, βB1-3 γA-F, γS	*M.xanthus Protein S* *P.polycephalum spherulin 3a* *EDSP of Cynops*	osmotic stress?	none	all vertebrates: γA-F not in birds
Taxon-specific Enzyme Crystallins				
δ1,δ2	**argininosuccinate lyase** (ASL)	δ2 has ASL activity	none	most birds, reptiles
ε	**lactate dehydrogenase B**	LDH activity	NADH	many birds, crocodiles
ζ	**NADPH:quinone oxidoreductase**	quinone reductase activity	NADPH	hystricomorphs, camels
η	**cytosolic aldehyde dehydrogenase**	retinal dehydrogenase	NAD+	elephant shrews
λ	*hydroxyacyl-CoA dehydrogenase*	?	NADH ?	rabbits, hares
μ	*ornithine cyclodeaminase* *glutamyl-tRNA reductase*	?	NADPH	some marsupials
π	**GAPDH**	GAPDH activity	NAD	geckos (*Phelsuma*)
ρ	*aldo-keto reductases*	?	NADPH	frogs (*Rana*)
τ	**α-enolase**	low enolase activity	none	some reptiles, birds several other species
Invertebrates				
S	glutathione-S transferase	weak GST activity in some subunits	GSH	octopus, squid
Ω	*ALDH*	no ALDH activity detected	NAD+?	octopus
J	?	?	none	jellyfish

Fig. 4.1. The crystallins.

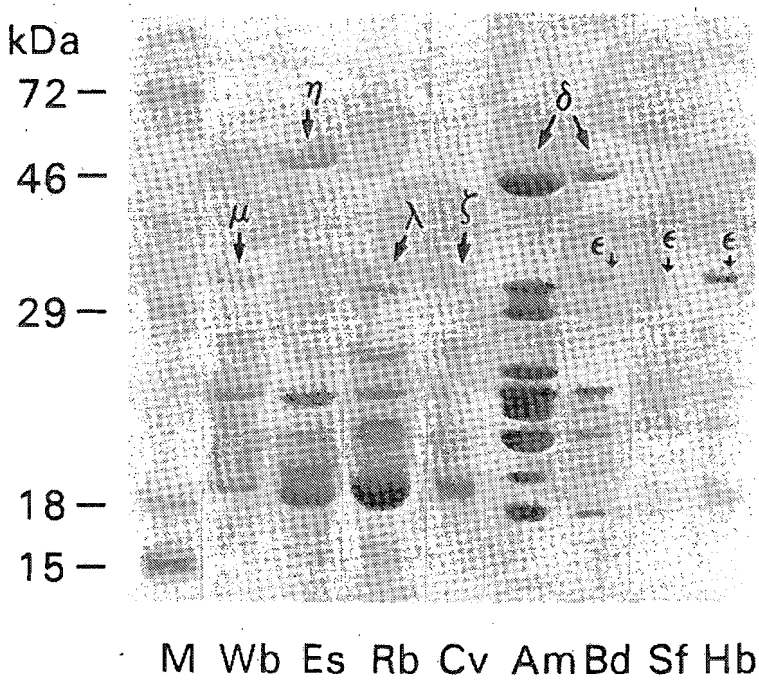

*Fig. 4.2. Taxon-specificity in crystallins. SDS PAGE[23] of lens extracts from some mammals and birds. Some major taxon-specific crystallins are indicated. M: size markers; Wb: tamar wallaby (*Macropus eugenii*); Es: elephant shrew (*Elephantulus rufescens*); Rb: rabbit (*Oryctolagus cuniculus*); Cv: rock cavy (*Kerodon rupestris*); Am: american merganser (*Mergus merganser*); Bd: black duck (*Anas rubripes*); Sf: chimney swift (*Chaetura pelagica*); Hb: Anna's hummingbird (*Calypte anna*).*

This was a remarkable and unexpected discovery. Instead of being a specialized lens structural protein, LDHB/ε-crystallin is the product of hundreds of millions of years of evolution as a glycolytic enzyme. It has become a crystallin by direct gene recruitment without prior gene duplication. This means that the same protein produced by a single gene is performing as a crystallin in the lens while still maintaining its normal pre-recruitment role as an enzyme in other tissues. Thus, in addition to the selective forces acting on this protein in its role as an enzyme, it now experiences another set of pressures from the new role in lens.

Sequence Changes and Adaptive Conflict

The effect of such pressures are apparent in LDHB/ε-crystallin itself. Although both enzyme and crystallin are identical in the same organism, sequence comparison with the LDHB polypeptides of other species reveal some unusual changes. In particular, two amino acid residues, Asn 114 and Phe 118, which are conserved in both LDHA

and LDHB sequences from species throughout the vertebrates, are changed to glycines in most species which have recruited the enzyme as a crystallin.[1,3,4] These two residues are close together on the surface of the protein, lying on the same side of an α-helix which runs across the top of the active site cleft in the LDH subunit (Fig. 4.3). The phenylalanine reside in particular forms an exposed, hydrophobic bump on each of the four subunits of the LDH tetramer. In duck LDHB/ε-crystallin, the substitution of glycines at positions 114 and 118 creates instead a flat patch on each subunit. This evidently has no beneficial effect on enzyme activity since it has never appeared in other vertebrate LDH sequences. Instead it must be due to the second role in lens. Given the critical aspects of protein-protein interactions in the lens it is likely that the modification serves to remove a potential site for protein aggregation.

This substitution of Phe 118 is found in many birds and in the crocodilian sequences which have been examined. Since one gene encodes both enzyme and crystallin, in those species which have recruited ε-crystallin, the LDHB enzyme in heart muscle also has this unusual sequence change. In most species which use ε-crystallin it appears that the modified LDHB functions well enough that these changes do not exert a significant evolutionary burden. However, there are two species of bird which have not followed this path. Both the chimney swift

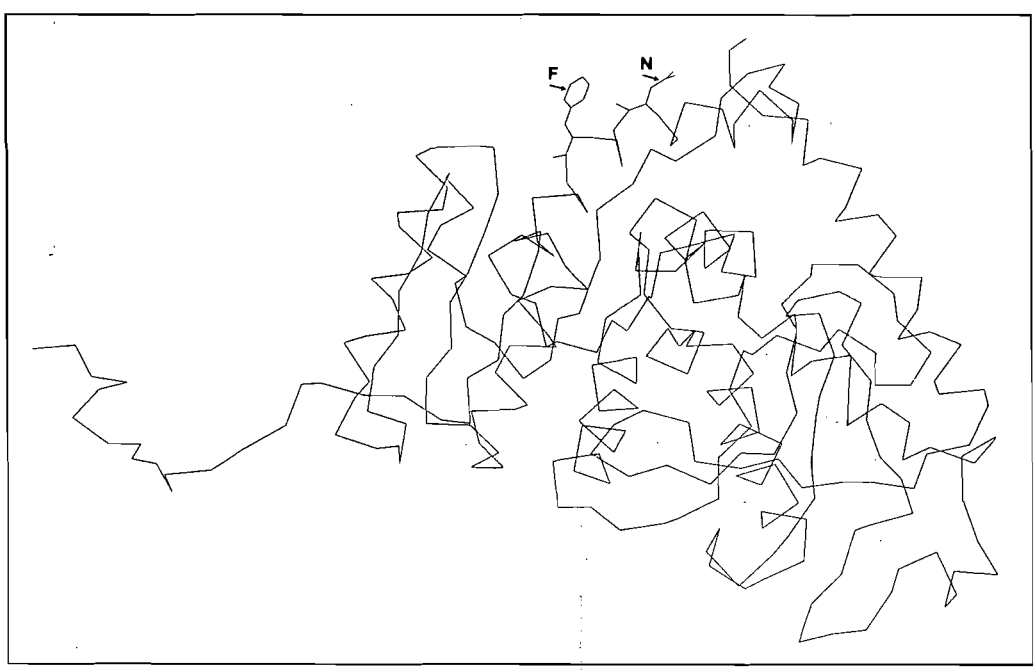

Fig. 4.3. Sequence modifications in an enzyme recruited as a crystallin. The exposed positions of Asn 114 (N) and Phe 118 (F) shown on a backbone trace of an LDH subunit.[150]

(*Chaetura pelagica*) and Ana's hummingbird (*Calypte ana*) have high levels of ε-crystallin in their lenses. These two species, although superficially very different, belong to the same order, Apodiformes.[6] They also share the characteristic of very energy-intensive life styles. Swifts spend a large part of their life on the wing at high speed in pursuit of insects while hummingbirds maintain an extremely high metabolic rate as they hover to collect nectar from flowers. When the ε-crystallins from the lenses of these two species were sequenced it was found that the Asn 114/Phe 118 combination of residues was still present.[2] Conceivably, in these species even a small decrease in the function of LDHB as an enzyme might have measurable consequences for the animal. As a result there may have been particular pressure for LDHB sequence to have been maintained to optimize its enzymatic role.

However, if the sequence changes in other species are indeed a response to selective pressures in the lens, then in swifts and hummingbirds there must have been a different response to accommodate these pressures. This may well have been the case, for both these species have reduced the content of another crystallin in their lens. In the case of the swift, δ-crystallin is completely absent as a detectable

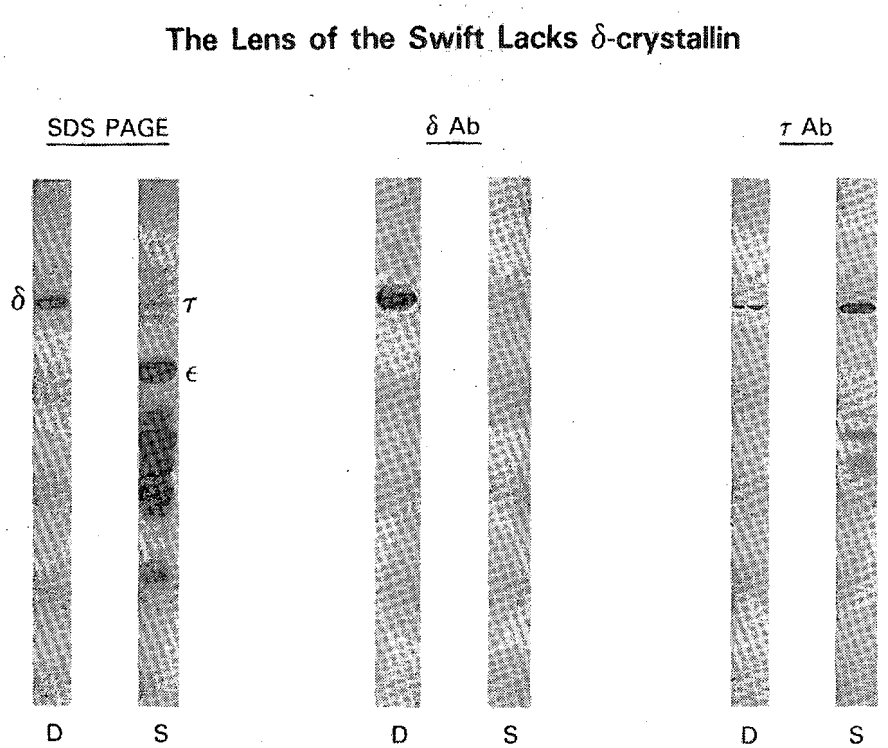

Fig. 4.4. The swift (Chaetura pelagica) *lacks δ-crystallin. SDS PAGE*[23] *and western blots of lens extracts from embryonic duck* (Anas platyrhynchos) *(as control) and from adult swift.*

crystallin (Fig. 4.4), while in the hummingbird it has been reduced to much lower levels than in other birds[2] (Fig. 4.2). One possible interpretation of this coincidence is that unmodified LDHB may interact with abundant δ-crystallin, perhaps through Asn114/Phe118, in a way which is detrimental to the lens. This can be overcome by a sequence modification to LDHB/ε-crystallin. However, if that solution is not advantageous an alternative would be to remove or reduce the δ-crystallin. A third solution which may also have been employed by many species of bird, is the reversion or loss of the ε-crystallin phenotype by LDHB. Many modern birds do not have abundant ε-crystallin in the lens. Since ε-crystallin is also present in crocodiles it was probably first recruited in a common ancestor of all archosaurs, including crocodiles, birds and dinosaurs. This implies that avian species which do not use this crystallin must have lost its expression after the divergence of birds and crocodiles.

This raises some interesting questions about the recruitment of ε-crystallin. It arose in lenses which already had δ-crystallin and which therefore had already adapted for the terrestrial environment. It must therefore have conferred some important secondary benefit to lens in order to have been retained by so many descendent species for so long. If the sequence modifications to LDHB were important they must have been made early on. This condition would have been retained in crocodiles, ducks and other birds to the present. Other birds, like chicken which lacks ε-crystallin,[3] must have lost ε-crystallin expression and undergone a reversion in their LDHB sequence, suggesting that there is indeed a real advantage to the function of LDHB in having the Asn 114/Phe 118 sequence. In contrast, as the ancestors of swifts and hummingbirds increased their energy budgets they too underwent a reversion in LDHB sequence but this was compensated by an alternative strategy which preserved high expression of ε-crystallin but reduced or removed δ-crystallin.

Protective Roles for Enzyme Crystallins

What special benefit could ε-crystallin bring to a lens? One suggestion is that although high levels of LDHB are not needed in lens for the purposes of glycolysis, the selective value of the protein comes from its ability to bind its cofactor NADH.[3] The reduced form of NAD+ (nicotinamide adenine dinucleotide) absorbs strongly in the near ultra violet at around 340 nm. Unlike those of mammals, bird retinas contain cone cell photoreceptors which have a peak sensitivity of 370 nm[7] allowing birds to see in the near UV. While this is undoubtedly useful under many circumstances it could also cause problems. Shorter wavelengths are scattered more efficiently by dust particles in the air, which is why the sky appears to be blue to our eyes. The blue-end of the spectrum thus contributes disproportionately to glare in bright light. For birds hunting insects against a brightly lit sky UV glare could be

a problem. It would also be a problem for birds and even crocodiles looking down through water for prey. UV glare would reflect off the surface while there would be little transmitted UV in the images from under the surface. Under these conditions it might be advantageous to filter out some of the UV. This could be achieved by sequestering NADH in the lens through binding to LDHB/ε-crystallin.

Strikingly, all the birds which use LDHB/ε-crystallin seem to fit this profile. They consist mainly of water hunters such as herons, gannets and gulls and bright light feeders such as swifts and hummingbirds.[1-3] Most birds which lack ε-crystallin, such as sparrows, chickens and owls feed on the ground, in low light or, in the case of penguins, entirely under water. Indeed, there is a fascinating correlation between the presence of ε-crystallin and an enigmatic feature of the avian retina, the pecten.[8] This is a folded, conical structure which protrudes from the back of the eye toward the lens. Unlike the rest of the avian retina it is vascularized and it is thought that it acts as a means of delivering nutrition to the eye. Interestingly, the degree of involution and hence the surface area of the pecten is higher in just the kind of bright light feeders which have ε-crystallin in their lenses.[8] Could one function of the pecten be to deliver nutrients to the lens to increase its content of NADH? Unfortunately there is no direct evidence to support this idea and the design of experiments to investigate it further has been daunting. However such a mechanism would allow birds dynamic control of the UV absorption of their lenses in response to environmental conditions.

δ-CRYSTALLIN

If ε-crystallin is the archetype for gene recruitment, gene sharing and the secondary benefits of recruitment, δ-crystallin has the same significance as a new model for gene duplication.

Although it was through ε-crystallin that the realization of the nature of taxon-specific enzyme crystallin came about, the first example of this class was already known though unrecognized as such. δ-Crystallin was observed as the first and most abundant of the soluble proteins of the developing chicken (*Gallus gallus*) lens.[9-11] Since these lenses also lack the γ-crystallins (as originally defined), δ-crystallin was seen a replacement for γ-crystallins in birds. In fact δ-crystallin is probably the most widespread and one of the oldest taxon-specific crystallins. Almost all birds, with the exception of swifts,[2] and probably all the reptiles which have been examined have abundant δ-crystallin (Fig. 4.2). It must therefore have been recruited at a very early stage in the reptile/bird lineage. At first it probably served to dilute the γ-crystallins which were present in reptilian lenses and are still present in at least some species today. Later, in the birds, δ-crystallin completely replaced the embryonic γ-crystallins and by itself formed as much as 90% of the soluble protein of the central, nuclear regions of the lens. The

difference in protein content and properties between the lens of a fish and that of a bird are striking. Bird lenses are among the softest and most hydrated known with typical protein contents of as little as 20%.[10] They often exhibit remarkable powers of accommodation and contribute to the unmatched visual acuity of birds.

δ-Crystallin is very different from γ-crystallin. It forms tetramers of 50 kDa subunits, giving it a native molecular size of 200 kDa, similar to that of the βH fraction. Unlike α-, β- and γ-crystallins which have predominantly β-sheet conformation, δ-crystallin has a high content of α-helix.[12,13] Only one form of δ-crystallin was ever cloned from chicken lens, but two similar genes, designated δ1 and δ2 were found closely linked in the chicken genome.[14-17] The predicted amino acid sequences of the products of these two genes showed 91% identity, and there was further strong similarity in non-coding sequences such as introns and untranslated regions of gene transcripts. δ1-Crystallin was the gene expressed at high levels in lens. δ2-Crystallin was named for its similarity to δ1 although there was no evidence that it actually served as a crystallin and its expression in lens was much lower than that of δ1-crystallin.[18]

When the first sequences for chicken δ-crystallin were produced no similarity to other proteins was noted. Coincidentally the sequence for yeast argininosuccinate lyase (ASL) was published at the same time.[19] However, at this early stage in the development of sequence databases the two sequences were not compared. It was not until the cDNA for human ASL was determined and entered into a database that the close similarity between chicken δ-crystallins and ASL was realized.[20-23] ASL is usually associated with the urea cycle in mammals but is also expressed in non-ureotelic tissues such as those of the eye,[24] and may be involved in various pathways such as nitric oxide synthesis.[25] Sequence comparisons showed that in fact it was δ2-crystallin which was most similar to the ASL enzymes of human and yeast.[20] Southern blot hybridization of chicken genomic DNA showed that the two δ-crystallin genes were the only ASL-like sequences present.[26] This suggested that δ2-crystallin was actually chicken ASL. But what about δ1-crystallin?

ASL enzyme activity was higher in embryonic chicken lens, which contains more than 80% δ-crystallin, than in other tissues[26] but this activity was very low compared to that of the purified human enzyme, showing that the chicken lens crystallin was not a fully active ASL enzyme. From these results it seemed that there had been a duplication of the ASL gene in birds. While one gene maintained the function of an enzyme, the other diverged and specialized as a crystallin. Since enzymatic activity was not essential for this new, structural role, δ1-crystallin lost its ancestral ASL activity. Thus chicken δ-crystallin appeared to follow a different model for taxon-specific crystallins from that exemplified by LDHB/ε-crystallin, a model without protein multifunctionality. Although the term "gene sharing" was first used in

connection with these proteins, the chicken δ-crystallins, unlike duck LDHB/ε-crystallin, have separate, specialized functions encoded by separate genes. However, chicken δ-crystallins do not tell the whole story of this family.

Previous work comparing the δ-crystallins of chicken with those of the duck (*Anas platyrhynchos*) had suggested that more isoforms of δ-crystallin were present in the duck.[27] Given the presence of two genes, this raised the possibility that in duck both genes were being expressed as crystallins. The first indication that this was correct came when crude duck lens extract was measured for ASL activity and was found to possess fully a quarter the activity of the purified human enzyme.[26] Thus in duck, unlike chicken, ASL/δ2-crystallin is indeed a multifunctional, taxon-specific enzyme crystallin. Later, full length cDNAs for both δ1- and δ2-crystallin were cloned from duck lens and both were found to be highly abundant.[28,29] Subsequently both duck[30] and chicken[31] δ1- and δ2-crystallins were expressed in different systems and for both species it was confirmed that δ2-crystallin is an active ASL while δ1-crystallin has no detectable activity.

ASL/δ2-crystallin is an enzyme crystallin in ducks, geese and swans, closely related members of the Anseriformes.[26,28,32] Most other birds which have been examined, such as pigeon (*Columba livia*)[33] have very low levels of lens ASL activity like the chicken. However, the ostrich (*Struthio camelus*), a ratite, also has high levels of ASL activity in its lens suggesting that in this flightless bird ASL/δ2-crystallin is expressed as a crystallin.[34] Since ducks and ostriches are so distantly related this suggests that the condition of two active δ-crystallin genes in the lens is ancestral. Following the model of LDHB/ε-crystallin and several other examples of taxon-specific enzyme crystallins it is very likely that ASL was recruited as a crystallin in an ancient ancestor of reptiles. Initially, like most other enzyme crystallins, one gene would have served two functions, with low level expression of the enzyme in various tissues and very high level expression in the lens.

The sequence changes forced on LDHB by the secondary role as ε-crystallin show how competing selective pressures can act on a bifunctional recruited gene. This can set up an adaptive conflict in which changes beneficial for one role are deleterious for the other. Under these circumstances there is selective advantage in gene duplication and specialization. This is what probably occurred at some point in the evolution of δ-crystallin. Duplication allowed one gene to adapt to whatever extra requirements the lens environment dictated. However it is clear that ASL itself can still function as a crystallin although it may require the presence of the more specialized δ1-crystallin to do so.

Although expression of ASL/δ2-crystallin can continue in the presence of δ1-crystallin it is evidently not required. Thus expression of ASL/δ2-crystallin in lens may be lost over time. In the same way non-lens expression of δ1-crystallin is also non essential and it too would be

expected to decline with time. Indeed this is essentially what seems to have happened in most birds which use only δ1-crystallin as a structural protein in the lens. Outside the lens, levels of mRNA for both δ1 and δ2 are generally very low but that for ASL/δ2-crystallin predominates, especially with age.[35,36] With the exception of another chicken eye tissue, the cornea, there is no evidence for expression of δ1-crystallin protein in non-lens tissues.[37] However if δ1-crystallin was expressed, its subunits would be capable of forming mixed tetramers with the enzymatically active ASL/δ2.[30] Indeed, when recombinant chicken δ1-crystallin was expressed in cultured mouse cells endogenous ASL activity was actually reduced, presumably by formation of mixed tetramers with lower activity.[31] Whether there is any benefit from such a non-lens role for δ1-crystallin in birds is unknown and perhaps unlikely. Certainly mammals are able to regulate ASL activity perfectly well by other means.

One prediction of the scenario presented here for the evolution of δ-crystallin is that at one time ancestors of birds had only one ASL/δ-crystallin gene. Recently we have obtained peptide sequences from the δ-crystallin of a reptile, the tuatara, a survivor of an ancient group, the sphenodonts. All the peptides of tuatara δ-crystallin examined seem to come from one sequence which more closely resembles δ2-crystallin and human ASL than it does δ1-crystallin. This is at least suggestive that this reptile expresses ASL as a crystallin and may not have acquired the lens-specialized δ1-crystallin. DNA analysis is now needed to investigate the number of ASL/δ-crystallin-like genes in this animal.

His89/Gln89

The lack of ASL activity in δ1-crystallin is rather surprising, considering that in the duck, δ1- and δ2-crystallins are 94% identical in amino acid sequence.[28] In both chicken and duck the two genes are closely linked in the same orientation and separated by only 4-4.5 kb.[16,29] This seems to have made them prone to gene conversion, and for both species the two δ-crystallins are more similar to each other than either is to its homologue in the other species. However, the enzymatically inactive δ1-crystallins in chicken and duck conserve at least one key amino acid difference from δ2-crystallins and from other ASL sequences. While active enzymes have histidine at position 89 this is replaced by glutamine in δ1-crystallins. Mutation of His89 to glutamine in human ASL reduces enzyme activity by over 90%.[38] Since activity is not eliminated, this residue is probably not involved in the central reaction of catalysis but has some important associated role. Surprisingly, a cDNA clone for a pigeon δ-crystallin was found to code for His89 and to be more similar to δ2-crystallins than to δ1-crystallins.[33] Since pigeon lens extract and total δ-crystallin has low ASL activity the authors concluded that His89 does not have an important enzy-

matic role. However since the clone was derived by PCR there is no evidence that it corresponds to the majority of expressed protein in pigeon lens and may in fact represent a low abundance δ2-crystallin.

Since the His89/Gln89 sequence change in chicken and duck δ1-crystallins is maintained in spite of gene conversion it may be under strong selection for some aspect of crystallin function. It seems unlikely that the selection is specifically for the loss of enzyme activity, since high levels of ASL activity in lens are not per se a problem for several species. The structures of turkey δ1-crystallin[13] and a modified form of duck δ2-crystallin[39] have now been determined by x-ray crystallography. Both reveal highly symmetrical tetramers arranged to form remarkable core bundles of 20 α-helices. These structures also show that His89 is not part of the active site of the enzyme although it is exposed on the surface nearby. Perhaps this residue has an accessory role in facilitating binding or release of substrates or products or a role in a conformational change necessary for function. Since the residue is surface exposed its change from histidine to glutamine may optimize or stabilize an interaction beneficial to its role as crystallin, such as a specific interaction with lens cytoskeleton. Alternatively this change might help maintain a more stable, less flexible structure for δ-crystallin. As a by product, this change contributes to a loss of enzymatic function but this is probably not the important, selected outcome.

Elements of Neutrality

δ-Crystallins also serve to illustrate another important point about enzyme crystallins in general. That is that these proteins are not recruited for their enzyme activity. Bird lenses do not need high levels of ASL activity but neither are such high levels of activity harmful. Birds do not even have a specific structural requirement for δ-crystallin. As shown by the chimney swift, δ-crystallin gene expression in lens can be entirely eliminated, perhaps as a response to adaptive conflict.[2] Its place in the swift lens is apparently taken quite adequately by LDHB/ε-crystallin while in the barn swallow (*Hirundo rustica*), a bird with rather similar hunting methods to those of swifts, δ-crystallin is abundant as normal in most other birds and reptiles. In other words the requirement is for a structural protein, not an enzyme, but even at this level the choice of a particular protein is at least partially neutral.

Intron-Slippage in Duck ASL/δ2-crystallin

While the origins of introns are controversial,[40-42] it seems to be accepted that they may contribute to protein and genome evolution in many ways, including acting as sites for insertion of new coding sequences into existing stable structures. In duck ASL/δ2-crystallin additional protein sequence has been gained through a recent splice-site slippage. A single base change (GT->GC) in the splice site recognition

sequence has led to use of a cryptic site six nucleotides further into the intron.[28,29] This causes the in frame insertion of two amino acids into the N-terminal region of the enzyme sequence. This insertion is unique in the ASL family in eukaryotes.[28] However it has evidently not had any major deleterious effect on enzyme activity since duck δ2-crystallin is an active ASL.[30] Whether, on the other hand, the insertion has any beneficial effects for the role of the protein in the lens is not known, although it is interesting that it creates an RGD tripeptide, a cell attachment motif.[43] In many cases, homologous genes of very distantly related species have introns in similar but non identical positions. This could reflect either independent insertion of introns at susceptible regions or slippage of the whole intron. Duck ASL/δ2-crystallin illustrates the first stage in such slippage. A reciprocal slip at the 3' end of the same intron could restore the number of amino acid residues but move the intron in a 3' direction.

ρ-CRYSTALLIN

ρ-Crystallin, the only taxon-specific crystallin identified so far in amphibians, is found in the lenses of frogs of the genus *Rana*. Although its enzymatic specificity is not known, ρ-crystallin binds NADPH (nicotinamide adenine dinucleotide phosphote) and belongs to an aldo-keto reductase superfamily which includes aldehyde and aldose reductase, prostaglandin F synthase and several detoxification enzymes.[44-47] The x-ray structure analysis of aldose reductase shows that this superfamily uses a structural motif consisting of eight parallel βα units which was first observed in triose phosphate isomerase (TIM) and now known to be very common among enzymes.[48]

τ-CRYSTALLIN

α-Enolase/τ-crystallin is in many ways atypical of enzyme crystallins. However it also serves to illustrate some general processes of crystallin gene recruitment. Unlike every other known example of enzyme crystallins, α-enolase/τ-crystallin has a rather widespread and patchy distribution. Furthermore while all other enzyme crystallins in vertebrates are associated with terrestrial species it is the only one known to be prominently expressed in some aquatic species. It was first discovered as the 48K protein in sea lamprey (*Petromyzon marinus*), where it was estimated to make up 13% of total lens protein, and as τ-crystallin in a turtle (*Pseudemys scripta*) where it was also very abundant[49,50] (Fig. 4.5). A survey of other species detected 48K/τ-crystallin in numerous species, including several reptiles, birds and fish such the air-breathing gar (*Lepisosteus oculatus*).[49] Its abundance varied among species, such that it was easily detected in domestic duck lenses but was barely detectable in chicken lens. At this time it was not thought be present in mammalian lenses.

Protein sequencing showed that turtle τ-crystallin was probably identical to α-enolase,[20] and this assignment allowed the interpretation

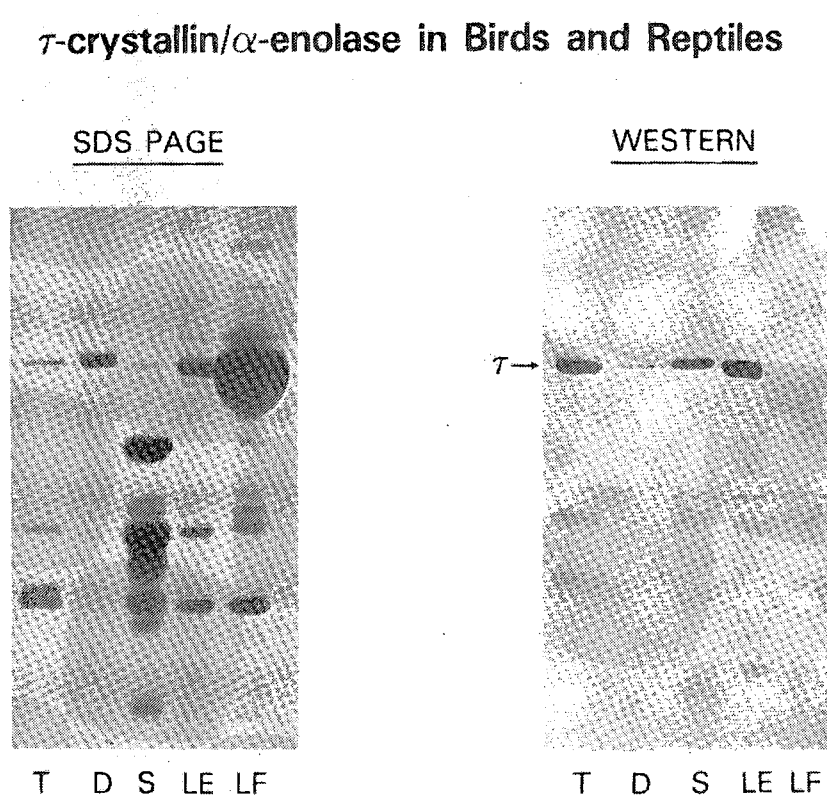

*Fig. 4.5. α-enolase/τ-crystallin (τ). SDS PAGE of lens extracts[23] (left) and western blot with anti-lamprey τ-crystallin antiserum (right). T: turtle (*Pseudemys scripta*); D: embryonic duck (*Anas platyrhynchos*); Sw: chimney swift (Chaetura pelagica); LE: embryonic chicken (*Gallus gallus*) lens epithelial cells; LF: embryonic chicken lens fiber cells.*

of peptide composition data from the lamprey protein which was also seen to match α-enolase.[51] Purified turtle τ-crystallin was shown to have enolase activity, albeit at a low level.[20,51] Since the previous survey had identified fairly high levels of τ-crystallin in duck lens, full-length cDNA for τ-crystallin was cloned from this source. Duck lens τ-crystallin was shown to be the product of a single gene and both τ-crystallin and human α-enolase cDNA probes hybridized to identical band in southern blots of duck genomic DNA.[51]

But is α-enolase/τ-crystallin a crystallin? In domestic duck, mRNA for α-enolase is more abundant in embryonic lens than in liver.[51] However the very high levels of expression in the lens seem to be a rather transient feature of embryogenesis. Analysis of the gene promoter for τ-crystallin found it to be highly active in all cultured cells with no tissue preference.[52] When the entire duck gene was expressed in transgenic

mice elevated levels of α-enolase were found in all tissues.[53] This experiment illustrated the point that lenses are capable of acquiring large increases (about seven fold in this case) in concentration of an enzyme in one step without serious problems. Indeed in the transgenic mice the duck transgene increased the level of α-enolase to close to parity with some individual β-crystallin subunits.[53] However in terms of expression patterns the duck gene behaved in adult mice in a very similar way to the endogenous α-enolase with no preference for lens.

These observations may be explained by the discovery that α-enolase is expressed at high levels in vivo in many stem cell populations, such as corneal limbus.[54] The function, if any, of high levels of α-enolase in stem cells is not known. It is possible that enolases may play structural roles in addition to their role in glycolysis. For example, γ-enolase has been found to be associated with the centrosome in HeLa cells[55] while several glycolytic enzymes are believed to form a cytomatrix with cytoskeletal proteins.[56-58]

Among the stem cells with high levels of α-enolase are lens epithelia (Fig. 4.5). α-Enolase/τ-crystallin has been measured at 9% of total protein in adult chicken epithelia and 12% in adult duck,[59] and it is likely that levels are higher in embryonic tissue. α-Enolase is at much lower levels in differentiated fiber cells (Fig. 4.5). It seems that α-enolase is an enzyme which is necessarily expressed at high levels in lens epithelia. In a small lens such as that of the lamprey, where epithelium constitutes a large fraction of total lens mass, α-enolase will accordingly achieve high overall levels. In other species such as mammals α-enolase is a prominent abundant enzyme but in a larger lens may not exceed .1% of total protein.[60]

Thus in many species α-enolase is intermediate between the low levels of many enzymes and the high levels of crystallins. Even then, it achieves concentrations in epithelial cells which are certainly in the structural range. Furthermore, overexpression of α-enolase does not seem to have any harmful effects in transgenic mice.[53] Thus *a priori* this enzyme is a good candidate for recruitment to even higher level expression in the lens. Indeed, there are clearly examples in which α-enolase unambiguously achieves the level of a crystallin. In turtle lens, for example, α-enolase/τ-crystallin is 46% of total protein in the epithelial cells and 6.5% in the nucleus giving an overall abundance of about 10%.[50,59] Immunohistochemistry shows high levels of α-enolase/τ-crystallin throughout the embryonic lens, including fiber cells.[51] The occurrence of τ-crystallin in diverse taxa may either reflect its "normal" high levels in epithelial cells or independent parallel recruitment of the same suitable gene in different lineages.

Intron Positions and Protein Structure

The gene for α-enolase/τ-crystallin from the domestic duck (*Anas platyrhynchos*)[52] and the homologous human α-enolase gene[61] have the

same pattern of intron positions. The structure of enolase from yeast has been determined by x-ray crystallography[62,63] and the sequence of enolase is sufficiently well conserved that a model of duck α-enolase can be built on the yeast enzymes' coordinates (unpublished). Enolase has two domains, an N-terminal domain of about 150 residues linked by a connecting peptide to a C-terminal domain of about 280 residues. The C-terminal domain was first thought to conform to the 8-fold β-strand/α-helix supersecondary structure repeat, $(\beta\alpha)_8$, typified by triose phosphate isomerase (TIM).[48,62] More detailed analysis showed that enolase actually has a different folding topology, $\beta\beta\alpha\alpha(\beta\alpha)_6$.[63] The TIM structural family, including other enzymes such as pyruvate kinase, has been used to illustrate the idea that all exons represent discrete structural elements and that genes were assembled from such units by intron-mediated mechanisms.[64] This was supported by the observation that introns in TIM map near the ends of β-strands or α-helices and not in the middle.

Enolase seems to contradict this model. First it has achieved a very similar tertiary structure through a different folding pattern and no significant sequence similarity. Second, introns mapping to the TIM barrel-like C-terminal domain of enolase do not neatly delineate secondary structures and three map to the sequences within α-helices (Fig. 4.6). These observations suggest that the βα barrel may simply be a thermodynamically stable structure available to many protein sequences regardless of their evolutionary origins.

However, the N-terminal domain of enolase is different. In this region introns fall neatly between supersecondary structures and one intron exactly corresponds to the join between the two domains (Fig. 4.6). Thus this domain could very well reflect the structures of ancestral motifs. Since enolase is present in prokaryotes[65] where it lacks introns the question again arises of whether the vertebrate introns were inserted or whether ancient introns were deleted in bacteria. It is possible that the N-terminal domain is descended from a well-structured RNA which directed intron insertion accordingly while the insertion into the C-terminal domain was essentially random.

GECKO CRYSTALLINS: A RESPONSE TO LIGHT?

The acquisition and loss of taxon-specific crystallins has been associated with changes such as moving from diurnal to nocturnal habits.[66] This recruitment could be driven either by the primary pressures of modifying the optical properties of the lens or by secondary pressures such as UV or oxidative stress. This has prompted an examination of geckos, lizards which adopt several different habits and which in some lineages may have moved from diurnality to nocturnality and back again.[8,67] Indeed, in two genera of diurnal geckos taxon-specific crystallins were found while none were apparent in nocturnal species.[68,69] In both *Phelsuma* and *Lepidodactylus* taxon-specific subunits of about

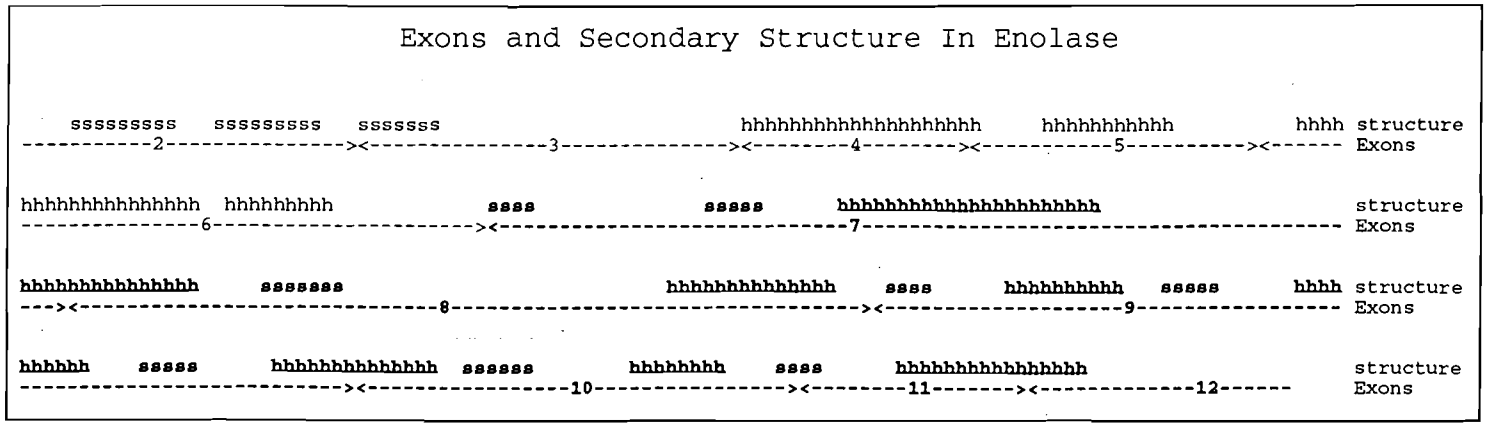

Fig. 4.6. Exons and secondary structure in enolase. Secondary structures of yeast enolase and a model of duck α-enolase/τ-crystallin are shown. (s) Residues in β-strands; (h) residues in α-helices. One character represents one residue. The relative positions of exons in the duck gene are shown by arrows.[52]

Exons 2-6 correspond to the N-terminal domain of the protein, exons 7-12 (bold face) correspond to the C-terminal αβ barrel.

36 kDa were observed, similar in size to several known crystallins. The protein in *Phelsuma* has been named π-crystallin and identified as the enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH).[69] Interestingly GAPDH is expressed at moderately abundant but sub-crystallin levels in mammals. The protein in *Lepidodactylus* is also being characterized[68] and is different from π-crystallin (de Jong and Röll, unpublished).

These results suggest the independent recruitment of taxon-specific enzyme crystallins in response to a species moving into a brighter light environment. Since geckos are reptiles which already have high levels of "lens-softening" δ-crystallins, the new crystallins must be conferring secondary advantages, probably in protection against UV as was suggested for ε-crystallin and some other taxon-specific crystallins.[3,70] Interestingly, GAPDH, like LDH and enolase, is a glycolytic enzyme which has been shown to associate tightly with actin and other components of cytoskeleton.[56-58] These enzymes may be able to play a role in stabilization of cytoskeleton in lens fiber cells in addition to any other functions.

ENZYME CRYSTALLINS IN MAMMALS

The reptile/bird lineage presents a fairly uniform picture of the evolutionary processes by which lens composition is modified (Fig. 4.7). At some very early stage ASL/δ-crystallin was recruited and the adaptation of the lens to the terrestrial, diurnal environment began. Later, at least in birds, embryonic γ-crystallin expression was lost and in the archosaurs LDHB was recruited as ε-crystallin while diurnal geckos have also independently recruited different taxon-specific crystallins.[68,69] However the overall similarity in the retention of δ-crystallin in all descendants of early reptiles is remarkable.

Mammals too are descended from a major group of early terrestrial vertebrates, the synapsids, the so-called "mammal-like reptiles." This lineage includes the pelycosaurs which formed one of the dominant group of land animals in the early permian, the therapsids of the later permian and the cynodonts of the early triassic.[71] If it was useful for the reptile/bird line to modify their lenses with a recruited enzyme crystallin it seems reasonable to expect that the same applied to the ancestors of mammals. However, most modern placental mammals do not express taxon-specific crystallins and are instead limited to the ancient α- and β-crystallins and a group of very highly conserved γ-crystallins whose genes are closely clustered on one chromosome. Where taxon-specific crystallins do exist in mammals they are rather tightly limited in phylogenetic distribution. If the ancestors of mammals indeed had a taxon-specific crystallin it must have been lost by most descendent species which, in at least some cases, have independently recapitulated the process of gene recruitment (Fig. 4.7). Candidates for the role of ancestral synapsid taxon-specific crystallin are δ-crystallin itself which

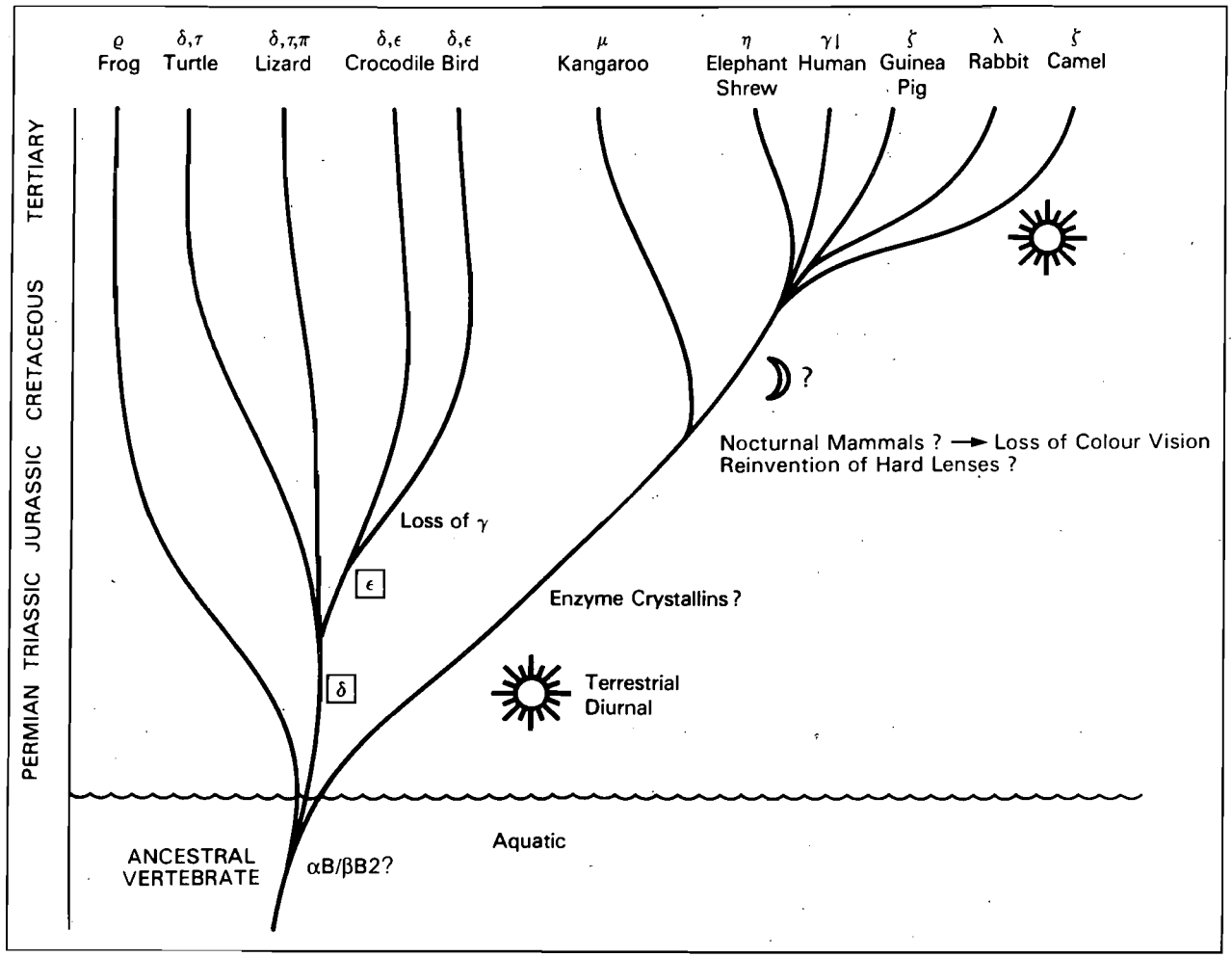

Fig. 4.7. An evolutionary tree for vertebrates illustrating the distribution of taxon-specific crystallins. Up-dated from Wistow G, Trends Biochem Sci 1993; 18:301-306.

might have been recruited in a common ancestor of both major reptilian lineages, μ-crystallin which has a wide but patchy distribution in marsupials, η-crystallin which is found in an ancient diurnal group of placentals and ζ-crystallin which is found in two separate lineages of modern placentals.

η-CRYSTALLIN

The most abundant taxon-specific crystallin in placental mammals is η-crystallin[60] which is probably the single major protein component in the lenses of macroscelids, elephant shrews (Fig. 4.2). These small mammals are active, diurnal insectivores. They are generally regarded as being relatively primitive and have even served as an illustration in a popular text for what some early mammalian ancestors might have been like.[72] Most experts have placed macroscelids in phylogenetic groups such as the Insectivora, which would include shrews, or the Glires, which in most classifications includes rodents and lagomorphs.[73] However, protein sequence data from the αA-crystallin of *Elephantulus rufescens*, the rufous elephant shrew, show identity with those of the hyrax.[60,74] This would place the macroscelids in an early offshoot of the placental mammal family tree which appropriately also includes elephants as part of a group called the paenungulates.[74] Whatever their affinities, elephant shrews are not shrews.[75] They have large eyes and soft lenses.

η-Crystallin accounts for about a quarter of total lens protein in both *E.rufescens* and *E.edwardi* while in a species of another genus, *Macroscelides proboscideus*, it contributes about 10% of total protein.[60] Whether this represents generic or individual difference is not known. In the one example of *E.rufescens* which was examined, η-crystallin seemed to have largely supplanted γ-crystallins in a manner reminiscent of δ-crystallin in birds.[60] In *M.proboscideus*, with its lower content of η-crystallin, γ-crystallins could be detected. Again, individual differences may be significant and the loss of γ-crystallins in the specimen of *E.rufescens* may have been due to aging effects in an old animal.

Partial protein sequence of η-crystallin and immunochemical reactivity suggested possible identity with cytoplasmic aldehyde dehydrogenase (ALDH1).[60] The very close sequence similarity of η-crystallin to ALDH1 has now been confirmed by cloning η-crystallin from the lenses of *E.edwardi* and *M.proboscideus*.[76] Sequence data show that η-crystallins clearly group with ALDH1 of other vertebrates and that all the residues required for ALDH enzymatic function[77] are conserved.

The cytoplasmic enzyme ALDH1 has very low activity towards the soluble aldehydes which are good substrates for related enzymes such as the mitochondrial ALDH2. However, ALDH1 is widely expressed and highly conserved suggesting that it has an important function.[77,78] It now appears that at least one role for this enzyme is as retinaldehyde (retinal) dehydrogenase,[79,80] an activity which converts the aldehyde retinal to retinoic acid, an important activator of gene expression and a po-

tent morphogen in development.[81,82] In the mouse eye, ALDH1 expression is an early marker for development in the retina with preferential expression in dorsal retina.[79,83] Indeed, it has been suggested that ALDH-derived retinoic acid might have a role in inducing *Pax-6* expression itself in the determination of the eye as an organ.[83]

When recombinant *E.edwardi* η-crystallin was tested for ALDH activity it was found to be enzymatically active using 11-*cis*-retinal as substrate.[76] Together with its close sequence similarity to ALDH1 this enzyme activity shows that η-crystallin is indeed an ALDH1. However, when non-lens expression of η-crystallin was examined there was a surprise. PCR analysis of *M.proboscideus* liver detected mRNA for both η-crystallin and a second, more abundant ALDH1 in liver.[76] Further PCR analysis of elephant shrew tissues suggests that η-crystallin is the predominant form of ALDH1 in retina and iris as well as in lens. Thus η-crystallin may have been recruited directly from an eye-preferred ALDH1 isoform. Alternatively η-crystallin may be the second known example, after δ-crystallin, in which the gene recruitment of a taxon-specific enzyme crystallin is associated with gene duplication. This idea is supported by cladistic analysis which suggests that the separation of the two genes for ALDH1 occurred at an early stage in the evolution of elephant shrews but probably after their lineage had split off from those of most other placental mammals.[76]

The predominance of η-crystallin in eye tissues explains why this taxon-specific crystallin retains its retinal dehydrogenase activity while serving as a crystallin. ALDH activity is essential for normal development in many tissues. In lens itself ALDH1 is expressed from early stages[79] and is present at reasonably high sub-crystallin levels in lenses of many species.[60] Retinoic acid receptors have been implicated in expression of γ-crystallin genes[84,85] while overexpression of retinoid binding proteins in lens causes developmental defects.[86,87] High level expression of a hypothetical inactive η-crystallin might sequester the active enzyme in low activity heterotetramers and essentially eliminate ALDH activity with possibly serious consequences for lens development. These problems would be avoided through the retention of ALDH activity by η-crystallin even as it acquired a structural role in lens.

Like some other enzyme crystallins, η-crystallin binds a nicotinamide adenine dinucleotide cofactor. However in this case the preferred cofactor is NAD+, the oxidized rather than the reduced form, although it is not yet known whether levels of both NAD+ and NADH are elevated in elephant shrew lenses. The recruitment of η-crystallin may have been selected through its modification of the optical properties of the lens rather for a secondary role in protection against oxidative stress. If a protective role does exist it may be against the toxic effects of aldehydes rather than against more generalized oxidative stress.

ζ-CRYSTALLIN

It was essential for our recognition of the enzyme crystallins that some of the first to be discovered were well known enzymes which had already entered the sequence databases.[23] Others however were discovered first as crystallins and only later defined as enzymes. One such is ζ-crystallin which was first discovered in guinea pig (*Cavia porcellus*) lens where it accounts for 7-10% of total soluble protein.[88] A congenital cataract in this species was found to be associated with loss of a 35 kDa crystallin subunit.[88,89] This turned out to be a taxon-specific crystallin belonging to the alcohol dehydrogenase (ADH) superfamily.[90,91] However, unlike ADH, ζ-crystallin binds NADPH rather than NADH, suggesting a role as a reductase.[92,93] After testing a number of possible substrates it was found that ζ-crystallin is a novel NADPH:quinone oxidoreductase.[94] Like LDHB/ε-crystallin, ζ-crystallin is the product of a single gene which is also expressed in other tissues at lower levels.

ζ-Crystallin was also detectable at crystallin levels in some related South American hystricomorph rodents including degu (*Octodon degus*) and rock cavy or moco (*Kerodon rupestris*) but not in coypu (*Myocasor coypu*) or in other rodents.[93] Then most surprisingly it was also found in camelids, Old World camels and New World llamas.[88,93,95,96] Both hystricomorphs and camelids have their origins in South America. However they are so distantly related phylogenetically that the presence of ζ-crystallin in both groups must have been due to independent recruitment. Recent gene sequencing for guinea pig and llama ζ-crystallins confirms the independence of the recruitments, although there are some remarkable parallels in mechanisms used (see ref. 97 and below).

Independent recruitment of the same enzyme as a crystallin should not perhaps have been totally unexpected since the pool of suitable enzymes is obviously finite and some may be easier to recruit than others. Indeed, as discussed below, parallel recruitment in widely divergent species may have occurred several times in the case of α-enolase/τ-crystallin.[49]

λ-CRYSTALLIN

λ-Crystallin seems to be another previously unknown enzyme. It has been found only in rabbit (*Oryctolagus cuniculus*) and hare (*Lepus europaeus*).[98] λ-Crystallin is distantly related to hydroxyacyl- and hydroxybutyryl-CoA dehydrogenases and conserves the consensus NADH binding site, however its activity, if any, has not yet been determined.[98] From a phylogenetic standpoint, it is interesting that λ-crystallin was not seen in pika (*Ochotona princeps*) which is classified as lagomorphs with rabbits and hares. Pikas also differ from the other lagomorphs in that they express αA,[ins] the product of alternative splicing of αA-crystallin while rabbits do not.[99]

μ-CRYSTALLIN

μ-Crystallin was first observed as a 35 kDa subunit accounting for up to a quarter of total soluble protein in the lenses of some Australian marsupial mammals[60] (Fig. 4.2). So far it has been seen in all macropods (kangaroos, tree kangaroos and wallabies) which have been examined (unpublished). It was also identified in the lens of the only dasyurid examined, a carnivorous quoll, Dasyurops maculata.[60] It was not detectable in several other species, including various possums and wombat (unpublished) but a low level was seen in the sugar glider. It was not detectable by western blot in the only New World marsupial examined, the Virginia opossum (*Didelphis virginiana*).[60] Its pattern of occurrence suggests an early recruitment in a common ancestor of Australian marsupials perhaps 120million years ago and subsequent loss in several species. However more marsupial species, particularly from South America, need to be examined.

In contrast to placental mammals, marsupials retain in their retinas the cone cells with oil-drops found in diurnal reptiles and other vertebrate species.[8,67] This suggests that the ancestors of marsupials may not have experienced the nocturnal, burrowing phase which may have been an important part of placental mammal evolution (Fig. 4.7). Consequently marsupials may have retained other features from the eyes of their diurnal reptilian ancestors. If so, μ-crystallin may have an even more ancient origin than currently indicated and may be a candidate for the hypothetical ancestral enzyme crystallin of the reptilian ancestors of all mammals.

In grey kangaroo (*Macropus fuliginosus*) μ-crystallin is the product of a single gene which is expressed at high levels in lens and at lower levels in retina and brain, presumably in an enzymatic role.[100] Peptide sequences of μ-crystallin from kangaroo and quoll lenses could not convincingly demonstrate a relationship with any known proteins. However when kangaroo μ-crystallin was cloned it was found to be significantly similar (over 30% identity in predicted amino acid sequence) to ornithine cyclodeaminases (OCD) of the bacterium *Agrobacterium tumefaciens*.[100] The similarity was not apparent from peptide sequences because these all came from the more hydrophilic N- and C-terminal regions rather than the more hydrophobic central regions of the sequence which contain the highest similarity among OCDs and therefore presumably contain the active site.

OCD is an unusual enzyme involved in metabolism of opines, amino acid derivatives produced when *A. tumefaciens* invades plant cells.[101-103] OCD catalyses the conversion of ornithine directly to proline in the presence of NAD+.[102,103] In the more familiar biochemical pathways of standard textbooks this conversion requires two enzymes and passes through a glutamate semialdehyde intermediate. The mechanism of the OCD reaction is not known but it seems likely that it would involve a similar intermediate. Like OCD μ-crystallin binds a nicotinamide

adenine dinucleotide cofactor but in contrast to OCD it is NADPH rather than NAD+,[104] suggestive of a role as a reductase. Indeed both μ-crystallin and OCDs are related to another family of enzymes which are reductases, the glutamyl-tRNA reductases (GluTR) (Segovia and Wistow, in preparation). These unusual enzymes convert glutamyl-tRNA to a glutamate semialdehyde[105] using NADPH as cofactor. At this stage it seems reasonable to hypothesize that all three families of proteins are enzymes involved in unusual amino-acid metabolism and that they may share common affinities for derivatives of glutamate such as glutamate semialdehydes. This raises some interesting possibilities for the function of μ-crystallin.

Although μ-crystallin was discovered in marsupial lenses, it is conserved and expressed in other tissues in other vertebrates. Human μ-crystallin has been cloned from retina and brain and is over 80% identical to the kangaroo sequence. Northern blots of human mRNA detect μ-crystallin in neural, muscle and kidney tissue.[100] However, immunohistochemical detection of the protein shows a more restricted pattern. Although low levels are apparent in the epithelial and equatorial regions of human and rat lenses and may also be present as a general background in most nervous tissue, the highest levels of protein immunoreactivity are found specifically in the outer segments of the photoreceptors of the retina in human, rat and chicken (ref. 104 and in preparation). Indeed μ-crystallin is one of the earliest markers for photoreceptor development in embryogenesis. In this regard it is intriguing that another crystallin, η-crystallin, is derived from ALDH1 which is an even earlier marker for retinal development.[79]

The enzymatic function of μ-crystallin has still not been determined. All attempts to catalyze conversion of ornithine or proline with any nicotine dinucleotide cofactor have failed. Furthermore, although the protein, which appears to be a dimer, can easily be isolated from lens extracts using its ability to bind to blue sepharose it has proved very difficult to maintain its solubility (unpublished), an unexpected problem for a crystallin. However we do know that this protein is related to enzymes which metabolize derivatives of glutamate and that it is expressed in photoreceptors. It so happens that glutamate is the neurotransmitter of the photoreceptors[106] and that these cells are also highly susceptible to glutamate and ornithine toxicity.[78,107,108] Indeed, in the disease gyrate atrophy a systemic deficiency in ornithine metabolism is manifest as a rather specific syndrome of photoreceptor loss in the retina.[107,108] Perhaps μ-crystallin participates in metabolism of glutamate and as such plays an unanticipated role in the normal function of photoreceptors? Indeed, the human gene for μ-crystallin maps close to a breakpoint on chromosome 16 associated with cataract and micro-phthalmia.[109,110] Human and kangaroo genes for μ-crystallin have now been cloned and are being analyzed (unpublished).

Since gene expression in the retina may involve transcription factors which are shared by lens, such as Pax-6, μ-crystallin may have been a facile target for gene recruitment as a crystallin. The gene for μ-crystallin may have required only minor modifications to promoter sequences to allow high expression in lens.

CRYSTALLINS ELSEWHERE: INVERTEBRATE LENSES, LIGHT ORGAN LENS AND CORNEA

S-CRYSTALLINS

A variety of lenses are also found in the exceedingly diverse eyes of invertebrates. Most cephalopod mollusks such as octopus, squid and cuttlefish have eyes which are superficially very similar to those of vertebrates[8,111,112] (see Fig. 1.1). They have cellular lenses which seem to have arisen by convergent evolution to produce a similar solution to the problem of refining the optical properties of the eye. Like the lenses of fish, cephalopod lenses are extremely hard and relatively dehydrated, with protein content up to 70% wet weight.[113] Again like vertebrate lenses, most of the protein in these lenses consists of highly abundant soluble proteins with subunit sizes between 20 and 30 kDa which have been called S-(for squid) crystallins.[113] Although they are not related to any vertebrate crystallins S-crystallins share with taxon-specific crystallins a derivation from enzymes, in this case glutathione S-transferases (GST).[20,114] Unlike most examples of vertebrate enzyme crystallins, S-crystallins are generally lens-specific. They are also ubiquitous in cephalopod lenses, like α-, β- and γ-crystallins in vertebrates, rather than taxon-specific. S-crystallins are encoded by large gene families, again reminiscent of the multiple β- and γ-crystallins. Furthermore, most S-crystallins lack detectable enzymatic activity, although at least one squid protein does have some GST activity.[115]

It appears that the cephalopod lens is very ancient. This has allowed a greater degree of specialization for lens than is seen in vertebrates. The common ancestor of modern cephalopods must have recruited a GST as its original crystallin. Possibly this detoxification enzyme was already being expressed abundantly in the ocean-exposed eye in a protective role. Subsequently there was gene multiplication and specialization to produce more than a dozen lens-specific crystallins in the modern squid. Indeed, the specialization is such that the major GST enzyme of the squid is not expressed at high levels in lens. The antiquity of the cephalopod lens is further emphasized by the presence of other specialized components, such as a lens-specific tubulin.[116]

Sequence analysis of cephalopod S-crystallins shows they have the same exon-intron structure as mammalian GSTs.[117] This similarity, together with the degree of sequence similarity seen in various genes is consistent with the idea that these mollusks are more closely related to vertebrates than are other invertebrates such as arthropods.

ALDH: A Crystallin for All Seasons?

Although the predominant S-crystallins are ubiquitous in cephalopod lenses there is also at least one taxon-specific crystallin in this group. In addition to the S-crystallins, the octopus lens also contains a fairly prominent 59 kDa subunit which was named Ω-crystallin.[118] Surprisingly, when it was cloned, Ω-crystallin was found to be related to class 1 and 2 aldehyde dehydrogenases of vertebrates[119,120] and therefore distantly related to η-crystallin in elephant shrews.[60,76] However, unlike η-crystallin, Ω-crystallin has not conserved all the residues thought to be essential for enzymatic function and lacks detectable ALDH activity.[119,120] Again, it seems that the recruitment of this protein occurred sufficiently long ago that specialization has occurred.

Remarkably another member of the ALDH superfamily is also expressed abundantly in another lens-like tissue of the squid. The light organ of some squid emits light for signaling or camouflage purposes. Light is produced by luminescent bacteria and is diffused through a translucent "lens" derived from muscle tissue. A major soluble protein in this tissue has been named L-crystallin.[121] It too belongs to the ALDH superfamily, although it is not closely related to either η- or Ω-crystallins. This is another example in which soluble structural proteins of a functional lens have been recruited from an available enzyme.

The involvement of the ALDH superfamily in transparent tissues does not stop with the squid light organ. The vertebrate cornea consists mainly of a stroma of aligned bundles of collagen.[122] This is maintained by thin layers of cells on each surface, the outer epithelium exposed to the air and the endothelium which contacts the aqueous of the eye. Damage to these cells can lead to osmotic swelling of the stroma and opacity. Analysis of bovine corneal epithelium found a single major soluble protein component, BCP54.[123] Analysis of chicken cornea showed that this protein was not abundant,[37,124] showing that corneal proteins too may be taxon-specific. When BCP54 was characterized[125,126] it proved to be identical to ALDH III, the so-called tumor-inducible ALDH which is about 30% identical to the class I and II enzymes.[127] Surprisingly, the major site of constitutive expression of this inducible detoxification enzyme seems to be mammalian cornea.

Does this mean that ALDH superfamily members are in some way inherently transparent proteins? After all, the name "transparentin" was once proposed for BCP54.[128] In fact there is no reason to believe that these enzymes are "more transparent" than other proteins. Transparency depends on a medium lacking light absorbance and light scattering.[129,130] Absorbance is due to chromophores while scattering is due mainly to irregularly distributed objects or discontinuities which have sizes on the order of the wavelength of incident light. Since proteins are small it is more reasonable to talk of transparent solutions than transparent proteins.

So why are ALDH superfamily members so frequently recruited to transparent tissues? All these tissues are subject to osmotic stress, either in the swelling process which creates them or in maintaining their structure. Perhaps ALDH enzymes have some involvement in responses to such stress? In any case, it is likely that for some functional reason, these enzymes are easy to recruit under the conditions which give rise to transparent tissues. With their role as detoxification enzymes they may also play a protective role against toxic aldehydes which might result from oxidative insult, particularly to membranes. At least in terrestrial species they may also have a role in filtering UV radiation[125] and in this part of the spectrum they may actually be inherently opaque proteins.

What is clear is that overexpressed aldehyde dehydrogenases are not essential for lens or for cornea. Their recruitment is taxon-specific and many other species survive with only normal enzymatic levels of these proteins. BCP54/ALDHIII is only prominent in mammalian cornea while in chicken no single protein dominates to the same extent.[124] Instead several enzymes are quite abundant, including α-enolase[37] which other data show is localized to corneal limbus in mouse.[54] The most interesting discovery in chicken cornea was that enzymatically inactive δ1-crystallin, the major component of chicken lens is present at detectable levels.[37] There are two possible reasons why this lens-specialized protein might be abundant in cornea. The most likely is that cornea and lens share certain transcription factors, such as Pax-6.[131-136] Expression of a crystallin in the cornea could thus be due to overlap in transcriptional specificity of related tissues. Alternatively, whatever role δ1-crystallin has specialized to perform in lens, which might be something like stabilization of a particular kind of cytoskeleton or a role in osmoregulation, could also be beneficial for cornea. Again such a role cannot be essential for transparency since mammalian corneas survive quite well without expression of this protein.

OTHER CRYSTALLINS

Cellular lenses are also found in hydromedusan and cubomedusan jellyfish. The lens of the cnidarian *Cladonema radiatum* contains two major soluble proteins of 40 kDa and 70 kDa subunit size,[137] while certain cubomedusan jellyfish contain crystallins of 20 kDa and 35 kDa subunit size which have been named J-crystallins.[138] Sequence analysis of J1-crystallins, including cloning three intronless genes,[139] revealed no obvious identity with known proteins, although there is a weak but intriguing similarity to a region of the HSP60 family of molecular chaperones (Wistow, unpublished).

Other species of invertebrate have various kinds of acellular lenses. Some use inorganic materials while others use secreted structural proteins. Since crystallins have always been thought of as soluble proteins, these invertebrate eye proteins may not strictly qualify for this classification. Little is known about these proteins. Some at least are

probably conserved among widely divergent species since immunochemical methods suggest that the compound eyes of diverse arthropods contain related proteins.[140] A 52 kDa calcium-binding glycoprotein of the extracellular corneal lens of *Drosophila* compound eye has been partially characterized and named drosocrystallin.[141] It is not clear what the superfamily relationships of this protein might be or whether it is related to the common arthropod protein. Finally, there has been some analysis of the acellular lens of the mollusk *Aplysia californica* which contains protein subunits of two size ranges, about 60 kDa and 80 kDa.[142] However, since these subunits have identical N-terminal protein sequences they are likely to be products of the same gene, perhaps derived by post-translational modification. The limited sequence available does not reveal the relatedness, if any, of these proteins.

RECRUITMENT THROUGH MODIFIED GENE EXPRESSION

In the broadest sense of recruitment all that is necessary is for a protein to achieve very high concentrations in the lens. This could be accomplished by either transcriptional or post-transcriptional events. The latter are purely hypothetical but might in principle include tissue-specific enhancement of protein stability, or the stability or translational efficiency of mRNA. In fact no such mechanisms have yet been observed in the lens. Instead all the examples of recruitment hinge on tissue-specific increases in gene transcription (see Fig. 2.3).

TATA-Box Promoters

In some cases it is possible to compare homologous genes from species in which recruitment has and has not occurred. One intriguing observation which arises from this is that most of the enzyme crystallin genes, like those which encode the ubiquitous α-, β- and γ-crystallins, make use of TATA boxes to define the starting point for transcription while their non-recruited homologues lack TATA boxes and instead have GC-rich promoters of the kind associated with "housekeeping" genes (Fig. 4.8).

For example, the α-enolase/τ-crystallin gene of the duck has a TATA box and a single predominant transcription start site.[52] In contrast the promoter for human α-enolase, the only other α-enolase gene to have been sequenced, has a GC-rich promoter and multiple transcription start sites.[61] Similarly in chickens and ducks the genes for both the non-enzymatic δ1-crystallin and the enzymatically active ASL/δ2-crystallin have TATA boxes[14,15,29,143] while the genes for human and rat ASL again have "housekeeping" promoters.[144,145] In the case of ζ-crystallin in both guinea pig and llama, which have been recruited through acquisition of an alternative lens promoter, the upstream "enzyme promoter" is a GC-rich type while the downstream lens promoter again has a TATA box.[97,146,147]

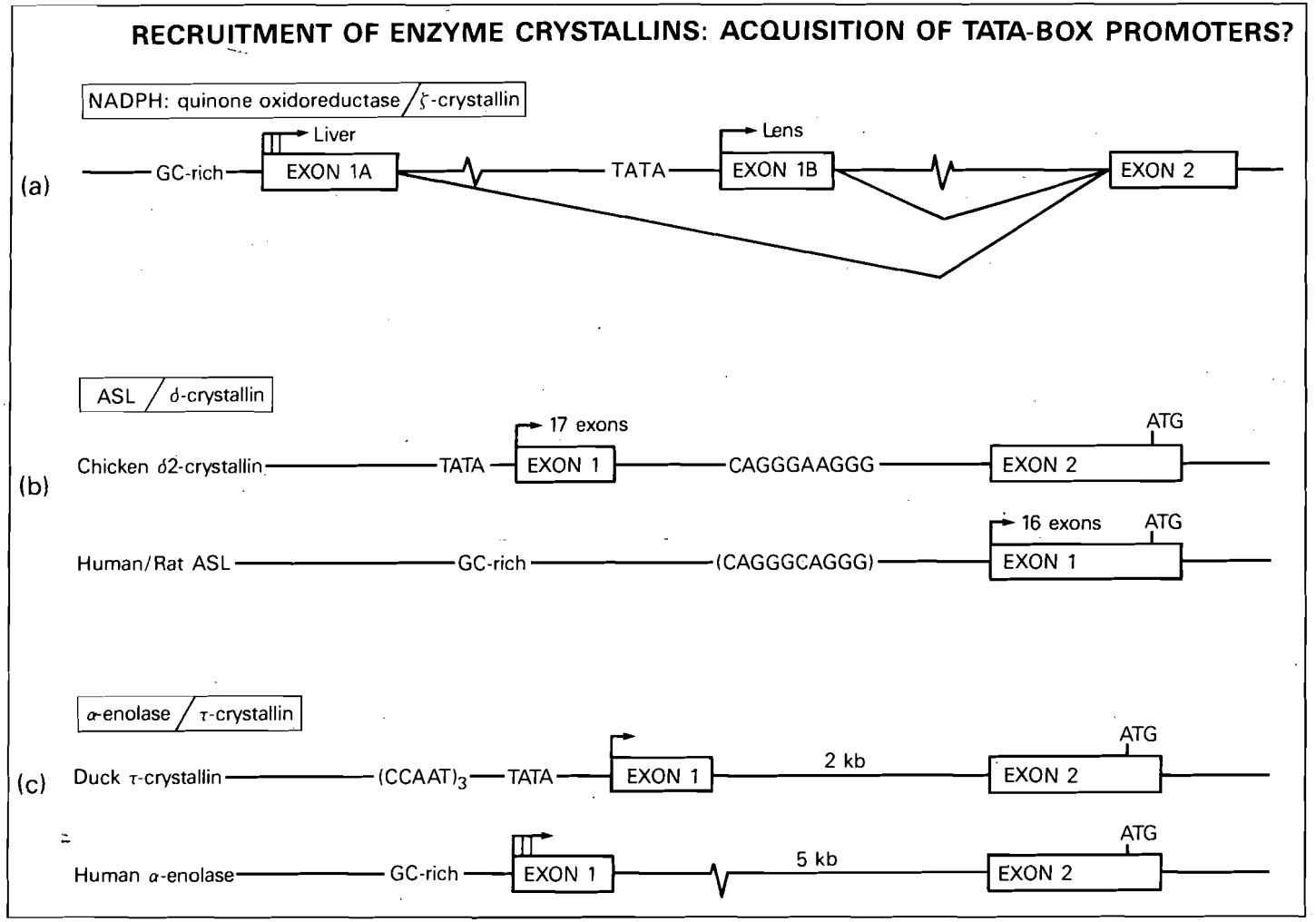

Fig. 4.8. Gene recruitment of enzyme crystallins is often associated with the presence of TATA box promoters. Taken from Harrington L et al, Dev Biol 1991; 148:508-516.

This suggests that at least one major route to recruitment requires the presence of a TATA box. This could reflect a preferred interaction between a lens-specificity factor and some component of the TFIID complex which is associated with binding to a TATA box but not to initiation complexes which form on non-TATA box promoters. However, there are always exceptions to any generalization about crystallins and it appears that duck LDHB/ε-crystallin lacks a TATA box altogether.[5]

New Promoters

In the case of the enzyme crystallins it is clear that genes expressed in many tissues have undergone sequence modification resulting in lens-specific overexpression. The most dramatic example of this is the acquisition of a second, lens-specific promoter by the NADPH:quinone oxidoreductase gene which gave rise to ζ-crystallin.[97,146,147] As described above, the 5' UTRs of ζ-crystallin mRNA in guinea pig lens and liver are different. They arise from alternative first exons spliced to a common second exon. Sequence analysis shows that the lens first exon lies downstream of that used in liver. Thus it appears to have been inserted into what would have been the first intron of the enzyme gene (Fig. 4.8). The guinea pig promoter has been defined by functional analysis.[134,137] It was noted that the complete promoter was neatly flanked by 9 bp direct repeats, one of which was upstream of all the functional elements while the other was in the first exon. When the ζ-crystallin promoter region was compared with the first intron of the homologous gene from mouse in which it has not been recruited as a crystallin there was no sequence similarity within the promoter itself.[146] However just downstream of the promoter region there was some conservation of sequence between species. This similarity begins close to the position of the direct repeat in the first exon of guinea pig ζ-crystallin.[147] This is at least consistent with the possibility that the lens promoter derives from a sequence which was inserted by transposition into the enzyme gene intron in an ancestor of guinea pigs.

ζ-Crystallin is also expressed in the lenses of camelids, including llama. Hystricomorph rodents like guinea pig and camelids like llama are sufficiently distant in evolutionary terms that the recruitment of ζ-crystallin in both must either represent an ancient ancestral feature of most mammals or else independent recruitment in two lineages. Yet gene sequencing shows that llama ζ-crystallin has also been recruited by insertion of an alternative promoter and first exon into the first intron of the same gene.[97] The insertion is close to the same position in guinea pig but not identical. Furthermore sequence alignments show that there is little conservation of promoter sequences but some conservation of both the alternative first exons. At present it is difficult to unravel the histories of the two genes. Their similarities are striking but so are their differences and many questions are yet to be answered.

Was ζ-crystallin ancestral to many mammals but lost in all but two lineages? Was the recruitment independent but directed in a similar way because of some feature of the enzyme gene which made it particularly prone to accepting transposons in its first intron? Both guinea pigs and camelids are of South American origin. Were they both subjected to a similar environmental pressure which led to the recruitment of a particular gene as a crystallin, or is it possible that they actually share a closer ancestry than expected?

Whatever the full story of ζ-crystallin recruitment turns out to be, it is clear that possession of two promoters has certain advantages for an enzyme crystallin gene. The two functions of enzyme and crystallin are separated allowing each promoter to specialize for its role without setting up an adaptive conflict at the level of gene expression. In spite of this, however, most other crystallin genes seem to make use of a single promoter for both modes of expression. Thus duck δ1-, δ2- and ε-crystallins all use the same transcription start site in lens and non-lens expression.[5,148]

However it is not out of the question that the δ-crystallins at one time made use of alternative promoters. When bird δ-crystallin genes are compared with their human homologues the most striking difference is in the number of exons.[66] The δ-crystallins have 17 while the mammalian ASL genes have only 16.[14,15,143-145] The difference lies in the presence of a 5' UTR exon in the bird genes (Fig. 4.8). It is conceivable that the recruitment of ASL occurred when the enzyme gene in an ancestral reptile gained a TATA-box containing lens promoter 5' to its housekeeping promoter. Both promoters could have co existed for a time. Eventually however the older, downstream promoter was lost. Intriguingly, a sequence proposed as a functional element for ASL and related genes in mammals can be found in the first intron of δ-crystallin genes in chicken and duck.[66,144] Could this be a sequence required for non-lens expression of these genes, a leftover from the original promoter which now works in cooperation with the newer upstream promoter?

Lens Specificity

Whatever the mechanism for promoter modification in crystallin gene recruitment, the result is that the gene acquires regulatory elements which respond to the peculiar transcriptional environments of the lens with high expression. In spite of the technical problems inherent in studying a tissue in which much of the gene expression occurs in terminally differentiated cells which will not grow in culture, the last few years have seen a great increase in our understanding of the detailed molecular mechanisms of crystallin gene expression. In particular there are exciting indications that lens expression depends upon tissue-restricted DNA binding proteins with roles in tissue determination, such as Pax-6 and SOX-2.[133-136,149] These results are described in the next section.

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