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Crystallins: The Over-expression of Functional Enzymes and Stress Proteins in the Eye Lens

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Introduction

The ocular lens in vertebrates, and even some invertebrates, is a transparent, avascular, cellular tissue whose principal function is light refraction. The necessary refractive index is achieved by the accumulation of a few soluble proteins which, from their role in the 'crystalline' (clear) lens, are known as crystallins (Bloemendal, 1981). In some species crystallins may account for 90% of total lens protein, which in turn may make up 60–70% of the wet weight of the tissue. The lens grows throughout life, with new layers of cells continually overlaying the old (Piatigorsky, 1981). As the differentiated lens fibre cells mature they lose cell nuclei and other organelles, in a process resembling early stages of apoptosis (Gao, Bassnett and Zelenka, 1994). There is thus very little protein turnover in the mature fibre cells. Since the lens must serve throughout life, crystallins must be maintained in a stable, benign environment.

Remarkably, in spite of their unusual role in maintaining lens transparency for years on end, crystallins are not highly specialized structural proteins. Instead they are generally proteins with non-lens functions which have acquired a new role in the lens by a novel process of gene recruitment through tissue-specific enhancement of gene expression (Wistow, 1993b). Some crystallins are quite ancient; their gene recruitment must have occurred in a common ancestor of all vertebrates and they are

essentially ubiquitous in their modern distribution. These crystallins are either identical to, or derived from, proteins with a role in stress responses. Some of these proteins, the a-crystallins, are members of the small heat-shock protein family (Klemenz *et al.*, 1991b; de Jong, Leunissen and Voorter, 1993) and have chaperone-like functions (Horwitz, 1992) which could contribute to the protection and solubilization of other lens proteins. Other crystallins (*Figure 1*) have arisen more recently as evolutionary pressures acted on the visual systems of various species (Wistow, 1993b). These taxon-specific crystallins are enzymes, and most of them are products of a single gene which has enormously increased expression in lens while still serving the ancestral catalytic role with low expression in many tissues. Because of this phenomenon, the lenses of some natural species are already very rich sources of active enzymes. For example, crude soluble extract of duck lens has fully one-quarter the argininosuccinate lyase (ASL) activity of the purified human liver enzyme (Piatigorsky *et al.*, 1988), and as such is considerably more active than most commercial preparations of this enzyme.

Recent advances in the molecular biology of crystallins has led to the identification of tissue-specific or strongly tissue-preferred gene promoters, which are capable of directing the expression of transgenes in various species (see below and Piatigorsky and Zelenka, 1992). Although determination of the basis of tissue-specific expression has proved to be a more complex problem than expected, the targeting of genes to the lens is a practical reality. This article reviews the nature of those proteins which already serve as crystallins, the tools available for manipulation of gene expression in transgenic animals and considers the implications for biotechnology and gene therapy in this system.

Ubiquitous stress-protein crystallins

α-CRYSTALLINS: STRESS PROTEINS AND CHAPERONES

α-Crystallins are abundant in the lenses of all vertebrates (de Jong, Leunissen and Voorter, 1993). Indeed, in some species, including humans, the αA-crystallin gene may be the single largest contributor of protein in the lens. There are two similar α-crystallin genes, αA- and αB-crystallin which are unlinked in those species in which they have been localized (Wistow and Piatigorsky, 1988). The first suggestion of a non-lens role for α-crystallins arose when the sequences of *Drosophila* small heat-shock proteins (shsp) revealed close similarities with αB- (Ingolia and Craig, 1982) (and hence with the closely related αA-) crystallin. This relationship was later extended to the whole shsp family, which is ubiquitous in eukaryotes. The significance of this became clearer when αB-crystallin was detected in a variety of non-lens tissues, showing that it has a non-lens role (de Jong, Leunissen and Voorter ,1993). It was first detected in scrapie-infected hamster brain (Duguid, Rohwer and Seed, 1988) and since then elevated levels of αB-crystallin have been shown to be associated with a number of neurological disorders, including Alexander's disease (Iwaki *et al.*, 1989), Lewy body disease (Lowe *et al.*, 1990) and Creutzfeldt–Jakob disease (Renkawek *et al.*, 1992). Indeed, αB-crystallin is now a valuable neural-tissue marker and antisera to αB-crystallin are commercially available. Lower amounts of αB-crystallin are also expressed constitutively in many tissues in both mammals and

Table 1. Crystallins

	Related or identical	Activity	Cofactor	Species
Ubiquitous stress-protein crystallins				
αA, αB	**Small heat-shock protein**	Solubilize heat-stressed protein	None	All vertebrates
βA1–4, βB1–3 } γA–F, γS }	*Micro-organism spore proteins,* *M. xanthus protein S,* *P. polycephalum spherulin 3a*	?	None	All vertebrates: γA–F not in birds
Taxon-specific enzyme crystallins				
δ1, δ2	**Argininosuccinate lyase**	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**	?	NAD+ ?	Elephant shrews
λ	*Hydroxyacyl-CoA dehydrogenase*	?	NADH ?	Rabbits, hares
μ	*Ornithine cyclodeaminase* *glutamyl-tRNA reductase*	?	NADPH	Some marsupials
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 in some subunits	GSH	Octopus, squid
Ω	*ALDH*	No ALDH activity detected	NAD+ ?	Octopus

birds (see, for example, Dubin, Wawrousek and Piatigorsky, 1989; Iwaki, Kume-Iwaki and Goldman, 1990; Kato *et al.*, 1991b; de Jong, Leunissen and Voorter, 1993; Lee, Kim and Wistow, 1993). Most significantly, it is induced by heat-shock in NIH 3T3 cells (mouse fibroblasts) (Klemenz *et al.*, 1991b), showing that it is in fact a small heat-shock protein; it can also be induced by other insults such as osmotic stress (Dasgupta, Hohman and Carper, 1992), ischaemia (Chiesi, Longoni and Limbruno, 1990) and by some proto-oncogenes (Klemenz *et al.*, 1991a). Inducibility of αA-crystallin has not yet been demonstrated, but the protein is present, albeit at very low levels in non-lens tissues, particularly in spleen and thymus (Kato *et al.*, 1991a), hinting at a possible role in the immune system.

The function of the shsp family is not well understood. Members of other heat-shock protein (hsp) families are known as chaperones and have dynamic roles in directing protein-folding pathways and the transport and assembly of multisubunit complexes (Hartl, Hlodan and Langer, 1994). The ubiquitous HSP70 proteins are ATPases with structural similarity to actin (Flaherty *et al.*, 1991). A chaperone-like role for the shsp family has now been suggested by observations that α-crystallins are capable of preventing the insolubilization of heat-denatured proteins (Horwitz, 1992). There is also some evidence to suggest that shsps, which have been identified as actin-depolymerizing proteins, and α-crystallins may have a role in assembly/disassembly of cytoskeleton in lens and in other cell types (Miron *et al.*, 1991; Nicholl and Quinlan, 1994).

α-Crystallins undergo a number of post-translational modifications, including cAMP-dependent phosphorylation (Voorter *et al.*, 1986; Chiesa, Gawinowicz-Kolks and Spector, 1987; Chiesa *et al.*, 1987; Voorter *et al.*, 1989), autophosphorylation (Kantorow and Piatigorsky, 1994) and cytoplasmic glycosylation (Roquemore *et al.*, 1992) which could influence their states of aggregation and other interactions. The quaternary structure of α-crystallins continues to be controversial with many competing models (see de Jong, Leunissen and Voorter, 1993 for references). It has been suggested that, given their apparent role as chaperones, α-crystallins and other shsp family members may have multiple modes of assembly and dynamic interchanges between alternative structures (Wistow, 1993c). This could explain some of the apparent contradictions in the biochemical and biophysical data. In spite of the growing interest in this family, there is little tertiary structural data. CD and ORD spectroscopy both suggest that α-crystallins have predominantly β-sheet structure (Li and Spector, 1967, 1974). Sequence comparison has recently suggested a region of similarity between shsp and HSP70 including a conserved α-helix (Lee *et al.*, 1993). However an X-ray structure determination is urgently needed.

β- AND γ-CRYSTALLINS: OSMOTIC-STRESS PROTEINS?

The other two recognized classes of ubiquitous crystallins, the β- and γ-crystallins, belong to the same protein superfamily, the βγ-crystallins (Driessen *et al.*, 1980; Wistow *et al.*, 1981). All vertebrates seem to have six or seven β-crystallin polypeptides (Ostrer and Piatigorsky, 1980; de Jong, 1981; Berbers *et al.*, 1984; Wistow and Piatigorsky, 1988). They form dimers and higher aggregates of mixed composition (Harding and Crabbe, 1984). Most species have a similar number of γ-crystallins which, in contrast, are strictly monomeric (Harding and Crabbe, 1984). The best-

defined γ-crystallins are those found in mammals, which are principally expressed early in development so that they predominate in the central (or 'nuclear') regions of the lens consisting of cells of embryonic origin. This group of γ-crystallins is apparently completely absent from birds (Treton *et al.*, 1984). However bird lenses do contain one ubiquitous protein, now called γS-crystallin, which is a more distantly related member of the γ-crystallin family and which, in mammals, is expressed only later in development in the less-dense cortical regions of the lens (Harding and Crabbe, 1984; Quax-Jeuken *et al.*, 1985; van Rens, de Jong and Bloemendal, 1991). The confusion of identity for this protein is such that it was originally called βS-crystallin. Like β-crystallins, and unlike classic γ-crystallins, this protein has an N-terminal extension and is N-terminally acetylated.

The tertiary structures of β- and γ-crystallins are known in considerable detail thanks to a number of X-ray crystallographic studies. These were first carried out on bovine γII-crystallin (Blundell *et al.*, 1981; Wistow *et al.*, 1983; Summers *et al.*, 1984) (which now has the systematic name γB-crystallin). The protein has a remarkably symmetrical structure of two domains, each containing a further twofold repeat for a total of four characteristic structural motifs, modified 'greek-keys', with a distinctive pattern of required amino-acid residues. This same pattern is seen in the β-crystallins for which X-ray structures are now also available (Bax *et al.*, 1990). The existence of the structural signature in the sequence of this superfamily allowed the identification of two non-lens members. Both these proteins are expressed specifically in micro-organisms which are undergoing spore or cyst formation in response to stress, typically osmotic stress or dehydration. The first to be noticed was protein S of the bacterium *Myxococcus xanthus* which has the same four-motif, two-domain structure as the β- and γ-crystallins (Wistow, Summers and Blundell, 1985). The predicted structure of protein S has now been confirmed by an elegant NMR study (Bagby *et al.*, 1994a,b). The major difference between this structure and that of a γ-crystallin is that the interdomain contact in the bacterial protein is unsymmetrical and principally involves motifs 2 and 3, while that in γ-crystallin is highly symmetrical and involves motifs 2 and 4. The difference in interaction may relate to the calcium-binding properties of protein S. The second protein, spherulin 3a, is found in a eukaryotic slime mould, *Physarum polycephalum* (Wistow, 1990). Interestingly, this eukaryotic protein represents a more primitive stage of molecular evolution than its bacterial relative and the crystallins. Instead of four motifs and two domains it has only two motifs and one domain and seems to have missed out on the last internal duplication of sequence in the evolution of this superfamily. Intriguingly, an unusual feature links prokaryotic *M. xanthus* and eukaryotic *P. polycephalum*. Unlike more closely related sporulating species, both these, otherwise very distantly related, micro-organisms sporulate in response to osmotic stress induced by polyols, sugar alcohols. Thus both organisms share an unusual stress response and both express members of the βγ-crystallin superfamily under such stress. This has led to the suggestion that the ancestral non-lens role of the β- and γ-crystallins may have been as osmotic stress proteins (Wistow, 1990, 1993b).

This may also point to a continuing non-lens function of one or more of the modern β- or γ-crystallins. Recently, very sensitive RNA protection methods have detected non-lens expression of γ-crystallin family genes in *Xenopus* larvae, although there has been no detection of non-lens protein (Smolich *et al.*, 1994). There is stronger

evidence of significant expression of β-crystallins, particularly βB2-crystallin, in non-lens tissues of newly hatched chickens, especially in retina, brain and kidney (Head, Peter and Clayton, 1991). The possibility of a non-lens function for some β-crystallins, perhaps as constitutive stress proteins in certain tissues, is therefore quite real.

In contrast, the main role of the γ-crystallins seems to be as specialized lens proteins. Their fundamental role in the lens seems to be the maintenance of a stable, low-water, high protein concentration environment suitable for the densest, highest refractive index regions of the lens. They are most abundant in very hard lenses, such as those of fish or nocturnal, burrowing rodents such as mice and rats (de Jong, 1981; Harding and Crabbe, 1984). They are much less abundant, or even absent, from softer lenses of diurnal, terrestrial species such as birds. Decreased γ-crystallin content may be due to reduced gene expression, as in humans (Russell *et al.*, 1987; Brakenhoff *et al.*, 1990), or gene loss, as in birds (Treton *et al.*, 1984). However, γ-crystallin content may also be reduced by another mechanism, namely the 'dilution' of γ-crystallins in the lens fibre cells by overexpression of other proteins through the direct gene recruitment of metabolic enzymes to a new structural role.

Gene recruitment: taxon-specific enzyme crystallins

Vertebrates with eyes originally evolved under water. This optically dense medium necessitates lenses with high refractive indices, just as is observed in lenses of fish and those produced by convergent evolution in cephalopods such as squid and octopus. However, when vertebrates emerged from water on to the land during the course of evolution there would have been strong pressure to modify the properties of their hard aquatic lenses for this new, low refractive index environment. Lenses with the high refractive index most useful under water would not be able to focus more distant objects, such as food or predators, in air. This has been hypothesized to be the basis of the remarkable diversity of taxon-specific crystallins in vertebrates (Wistow, 1993b) (*Table 1, Figure 1*). Different lineages of modern vertebrates must have made multiple independent modifications to their repertoire of crystallins in response to selective pressures of environment and habit.

Frequently this adaptation was achieved by the direct gene recruitment of enzymes as crystallins (*Figure 2*). In this novel evolutionary process, genes already being expressed in the lens for metabolic or other reasons underwent modification in their expression specifically in the lens. This led to a huge increase in the concentration of the recruited protein in the lens and thereby to the acquisition of a new structural role. It is important to recognize that after recruitment the same gene continues its normal pattern of expression in other tissues. This means that a recruited enzyme crystallin becomes a dual functional protein, retaining its well-adapted, ancient, catalytic role while, at the same time, acting as a crystallin, interacting with other proteins at high concentration to maintain lens transparency. These dual roles subject the protein to two different sets of evolutionary selective pressures. In some cases, as for example seen in lactate dehydrogenase B/ε-crystallin (Wistow, Mulders and de Jong, 1987; Hendriks *et al.*, 1988; Wistow, Anderson and Piatigorsky, 1990), the new role in the lens may drive unusual protein sequence modifications in the enzyme. It has been suggested that such competing selective pressures can lead to an adaptive conflict, in
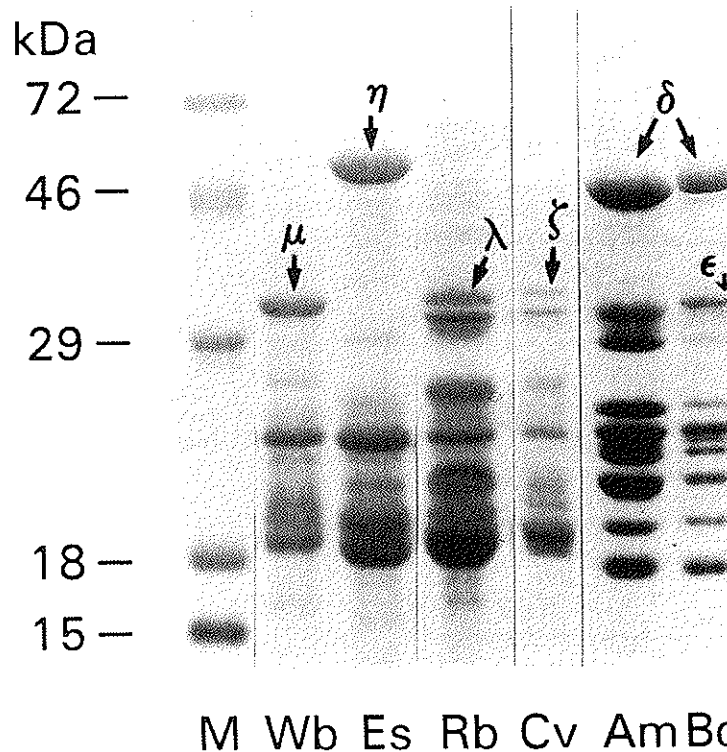
Figure 1. Diversity and similarity of abundant lens crystallins. SDS-PAGE of total soluble protein (Wistow, 1993a) from lenses of several species. Taxon-specific crystallins are marked (see *Table 1*). Species are: Wb, tamar wallaby; Es, elephant shrew; Rb, rabbit; Cv, rock cavy; Am, American merganser; Bd, black duck.

which changes beneficial for one role are deleterious for the other (Wistow, Anderson and Piatigorsky, 1990; Wistow, 1993b). This can be solved in various ways, including loss of the recruited function (revision) or, more rarely, by gene duplication followed by specialization and separation of enzyme and crystallin functions.

RECRUITED ENZYMES

The enzymes which have so far been identified as crystallins are listed in *Table 1*. The archetype of this phenomenon is ε-crystallin. This protein is extremely abundant in the lenses of many birds and crocodiles, two groups which are closely related (Stapel *et al.*, 1985). ε-Crystallin accounts for as much as half the total protein in the lens of a humming-bird (*Calypte ana*) (Wistow, Anderson and Piatigorsky, 1990), and is 10–25% of total protein in other species, particularly sea-birds, waterfowl and others that hunt their food in bright light (Stapel *et al.*, 1985; Wistow, Mulders and de Jong, 1987). When ε-crystallin from duck (*Anas platyrhynchos*) lens was partially sequenced, it became clear that it was very closely related to lactate dehydrogenase B (LDHB), the predominant heart isoform of this glycolytic enzyme (Stapel *et al.*, 1985; Wistow, Mulders and de Jong, 1987). Indeed, peptide sequences of authentic heart LDHB showed identity to the lens protein (Wistow, Mulders and de Jong, 1987) and, later,
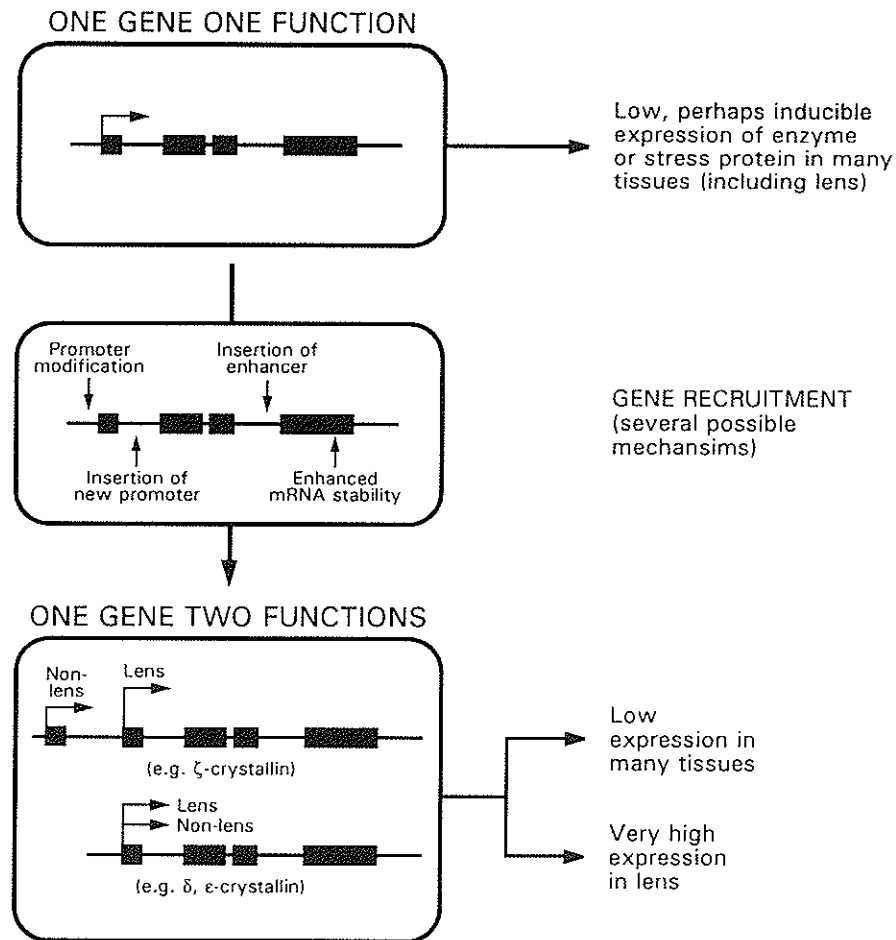
ONE GENE ONE FUNCTION

Low, perhaps inducible
expression of enzyme
or stress protein in many
tissues (including lens)

Promoter modification   Insertion of enhancer

Insertion of new promoter   Enhanced mRNA stability

GENE RECRUITMENT
(several possible
mechansims)

ONE GENE TWO FUNCTIONS

Non-lens   Lens

(e.g. ζ-crystallin)

Lens
Non-lens

(e.g. δ, ε-crystallin)

Low
expression in
many tissues

Very high
expression
in lens

Figure 2.   The gene recruitment of crystallins. A schematic gene is shown. Boxes are exons.

cloning experiments confirmed that the heart and lens proteins are the products of the same single gene (Hendriks *et al.*, 1988). In its new role in lens, LDHB/ε-crystallin must survive without turnover for very long periods, even years, while almost continually exposed to light and while interacting closely with other lens components. Peptide sequencing reveals that there is some post-translational modification of ε-crystallin in the lens as two asparagine residues become deamidated, probably as result of ageing in the lens (Wistow, Mulders and de Jong, 1987; Hendriks *et al.*, 1988). However, in spite of all this, duck lens LDHB/ε-crystallin is almost as enzymatically active *in vitro* as the purified heart enzyme (Wistow, Mulders and de Jong, 1987).

Duck lens is an even more impressive source of another enzyme, argininosuccinate lyase (ASL). This enzyme is usually associated with the urea cycle in mammals but is also expressed in non-ureotelic tissues such as those of the eye (Rao and Cotlier, 1984) and may be involved in various pathways such as nitric oxide synthesis (Hecker *et al.*, 1990). In birds, ASL is identical to δ2-crystallin (Wistow and Piatigorsky, 1987;

Piatigorsky *et al.*, 1988; Barbosa *et al.*, 1991). So far, δ-crystallins have been found in all reptiles and birds examined, with the interesting exception of the chimney swift, a bird which may have eleminated δ-crystallin gene expression as a response to adaptive conflict (Wistow, Anderson and Piatigorsky, 1990). In the swift and in a humming-bird (a closely related species) high levels of ε-crystallin replace δ-crystallin, and in these species the sequence of LDHB/ε-crystallin is not modified. It is possible that a potentially unfavourable interaction between these proteins is the basis for the adaptive conflict which has driven all these changes in sequence or gene expression.

In chickens and ducks there are two δ-crystallin genes (Piatigorsky, 1984; Nickerson *et al.*, 1985, 1986; Wistow and Piatigorsky, 1990; Li, Wistow and Piatigorsky, 1994). While one codes for ASL/δ2-crystallin (Barbosa *et al.*, 1991; Kondoh *et al.*, 1991) the other, δ1-crystallin seems to be an example of gene duplication forced by selective pressure in the lens. δ1-Crystallin is highly similar to ASL/δ2-crystallin but because of a few key amino acid changes, including a critical change of histidine to glutamine, has lost ASL activity (Barbosa, Cialkowski and O'Brien, 1991; Barbosa *et al.*, 1991; Kondoh *et al.*, 1991). Instead it seems to have become a more specialized crystallin. In some species, such as chicken, only δ1-crystallin is expressed at high levels as a structural protein (Parker, Wawrousek and Piatigorsky, 1988). However, in other birds, for example ducks, ASL/δ2-crystallin continues its role as both enzyme and crystallin (Piatigorsky *et al.*, 1988; Wistow and Piatigorsky, 1990; Li, Wistow and Piatigorsky, 1994). The result is that duck lenses have such high ASL activity that crude soluble extract of duck lens has fully a quarter the activity of purified human liver ASL (Piatigorsky *et al.*, 1988).

Another familiar enzyme, α-enolase, has been recruited as τ-crystallin in some species (Stapel and de Jong, 1983; Williams *et al.*, 1985; Wistow and Piatigorsky, 1987; Wistow *et al.*, 1988; Rudner, Katar and Maisel, 1990) and, at least in embryonic turtle lenses, also retains some enzyme activity (Wistow *et al.*, 1988). Elephant shrew η-crystallin, which may be the most abundant mammalian taxon-specific crystallin, is apparently identical to cytoplasmic aldehyde dehydrogenase (ALDH) (Wistow and Kim, 1991). However, no significant ALDH activity could be measured in lens extracts of elephant shrews obtained *post mortem* from zoos. Yet this could easily be due to the relative lack of freshness of these samples and the low inherent activity of the enzyme which, furthermore, has an active-site cysteine residue which could be sensitive to oxidation (see Weiner, 1990). Now that elephant shrew η-crystallin has been cloned (Graham, Hodin and Wistow, in preparation), the recombinant protein is being expressed in *Escherichia coli* to see if ALDH activity can be detected in freshly synthesized protein. It is also possible that η-crystallin, like δ1-crystallin, is the result of gene duplication and specialization, with subsequent loss of enzyme activity. However, sequence analysis shows that, unlike δ1-crystallin, all the residues required for enzyme activity (see Weiner, 1990) are present in η-crystallin (Graham, Hodin and Wistow, in preparation).

NOVEL ENZYMES

Although some taxon-specific crystallins were easily identified as familiar enzymes, others seem to be novel enzymes discovered for the first time as crystallins. ρ-Crystallin (originally named ε-crystallin) which is found in the lenses of frogs of the

genus *Rana* (Tomarev *et al.*, 1984), belongs to an aldo-keto reductase superfamily which includes aldehyde and aldose reductase, prostaglandin F synthase and several detoxification enzymes (Carper *et al.*, 1987, 1989; Watanabe *et al.*, 1988; Bohren *et al.*, 1989). ρ-Crystallin does bind NADPH, like other members of the superfamily, but its particular enzymatic specificity is not known (Carper *et al.*, 1989). Similarly, λ-crystallin, found in rabbits and hares, is another previously unknown enzyme. It is distantly related to hydroxyacyl- and hydroxybutyryl-CoA dehydrogenase and conserves the NADH-binding site, but its activity, if any, has not been determined (Mulders *et al.*, 1988).

A particularly interesting example of a novel enzyme discovered first as a crystallin is μ-crystallin. This protein may account for as much as 25% of total protein in the lenses of some Australian marsupials, specifically in macropods (kangaroos) and the one dasyurid (*Dasyurops maculata*) examined (Wistow and Kim, 1991). When the complete sequence of kangaroo lens μ-crystallin was determined by cDNA cloning (Kim, Gasser and Wistow, 1992) it became apparent that it has no close relatives among eukaryotic protein sequences. However, it is significantly similar (over 30% identical in amino-acid sequence) to ornithine cyclodeaminases (OCD) of the bacterium *Agrobacterium tumefaciens*. The similarity includes a putative ornithine binding site (Sans, Schindler and Schröder, 1988). OCD catalyses the conversion of ornithine to proline in one step in the presence of NAD^+ (Sans, Schindler and Schröder, 1988). Kangaroo μ-crystallin also is capable of binding pyridine dinucleotide cofactors but its highest affinity is for NADPH (Wistow and Segovia, 1994), in contrast to bacterial OCDs (Sans, Schindler and Schröder, 1988). *In vitro* assays of μ-crystallin with ornithine, proline and all four pyridine dinucleotide cofactors have so far revealed no enzymatic activity. However, μ-crystallin seems to be the product a single gene which is expressed in other tissues, presumably in an enzymatic role (Kim, Gasser and Wistow, 1992). In kangaroos the primary non-lens tissues containing μ-crystallin mRNA are retina and brain (Kim, Gasser and Wistow, 1992). μ-Crystallin is also present at enzymatic levels in other species. It has been cloned from human retina (Kim, Gasser and Wistow, 1992; Segovia and Wistow, in preparation) and seems to be expressed preferentially in photoreceptors in humans, rats and chickens (Wistow and Segovia, 1994). We have recently noticed that μ-crystallins and OCDs are both related to another family of enzymes involved in unusual amino-acid metabolism, the glutamyl-tRNA reductases (GluTR) (Segovia and Wistow, in preparation). These enzymes convert glutamyl-tRNA to a glutamate semialdehyde (Jahn, Verkamp and Söll, 1992) and use NADPH as cofactor. Since glutamate is the major neurotransmitter of the photoreceptors (Barnstable, 1993) there is the interesting possibility that μ-crystallin could be involved in the metabolism of glutamate or its derivatives and could play an important role in the visual process.

Finally, there is one example of a taxon-specific crystallin whose novel enzymatic function has been uncovered. ζ-Crystallin is found in guinea-pigs, some related animals and also in camels and llamas (presumably a rare example of independent recruitment of the same gene in two different lineages) (Huang *et al.*, 1987; Garland *et al.*, 1991; Lee *et al.*, 1993). Sequence analysis showed that ζ- crystallin is a distantly related member of the alcohol dehydrogenase superfamily (Borras, Persson and Jornvall, 1989; Rodokanaki, Holmes and Borras, 1989). Cofactor-binding studies showed further that ζ- crystallin binds NADPH, suggesting a role as a reductase (Rao

and Zigler, 1990). This led to an heroic effort to test substrates for the putative enzyme. Remarkably, an activity was found. ζ-Crystallin turns out to be a novel NADPH:quinone oxidoreductase (Rao, Krishna and Zigler, 1992). Unlike the familiar quinone reductases, ζ-crystallin operates through a single electron transfer mechanism.

INVERTEBRATE CRYSTALLINS

Lenses are also found in a variety of invertebrate eyes. In cephalopods, such as octopus and squid, the lenses are cellular and superficially similar to those of vertebrates. The principal structural proteins of these lenses, S-crystallins, are related to the enzyme glutathione S-transferase (GST) (Wistow and Piatigorsky, 1987; Tomarev and Zinovieva, 1988). Most do not have GST activity, but some minor subunits in the lens do seem to have detectable activity (Tomarev *et al.*, 1994). The recruitment events which led to cephalopod lenses may have been much more ancient than those in vertebrates. Consequently, there may have been more tissue-specific specialization over time. A minor component of octopus lenses has been named Ω-crystallin (Chiou, 1988). This protein has been cloned (Tomarev, Zinovieva and Piatigorsky, 1991) and shown to be related to aldehyde dehydrogenases. This draws a parallel with η-crystallin in elephant shrews, although Ω-crystallin has not conserved all the residues thought to be essential for enzymatic function (Tomarev, Zinovieva and Piatigorsky, 1991). This may be another example of greater specialization and more ancient recruitment in cephalopods. Remarkably, another ALDH-related protein has been recruited to an apparent structural role in another lens-like tissue. Some squid have an organ which emits light, probably for signalling or camouflage purposes. This organ has a diffusing lens derived from muscle tissue. The principal component of this tissue has been named L-crystallin and like η- and Ω-crystallins belongs to the ALDH superfamily (Montgomery and McFall-Ngai, 1992). The coincidence of multiple recruitment events for ALDH-like proteins is interesting. It suggests that these enzymes are easy to recruit under the conditions which give rise to lenses. As suggested below, this could relate to a role for ALDH in elongating cells, perhaps involving osmoregulation or interactions with cytoskeleton.

Some jellyfish also have cellular lenses. The major lens proteins of some cubomedusan jellyfish, named J-crystallins, have been cloned (Piatigorsky *et al.*, 1989). Sequencing revealed no obvious identity with known proteins, although there is a weak but intriguing similarity to the HSP60 family of molecular chaperones (Wistow, unpublished). N-terminal protein sequence has also been obtained for the major proteins of the eye lens from *Aplysia californica* (Cox, Glick and Strumwasser, 1991). The limited sequence available does not reveal the relatedness, if any, of these proteins. Finally, a protein of the extracellular corneal lens of *Drosophila* has been partially characterized and named drosocrystallin (Komori, Usukura and Matsumoto, 1992). Again, it is not clear what the superfamily relationships of this protein might be.

A common origin for crystallins?

While crystallins are clearly quite diverse, it is striking that they fall into only two

rather narrow categories (Wistow, 1993b). Even the taxon-specific crystallins are restricted to 35–54 kDa subunit metabolic enzymes, all with small molecule substrates, products and cofactors. Many of the enzymes bind NAD(P)H (*Table 1*). There are probably functional constraints on what kind of proteins will work well as crystallins. Size, thermodynamic stability and an ability to interact with other proteins to maintain short-range order (Delaye and Tardieu, 1983) without superaggregating (thereby creating centres for light-scattering) are all important characteristics. However, it is also possible that existing crystallins share common origins in some basic process of lens development.

It has been pointed out that all cellular lenses undergo enormous increases in cell volume and elongation (Wistow, 1993b). This must involve massive reorganization of cytoskeleton and either proactive or reactive control of osmolarity. Both groups of stress-protein crystallins have plausible connections with osmoregulatory events or with manipulation of cytoskeleton. αB-crystallin is induced by osmotic stress (Dasgupta, Hohman and Carper, 1992) and the βγ-crystallins are related to proteins which are also induced under similar conditions (Wistow, Summers and Blundell, 1985; Wistow, 1990). A protein isolated from turkey gizzard as an actin depolymerizing protein was found to be a small heat-shock protein related to α-crystallins (Miron *et al.*, 1991), suggesting that proteins of this class may have active roles in manipulating cytoskeleton. Indeed, there have been many observations of α-crystallin association with cytoskeleton and membrane fractions in lens, and it has recently been shown that α-crystallins exert a chaperone-like effect in assembly of cytoskeleton in lens (Nicholl and Quinlan, 1994).

In addition, several of the enzymes recruited as crystallins are able to bind substrates which are known to be potent osmolytes in certain systems, such as lactate and proline (see Kim, Gasser and Wistow, 1992 for references). ρ-Crystallin is related to aldose reductase, an enzyme which is expressed at high levels in the kidney and in the differentiating (and elongating) cells of the rat eye lens (Bondy and Lightman, 1989), which may both be subject to osmotic stress. There are also a number of examples in which enzymes, particularly glycolytic enzymes, are associated with cytoskeleton, forming a cytomatrix (Clarke *et al.*, 1985; Knull and Walsh, 1992). Indeed, in *Tetrahymena*, intracellular structures described as 14 nm filaments were found to be composed of the enzyme citrate synthase (Numata *et al.*, 1991), again showing that enzymes can serve as structural proteins. All this suggests the possibility that crystallins may represent proteins involved in important processes of cell elongation. Even in the absence of recruitment as crystallins, lens cells would need to express these proteins in order to be able to differentiate into fibre cells. The genes for such proteins with a fundamental role in an essential cellular process would necessarily be active in the lens and should thus be relatively facile targets for enhanced expression as crystallins. Higher expression of proteins capable of, for example, protecting cytoskeleton or exerting other protective effects, could indeed by beneficial for the long-term stability of the lens.

Gene expression: targeting the lens

Whatever their origin, all crystallins share the characteristic of very high gene expression in the lens. Several crystallin gene promoters and enhancers have been

analysed in some detail. What follows is a summary of promoter elements which have so far been identified in the expression of crystallin genes and a review of the transgenic animals which have been produced using these promoters.

αA-CRYSTALLIN

The αA-crystallin gene is expressed predominantly in the lens of all species examined. Promoter fragments from mouse (Chepelinsky, Sommer and Piatigorsky, 1987; Jaworski, Chepelinsky and Piatigorsky, 1991), hamster (van den Heuvel *et al.*, 1985), mole rat (Hendriks *et al.*, 1987), chicken (Thompson, Hawkins and Piatigorsky, 1987) and human (Jaworski, Chepelinsky and Piatigorsky, 1991) have been cloned and sequenced, and extensive regions of 5'- flanking regions have been examined in the mouse, human and chicken (1.8, 1.8 and 2.6 kbp, respectively).

The mechanisms regulating αA-crystallin gene expression in these different species appear to be similar, since mouse and chicken promoters show appropriate expression in a variety of heterologous systems. αA-Crystallin transcription has been studied using chick lens epithelial cells, an SV40-transformed cell line derived from mouse lens (αTN4-1 cells), a rabbit lens cell line (N1003A) and in transgenic mice. In general, the same promoter regions are implicated for function regardless of whether the transcription system is derived from rabbit, mouse or chicken. Despite this apparent evolutionary conservation of transcriptional control mechanisms, analysis of the promoters has revealed striking differences (*Figure 3*).

Global alignments of promoter sequences for mouse, human and chicken display only limited similarity; only four regions of significant sequence matches can be aligned in all three species (Jaworski, Chepelinsky and Piatigorsky, 1991; Sax and Piatigorsky, 1994). A number of short sequence motifs are common to all three species, but these are distributed differently with regard to location, orientation and copy number in each. An unexpected similarity among the αA-crystallin promoters is the presence of a repetitive element, characteristic of each species, at approximately 1 kbp upstream of the coding regions (Jaworski, Chepelinsky and Piatigorsky, 1991).

Definition of minimal promoter sequences for the mouse and chicken genes by transfection studies have established that −111 bp are sufficient for activity of the mouse promoter (Chepelinsky, Sommer and Piatigorsky, 1987), but a longer region, extending to −162, is necessary for functional activity of the chicken promoter (Yasuda *et al.*, 1988; Klement, Wawrousek and Piatigorsky, 1989). Other methods confirm the need for a greater extent of distal sequences in the chicken (Cooper, Constable and Davidson, 1984; Wawrousek *et al.*, 1990).

Functional elements of the mouse αA-crystallin promoter have been investigated in numerous studies. Genomic footprinting in αTN4-1 cells and *in vitro* footprinting with lens nuclear extracts showed corresponding patterns of protected regions (Kantorow *et al.*, 1993). Similar regions were also footprinted with nuclear extracts from L929 fibroblasts. Although the same sites were occupied, electrophoretic mobility shift assays (EMSA) demonstrated that different proteins were bound in the non-lens environment. The same promoter region may serve as an activating element in the lens and an inhibitory element in non-lens cells through the selective binding of different factors.

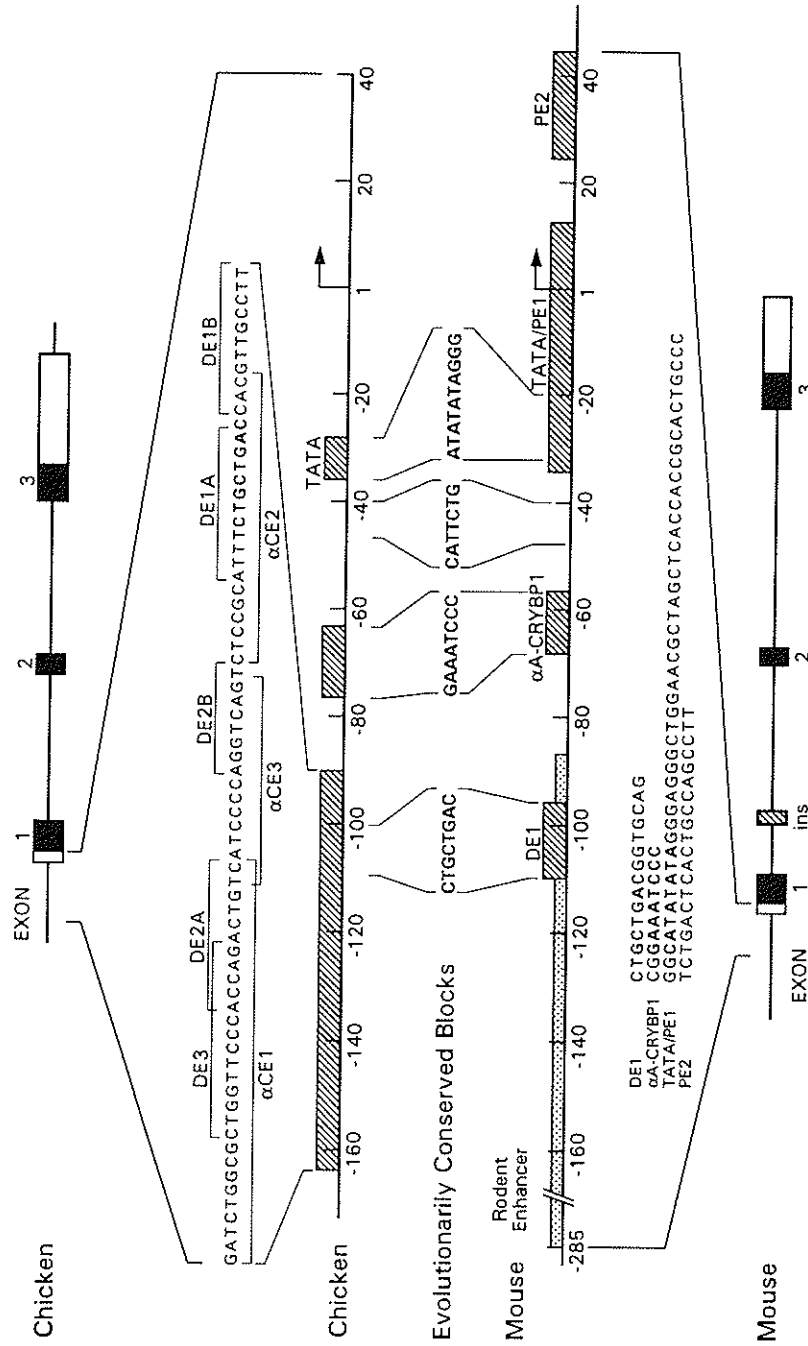The activity of the mouse promoter was originally shown to depend on two

Figure 3. The complex promoters of αA-crystallin genes from chicken and mouse. Identified functional elements are shown together with evolutionarily conserved blocks which occupy similar location in both genes. Hatched boxes indicate proposed binding sites for *trans*-acting factors. Sequences of identified regions are shown above and below the promoter schematics. The gene structures for bird and rodent αA-crystallins are also shown. Coding sequences of exons are shown in black. Some mammals have an alternatively spliced insert exon (ins) (see de Jong, Leunissen and Voorter, 1993).

interacting domains, a distal element (–111/–88), and a proximal element (–88/–60) (Chepelinsky, Sommer and Piatigorsky, 1987). Further definition of the proximal element led to the expression cloning of the transcription factor αA-CRYBP1 and αTN4-1 cells (Nakamura *et al.*, 1990). αA-CRYBP1 binds to the sequence 5'-GGGAAATCCC-3' at positions –66/–57 in the mouse promoter, overlapping one of the evolutionarily conserved sequences. The αA-CRYBP1 site is similar to enhancers in that it functions independently of orientation; but, unlike enhancers, spatial location is important to αA-CRYBP1, and the extent of activation is low (threefold) (Sax, Kelement and Piatigorsky, 1990). Enhancer activity of the αA-CRYBP1 site in transfection studies appears to operate only in αTN4-1, the mouse lens-derived cell line from which it was cloned, but multimerization of the site allows activity in non-murine and non-lens cells (Sax, Klement and Piatigorsky, 1990). Among the mutations of the αA-CRYBP1 site which abolish its activity is the change of GGG to GAG (Sax, Klement and Piatigorsky, 1990), which introduces the variant of the αA-CRYBP1 site found in humans, chickens and mole rats (Hendriks *et al.*, 1987; Jaworski, Chepelinsky and Piatigorsky, 1991). Thus, αA-CRYBP1 recognizes a site present in the mouse but not in the chicken or human promoters; nevertheless, the homologue of αA-CRYBP1 has been identified in chicken lens (Donovan *et al.*, 1992).

Other regions important for transcriptional activity include DE1 (a portion of the distal element spanning –111/–97), TATA/PE- 1 (–32/+12) and PE-2 (+24/+43). Like the αA-CRYBP1 site, the DE1 site overlaps a sequence block completely conserved in mouse, chicken and human αA-crystallins; PE-1 and PE-2 correspond to regions well conserved among mammals (Jaworski, Chepelinsky and Piatigorsky, 1991; Sax and Piatigorsky, 1994). DE1 lies within a rodent αA enhancer element, defined in the hamster as –180/–85 (Chepelinsky, Sommer and Piatigorsky, 1987; Yu, Tsui and Breitman, 1990), that can activate the minimal promoter for γF-crystallin. Interestingly, DE1 and αA-CRYBP1 appear to be functionally redundant sites in transgenic mouse experiments (Sax, Ilagan and Piatigorsky, 1993), since promoter activity is abolished only if both sites are simultaneously mutated.

As noted above, more upstream sequences, extending to –162, are required for activity of the chicken αA-crystallin promoter. This segment can act as an enhancer in a heterologous system using the minimal promoter for β-actin (Matsuo and Yasuda, 1992). Nearly the entire length of the enhancer region is covered by a series of neighbouring and overlapping sequences that have been implicated as protein-binding sites or functional *cis*-acting elements; varying nomenclature has arisen for these elements, depending on the laboratories and techniques used to identify them (*Figure 3*).

The linked elements DE1A and DE1B of the chicken gene (Klement, Cvekl and Piatigorsky, 1993), which coincide with the element αCE2 (Jahn, Verkamp and Söll, 1992), contain the same conserved sequence block covered by DE1 of the mouse αA-crystallin promoter. Both the mouse DE1 and chicken DE1A/B bind nuclear extracts in EMSA, but the mouse element fails to compete with that of the chicken for binding of chicken nuclear extracts (Klement, Cvekl and Piatigorsky, 1993). Like the analogous mouse sequence, the chicken DE1A/B element appears to co-operate with other promoter regions for activity, since a requirement for interaction between the regions defined as αCE2 and αCE1 has been demonstrated (Matsuo and Yasuda, 1992). A protein, αCE1, has been identified that binds specifically to the αCE1 element

(Matsuo *et al.*, 1991; Matsuo, Takeuchi and Yasuda, 1992). Recent work has suggested that several other factors, including USF, CREB and Pax6, bind to the chicken αA-crystallin promoter (Cvekl *et al.*, 1994). Pax6 binding may have particular significance for lens-specific expression (see below).

There are many differences between the αA-crystallin promoters of mouse and chicken. Substantially different minimal sequence lengths are required, and the extent of sequence similarity is meagre. Furthermore, analyses of sequences that are conserved between the mouse and chicken (the αA-CRYBP1 and DE1 regions) show little functional correspondence. The lack of concordance between the mouse and chicken αA-crystallin promoters is intriguing in view of the fact that each apparently functions appropriately in a heterologous system. Currently, the predominant similarity between the mouse and chicken promoters is that each operates through a multiplicity of factors whose interactions co-ordinately regulate transcription.

αB-CRYSTALLIN

Unlike αA-crystallin, αB-crystallin is expressed at significant levels in non-lens tissues such as kidney, lung, cardiac and skeletal muscle and in trace amounts in brain and spleen, in addition to high levels in lens (Duguid, Rohwer and Seed, 1988; Dubin, Wawrousek and Piatigorsky; 1989; Iwaki *et al.*, 1989; Iwaki, Kume-Iwaki and Goldman, 1990). There have not been extensive studies of the human αB-crystallin gene promoter, but it has been shown that a fragment from the promoter −537/+21 linked to the CAT (chloramphenicol acetyltransferase) reporter gene expressed well in lens cells but failed to express in an astrocytoma cell line, suggesting that nerve-specific elements may lie elsewhere (Dubin *et al.*, 1990).

Most functional studies of the αB-crystallin promoter have focused on the mouse gene (*Figure 4*). Primer extension of mouse lens αB-crystallin mRNA identifies a transcription start site 45 bp upstream of the ATG codon and 25 bp down from a TATA box (Dubin, Wawrousek and Piatigorsky, 1989). The same start site is used in heart, skeletal muscle and kidney. A minor transcription start site at −40 to −50 is also used in heart and skeletal muscle. αB-crystallin is induced by heat shock in NIH 3T3 cells and a perfect heat-shock consensus sequence is found at position −53 to −39 in the mouse and human genes (Klemenz *et al.*, 1991b).

A fragment of the mouse gene promoter (−661 to +44) linked to CAT was shown to express in lens and skeletal muscle in transgenic mice when present as one copy, while in mice with multiple copies significant CAT activity was also detected in heart and other tissues (Dubin *et al.*, 1991). Transfection experiments in mouse muscle and lens cells with deletion mutants of this construct showed that the region −426 to −257 is necessary for expression in myotubes while the sequence downstream of −115 is essential for lens expression, albeit at lower levels than for longer promoter fragments (Dubin *et al.*, 1991). Further study of this −426 to −257 fragment linked to the heterologous TK (thymidine kinase) promoter expressing human growth hormone (hGH) as reporter, defined this region as a transcriptional enhancer which stimulated hGH production in differentiated myotubes, but showed less stimulation in lens cells (Dubin *et al.*, 1991). DNase I footprint analysis showed protection of three areas of the αB promoter: αBE-1 (−407 to −397), αBE-2 (−360 to −327) and αBE-3 (−317 to

αBE-1   TCCACAAAGAGTTAATGTCCCTGG
αBE-2   CCAAGATAAGTTGCTGGCTCAATTCCCCTGGCATG
αBE-3   GGAGAGGAGGAGGGGCC
MRF   CCAGCAGCTGCTTGGGAATTCCA
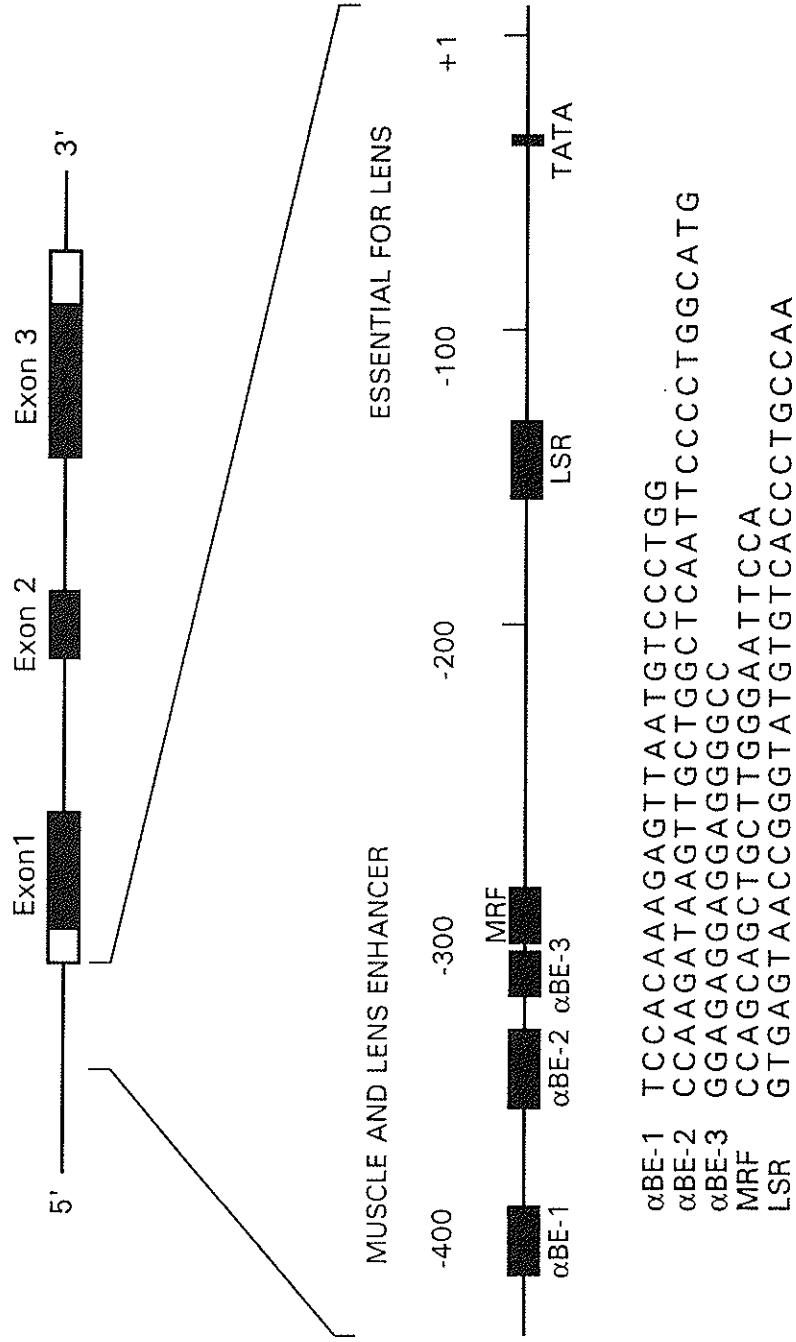LSR   GTGAGTAACCGGGTATGTGTCACCCTGCCAA

Figure 4. Functional elements of the mouse αB-crystallin gene promoter. The gene structure common to bird and mammal αB-crystallins is shown with coding sequences of exons in black. LSR, lens specific region.

–306) with myotube and αTN4 extracts, and a fourth region, MRF (–300 to –288), with myotube extract only (Gopal-Srivastava and Piatigorsky, 1993). This latter region contains a consensus E-box sequence, CANNTG. Site mutagenesis with transient transfection and gel retardation experiments confirms the importance of these four areas in αB activation in lens and skeletal muscle. In addition, gel shifts show that anti-MyoD and anti-myogenin monoclonal antibodies can alter the mobility of complexes formed between myotube extracts and the αB MRF sequence. Finally, transactivation experiments in which MyoD or myogenin expression constructs were co-transfected in NIH 3T3 cells with the αB enhancer linked to TK-hGH showed that either MyoD or myogenin can transactivate growth hormone expression. Mutations in the E-box region abolished this transactivation. Thus, the –426 to –257 region of the mouse αB gene has been shown to contain three regions (–407 to –306) with enhancer function in differentiated muscle and lens cells and a fourth region (–300 to –288) that is muscle-cell specific and responds to activation by MyoD and myogenin.

Most of these sequences are also well conserved in the human gene, although the human gene lacks the E-box found in the mouse MRF region. When compared to a more distantly related homologue, the duck αB-crystallin gene, the αBE-2 site and an AATAAA-containing sequence closer to the TATA box are found to be well conserved (unpublished). Unlike the human gene, the duck gene possesses an identical E-box in the same general region as the mouse MRF. Other sequences are much less well conserved. For example, a core motif of the αBE-1 site is found in complement at a position about 150 bp closer to the TATA box in duck than in mouse and human. More surprisingly, the heat-shock consensus sequences of the human and mouse genes are not present in the duck gene promoter.

Sequences essential for lens-preferred expression seem to lie closer to the transcription start site than those involved in muscle-preferred expression. Transgenic mice carrying the –426 to +44 fragment of the aB promoter linked to CAT showed high expression in the lens, heart and skeletal muscle with low expression in other tissues (Gopal-Srivastava and Piatigorsky, 1994). 5' deletion mutations of this transgene suggest that the –426 to –164 region is necessary for activity in cardiac and skeletal muscle while –164 to +44 confers lens specificity. DNase I footprint analysis using αTN4-1 (a transformed lens cell line) extracts, mutagenesis and transfection experiments in lens cells confirm the –147 to –118 region as a lens-specific element for αB regulation (Gopal-Srivastava and Piatigorsky, 1994).

In mammals there is also evidence for alternative upstream promoters in the αB-crystallin gene which give rise to longer transcripts. This is seen most prominently in lung, brain and spleen (Dubin, Wawrousek and Piatigorsky, 1989; Iwaki *et al.*, 1990). However, this phenomenon does not seem to be evolutionarily conserved, since there is no evidence for such upstream initiation in the duck, in which the predominant mRNA for αB-crystallin in lung is the same size as in other tissues (Lee, Kim and Wistow, 1993). Nevertheless, birds do have a longer mRNA, which is observed in duck and chicken lens (Dodemont *et al.*, 1985; Lee, Kim and Wistow, 1993). Instead of an upstream promoter, this longer mRNA in lens derives from alternative polyadenylation (Sawada, Agata and Eguchi, 1992; Wistow and Graham, unpublished).

β-CRYSTALLINS

β-crystallins have not been studied as extensively as some other classes. However, there has been some promoter analysis for two β-crystallin genes, both from chickens. Several deletion fragments of the chicken βB1-crystallin gene from −434/+30 to −126/+30 were able to drive expression of the CAT reporter gene more efficiently in primary explants of embryonic chicken lens epithelial cells than in muscle fibroblasts or HeLa cells (Roth, Das and Piatigorsky, 1991). Four functional elements, PL-1, PL-2, OL-1 and OL-2, defined by transfection studies, footprinting and EMSA, were located between positions −126 and −53 of the promoter.

Two β-crystallin polypeptides, βA3- and βA1-crystallins, are encoded by a single gene in both mammals and birds (Hogg *et al.*, 1986; McDermott, Peterson and Piatigorsky, 1992). The βA3/A1-crystallin gene has a similar structure in both human and chicken. A promoter construct containing the fragment −382/+22 of the chicken gene promotes expression of the CAT reporter gene in chicken primary lens epithelial cells but not in dermal fibroblasts (McDermott, Peterson and Piatigorsky, 1992). Deletion of sequences between −382 and −143 greatly reduces promoter activity.

γ-CRYSTALLINS

In mammals, there are six tightly clustered γ-crystallin genes with standardized names γA–γF (Breitman *et al.*, 1984; Lok *et al.*, 1984; Moormann *et al.*, 1985; Graw *et al.*, 1993). These are the classic γ-crystallins which are an important part of the embryonic lens and which, as described above, are absent from bird lenses. The proximal promoters of these genes are generally well conserved and have strongly lens-preferred expression in various systems. In rodents all six γ-crystallins are induced as fibre cells form in the embryonic lens, and their expression decreases differentially after birth until only γB-crystallin transcripts are detectable in the adult rat lens (van Leen *et al.*, 1987; Goring, Breitman and Tsui, 1992). Since γ-crystallins appear to be highly specific for the terminally differentiated lens fibre cells, and these cells cannot be grown in culture, a variety of approaches have been adopted for promoter analysis. For example, the expression of the mouse γF-crystallin gene (formerly designated γ2) has been examined in detail in primary explants of chicken lens epithelial cells (Lok *et al.*, 1989) (*Figure 5*). Surprisingly, this has been successful even though chickens do not possess homologous γ-crystallin genes. In this system the γF gene has been shown to have a lens-specific promoter consisting of two upstream enhancer-like elements (which can be successfully substituted by similar regions of the hamster αA-crystallin gene) and a proximal promoter (Yu, Tsui and Breitman, 1990). At early stages in lens development either the enhancers or the proximal promoter can direct gene expression, while later in development, co-operation between these elements is required for expression in fibre cells (Goring *et al.*, 1993). Within the proximal promoter, close to the TATA box are sequences, showing no homology to other lens-specific elements, which are able to bind a factor, γF-1, which can specifically direct expression to adult fibre cells (Liu *et al.*, 1991; Goring *et al.*, 1993). Expression of the γF-crystallin promoter in explanted chicken embryonic lens epithelial cells is enhanced by retinoic acid, a potent inducer of cell differentiation, and a novel type of retinoic acid response element, γF-RARE, has been identified in the mouse γF-crystallin gene enhancer region (Tini *et al.*, 1993).
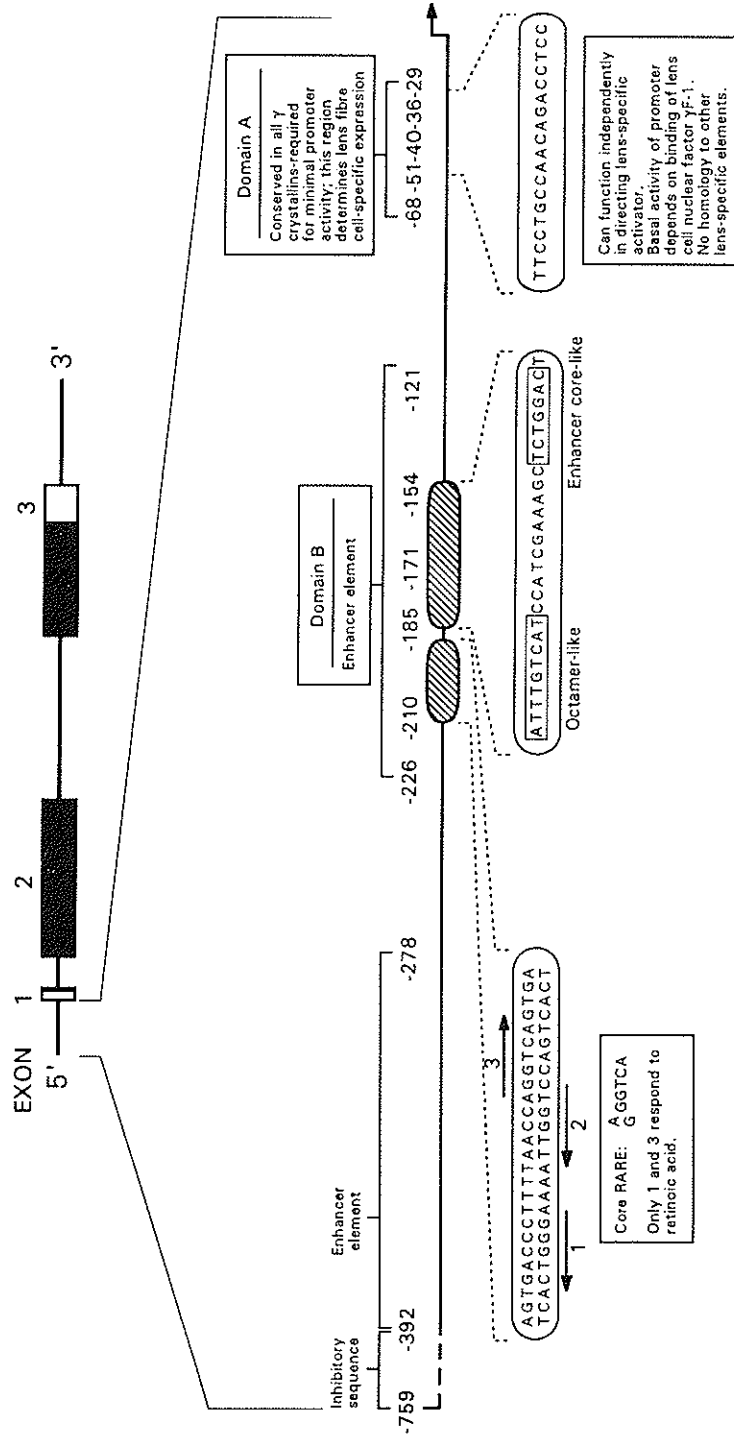
Figure 5.   Functional elements of the promoter of the mouse γF-crystallin gene. The structure of a typical γ-crystallin gene is shown with coding sequences of exons in black.
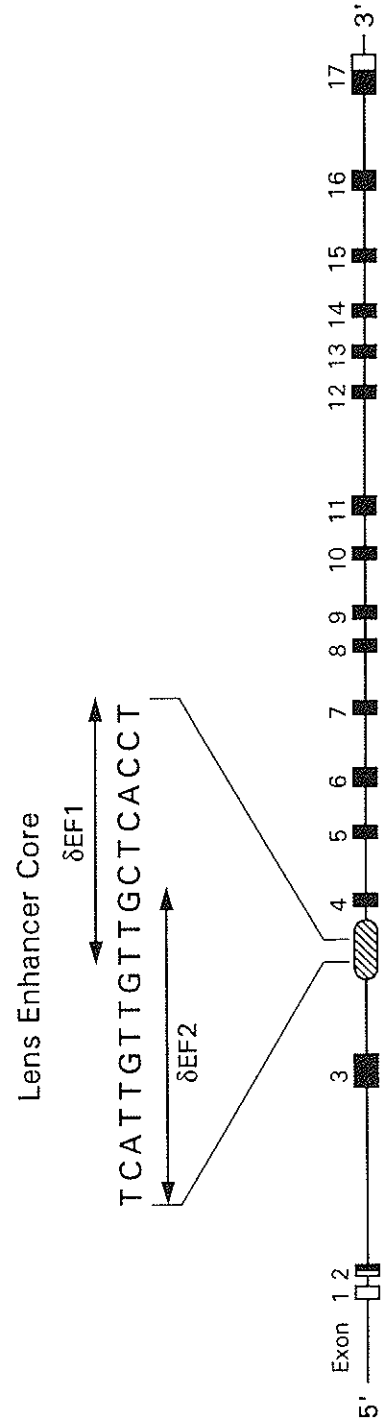
Figure 6.   Schematic of the chicken δ1-crystallin gene with the location of the enhancer element. Coding sequences of exons are shown in black.

Somewhat similar results have been obtained for the rat γD-crystallin gene. In this case the promoter was transfected either into primary mouse lens epithelial cells or into chicken neural retina cells which can be induced to transdifferentiate into lens-like cells (Peek *et al.*, 1990). This promoter was found to contain at least three important elements but with different requirements for these elements in the two cultured cell systems (Peek *et al.*, 1990). The presence of a non-lens silencer was also suggested (Peek *et al.*, 1992). Like the mouse γF-1 element, a 12-nucleotide sequence immediately upstream of the TATA box in the rat γD gene was found to bind a factor found in rat lens but not in retina or brain. Non-lens factors were found to bind to the putative silencing region (Peek *et al.*, 1992).

δ-CRYSTALLINS

As described above, there are two genes for δ-crystallins in chickens, one of which codes for ASL/δ2-crystallin and is not expressed in lens at crystallin-like levels (Parker, Wawrousek and Piatigorsky, 1988; Wistow and Piatigorsky, 1988; Kondoh *et al.*, 1991; Piatigorsky, 1992). Most work has concentrated on the more specialized δ1-crystallin gene which may give rise to as much as 90% of the soluble protein in the embryonic chick lens (Piatigorsky, 1984). Early work identified a lens-preferred promoter, with an upstream negative element (Borras *et al.*, 1985; Borras, Peterson and Piatigorsky, 1988). Subsequently, an enhancer was located in the third intron of the δ1-crystallin gene, which appears to be responsible for the high expression of this gene in lens (Hayashi *et al.*, 1987) (*Figure 6*). Surprisingly, similar sequences are also present in the much less active ASL/δ2- crystallin gene of the chicken, and combinations of promoters and enhancers from both genes were all found to be preferentially expressed in lens (Thomas *et al.*, 1990).

The δ1 enhancer contains at least two overlapping binding sites for factors designated δEF1 and δEF2 (Goto, Okada and Kondoh, 1990; Funahashi *et al.*, 1991; Kamachi and Kondoh, 1993). A protein capable of binding to the δEF1 site has been cloned and turns out to be a general factor which may have a role in suppressing non-lens expression (Kamachi and Kondoh, 1993). Binding of multicomponent complexes through the δEF2 site appears to be more lens-specific and may be responsible for high expression in the lens (Kamachi and Kondoh, 1993).

ζ-CRYSTALLIN

ζ-Crystallin is a novel enzyme with NADPH:quinone oxidoreductase activity, which has undergone gene recruitment in certain mammals, acquiring a second function as a lens structural protein (Huang *et al.*, 1987; Garland *et al.*, 1991; Lee *et al.*, 1993). The recruitment of this enzyme crystallin can be explained by the lens specificity of an alternative promoter which does not require host-specific factors (Lee, Gonzalez and Winstow, 1994). This promoter is located in what would otherwise have been the first intron of the enzyme gene (*Figure 7*). Both the upstream 'housekeeping promoter' and the lens promoter give rise to separate untranslated first exons which splice to a common, coding second exon (Gonzalez *et al.*, 1994). The lens promoter is flanked by direct repeat sequences and may have been inserted into the gene by some kind of transposon mechanism (Lee, Gonzalez and Wistow, 1994). The strong
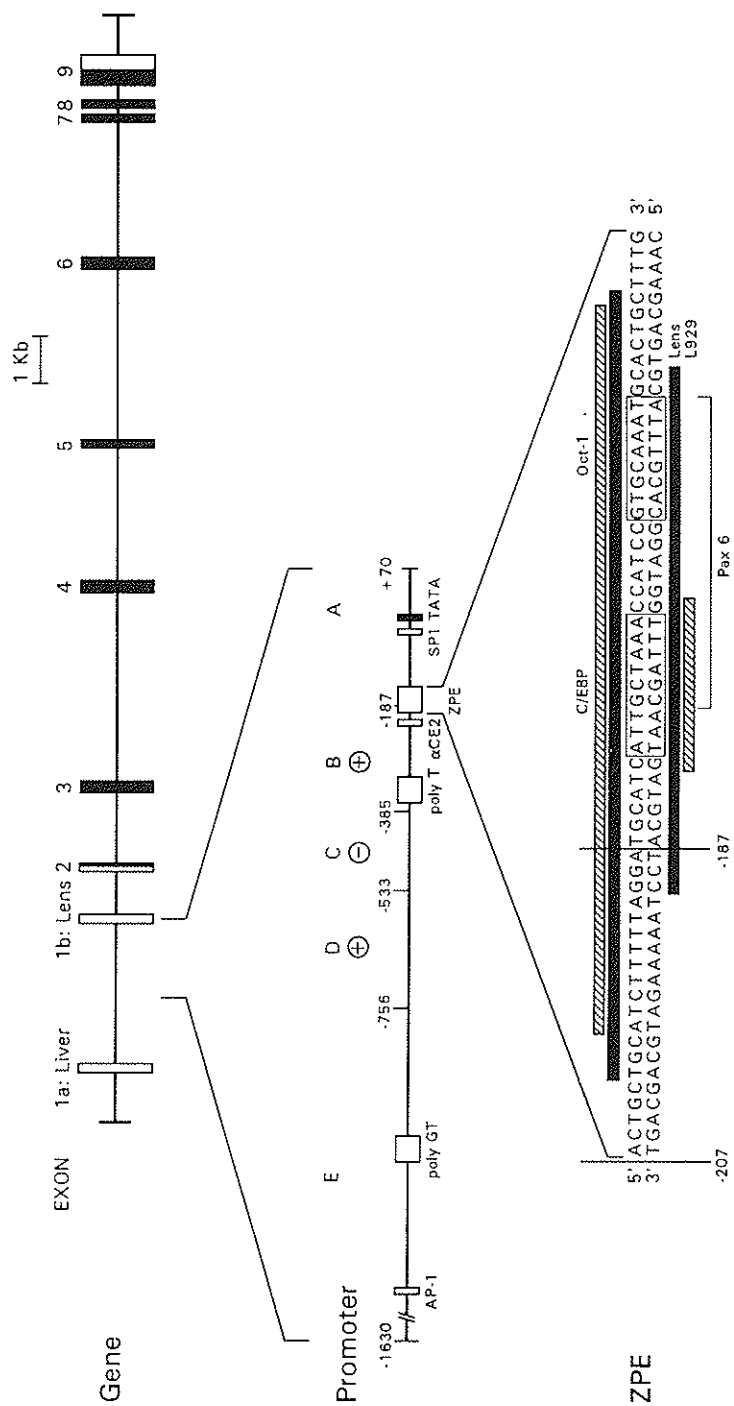
Figure 7. The guinea-pig ζ-crystallin gene has alternative promoters. Both alternative first exons are non-coding (white). Functional elements of the lens-specific promoter are shown. A–E show regions tested by transfection analysis (Lee, Gonzalez and Wistow, 1994).

tissue preference of the lens promoter is apparent in both cultured cell transfections and in transgenic mice (Lee, Gonzalez and Wistow, 1994). While proximal regions of the promoter have some activity in the brain of transgenic mice, this is abolished by the addition of more distal regions. The minimal active promoter is differentially footprinted by extracts from lens and non-lens cells. Deletion within the major region footprinted in lens, ZPE (ζ protected element), abolishes promoter function. More detailed analysis of this region by competitive EMSA has shown that this sequence gives rise to two major gel-shifted complexes, one of which is found in extracts of non-lens cells while a higher-mobility complex predominates in extracts of lens-derived cells (Richardson, Cvekl and Wistow, submitted). The lens complex depends on a sequence designated ZE-1 which is flanked by consensus binding sites for C/EBP and Oct-1 family transcription factors. The ZE-1 site is the core of a consensus Pax-6 binding site (Epstein *et al.*, 1994; Richardson, Cvekl and Wistow, submitted) and antisera to Pax-6 abolish the lens-specific EMSA complex.

α-ENOLASE/τ-CRYSTALLIN

The complete gene for duck α-enolase/τ-crystallin has been cloned (Kim *et al.*, 1991). Although the endogenous gene shows preferential expression in embryonic duck lens, no lens-preferred expression of the gene promoter has been observed in either transfected cultured cells or in transgenic mice (Kim *et al.*, 1991; Kim and Wistow, 1993). However, this gene does illustrate the way in which different regions of the lens express different complements of proteins. α-Enolase/τ-crystallin may be the most abundant protein component of avian lens epithelial cells (Rudner, Katar and Maisel, 1990). Its pattern of expression parallels that of the proto-oncogene c-*myc* in the same system (Harris, Talian and Zelenka, 1992). Furthermore, the duck gene promoter contains an E-box, a consensus c-Myc binding site (Blackwell *et al.*, 1990; Halazonetis and Kandil, 1991), which is conserved in the homologous human gene (Giallongo *et al.*, 1990), the only other α-enolase gene that has been sequenced. Nuclear proteins from chicken lens cells bind to the consensus c-Myc binding site in the duck α-enolase/τ-crystallin promoter in EMSA and DNase I protection assays (Warwar *et al.*, 1992). A monoclonal antibody detects c-Myc in the protein complexes involved in binding to this site and co-transfection of a chicken c-Myc expression vector into primary chick embryo lens epithelial cells increases the expression of a promoter/reporter gene construct fourfold. Transcriptional activation in transfected cells is abolished by a site-specific mutation of the E-box (Warwar *et al.*, 1992). This suggests that c-Myc is among the positive regulators of this promoter.

LACTATE DEHYDROGENASE B/ε-CRYSTALLIN

The gene for duck LDHB/ε-crystallin has been cloned and its expression examined in chicken lens and heart cells (Kraft *et al.*, 1993). Identical start sites for transcription were found in both tissues (Hodin and Wistow 1993; Kraft *et al.*, 1993), although a second start site 28 bp upstream was also used in heart. High expression in both cell types requires sequences from the first intron. The intron does not contain a classic enhancer since it is unable to enhance a heterologous promoter (Kraft *et al.*, 1993). Although the gene is expressed at higher levels in lens than in heart *in vivo*, no

difference in promoter activity was observed in cultured cells. This suggests that the cultured chicken lens cells do not appropriately mimic the characteristics of intact lens required for ε-crystallin over-expression.

Transgenics

Crystallin gene promoters have been used to direct expression of a number of foreign genes to the lens. These have been either the bacterial reporter genes chloramphenicol acetyltransferase (CAT) or β-galactosidase (lacZ), used for straightforward functional analysis, or else genes for transforming proteins or toxins used to disrupt normal lens development.

Most constructions have involved the use of the mouse αA-crystallin gene promoter. A promoter fragment containing 5' flanking and untranslated sequences of exon 1 (−366/+45) has been used to drive the expression of CAT (Overbeek *et al.*, 1985), SV40 large T antigen (Mahon *et al.*, 1987), diphtheria toxin A (DT-A) (Kaur *et al.*, 1989; Harrington *et al.*, 1991), ricin A (Landel *et al.*, 1988), retinoic acid receptor- α-lacZ fusion (Balkan *et al.*, 1992), cellular retinoic acid-binding protein I (Perez-Castro, Tran and Nguyen-Huu, 1993), polyoma virus large T antigen (Griep *et al.*, 1989), human immunodeficiency virus TAT gene (Khillan *et al.*, 1988), dbl oncogene (Eva *et al.*, 1991), human papilloma virus type 16 E6 and E7 oncogenes (Griep *et al.*, 1993), human or murine urokinase-type plasminogen activator (uPA) (Miskin *et al.*, 1990), γ-interferon (Egwuagu *et al.*, 1994) and FGF-3/Int-2 (Chepelinsky *et al.*, 1994). In a similar manner, a fragment of the hamster αA-crystallin promoter covering the same region (−347 to +43) has been fused to CAT and DT-A (Yu, Tsui and Breitman, 1990). These constructs have shown a clear tissue specificity and the expression of the transgenes is co-regulated with the endogenous αA-crystallin during lens development. It was also shown that the chicken αA-crystallin gene fused to CAT requires 162 bp of 5' flanking sequences to direct lens-specific expression in transgenic mice (Klement, Wawrousek and Piatigorsky, 1989).

The other principal system used is that of the mouse γF-crystallin promoter. γF-crystallin sequences −759 to +45, which include the core promoter and two upstream enhancer elements, contain sufficient information to direct gene expression to terminally differentiated lens fibre cells. This promoter fragment has been used to generate CAT, lacZ, DT-A and SV40 T-antigen transgenics (Breitman *et al.*, 1989; Yu, Tsui and Breitman, 1990; Bryce *et al.*, 1993; Goring *et al.*, 1993).

The chicken δ1-crystallin 5' flanking region (−2200/−51) fused to the xanthine–guanine phosphoribosyl transferase gene was found to be expressed primarily in the lens of chimeric mice (Takahashi *et al.*, 1988).

The promoter for an enzyme crystallin, guinea-pig ζ-crystallin, also shows clear lens preference, as evidenced by the expression of the CAT gene in the lens of transgenic mice (Lee, Gonzalez and Wistow, 1994). A line of transgenic mice was also constructed containing the complete duck α-enolase/τ-crystallin (αENO) gene with 5' and 3' sequences (Kim and Wistow, 1993). The duck αENO mRNA was expressed in the same pattern as the endogenous mouse isozyme, showing no apparent tissue specificity.

Is there a master lens-specific factor for crystallin expression?

The lack of an obvious common promoter sequence among crystallin genes has been striking and frustrating. Nevertheless, crystallin genes from species as divergent as chickens and mice will function in each other's lens cells (see Kondoh, Yasuda and Okada, 1983; Kondoh *et al.*, 1987; Lok *et al.*, 1985; Klement, Wawrousek and Piatigorsky, 1989; Piatigorsky, 1992). This suggests that there are lens-specific transcription factors which are essential for expression in any lens and which recognize all crystallin genes. An interesting candidate for one of these putative factors has now emerged. Pax-6 is a transcription factor of the paired-domain homeodomain family. It is essential for normal eye development (see Hill *et al.*, 1991; Glaser, Walton and Maas, 1992; Li *et al.*, 1994). As mentioned above, there are growing indications that Pax-6 may actually be playing an important role as a transcription factor for crystallin genes (Cvekl *et al.*, 1994; Richardson, Cvekl and Wistow, submitted) Since Pax-6 is also expressed in brain (Stoykova and Gruss, 1994), this could explain the overlapping expression in neural tissue which has been noted for some crystallin promoter constructs (see, for example, Lee, Gonzalez and Wistow, 1994). Since Pax-6 is very highly conserved throughout evolution (Hill and Hanson, 1992) and has such an important role in development of head tissues, it could be an ancient common factor of eyes even in vertebrates and invertebrates (Quiring *et al.*, 1994). This protein could be one of the long-sought unifying factors of lens-specific gene expression in all species.

Implications for biotechnology and gene therapy

Recent work on the lens has shed some light on basic questions of molecular evolution, development and tissue-specific gene expression. To this point there has been no prospect of applied spin-off from this basic research. However, this work does provide some interesting lessons for biotechnology and gene therapy, and more speculatively some distant possibilities for applied exploitation of the lens system.

A common objective in biotechnology is the over-expression of functional proteins. From that perspective, the eye lens is a natural biotechnological factory. Extraordinarily high concentrations of active enzymes can accumulate rapidly in developing lenses without problems such as inclusion-body formation, the results of successful evolutionary 'genetic engineering' in the lens. Future experiments which aim at over-expressing other proteins in the lens, mimicking the gene recruitment process, should tell us how robust the system is and how broad a spectrum of proteins can become crystallins.

One interesting feature of the lens which may contribute to its ability to maintain a high concentration of various soluble proteins is that the ubiquitous stress protein crystallins, α-crystallins, and maybe even β- and γ-crystallins, may act to 'chaperone' other components of the lens. If this is true, and it remains to be demonstrated, then it may be possible to achieve similar high levels of expression for a wide variety of foreign proteins targeted to the lens.

As for using the lens itself as a vehicle for recombinant protein production, this is at least distantly possible. The characterization of crystallin gene promoters has provided the tools for expression of foreign genes in transgenic animals. So far there has been little attention paid to achieving high-level expression but this goal is now

being pursued. It is conceivable that important proteins could be produced at crystallin-like levels in transgenic lenses. Such a system might be advantageous for the production of high concentrations of active cytosolic proteins which have problems of processing or solubility in micro-organisms and for which other techniques, such as secretory expression in mammals (Westphal, 1989; Janne *et al.*, 1992), are not suitable. A disadvantage of lens expression is that animals must be killed and dissected to harvest expressed protein. However this problem could be minimized by the use of transgenic birds.

Although mice are the best-studied transgenic hosts, their lenses are small, particularly at birth. Chickens are a more attractive choice. Only two weeks after fertilization chicken embryos *in ovo* have large, obvious eyes and much larger lenses than mice. In the laboratory it is possible to harvest hundreds of lenses in a few hours. Extraction of soluble protein is a simple matter of homogenization (Wistow, 1993a). The technology for production of transgenic birds is less advanced than that for mice but it is progressing rapidly (Love *et al.*, 1994). Established lines of transgenic chickens could be used for continuous production of eggs carrying the desired product.

Even more speculatively, another interesting but completely unexplored strategy for high expression of a foreign protein in chicken lens would be homologous recombination into the δ1-crystallin gene. Since this gene accounts for as much as 90% of the crystallin content of embryonic chicken lenses, introduction of a foreign coding sequence could achieve very high expression. This technology is quite advanced in mammals in which it is now routinely possible to introduce genes into embryonic stem cells and then transfer those cells to embryos to create chimeric animals which can then be bred to homozygosity (for example see Fung-Leung and Mak, 1992; Koller and Smithies, 1992). At present the same kind of techniques for manipulating birds are not so advanced, although homologous recombination in chicken B cells can be achieved (Buerstedde and Takeda, 1991).

Apart from any future possibilities of biotechnological applications, crystallins provide some important, if indirect, lessons for another area of genetic manipulation, namely gene therapy. Gene therapy in the lens itself seems an unlikely prospect in the near future. There are no diseases of the lens itself which are serious enough to warrant the extreme measure of gene therapy in that tissue when cataract surgery is a simple process with a good outcome. However, the lens could possibly serve as a reservoir for expression of a secreted factor, such as an anti-inflammatory protein, which might be beneficial to other parts of a diseased eye. If the therapeutic protein in question could pass through the lens capsule, it might be preferable to introduce an expression vector by injection into the lens than into the much more sensitive retina.

However, the more immediate impact of crystallin research on gene therapy may be in the form of general lessons to be considered when targeting therapies to specific genes. The crystallins illustrate the way in which a single gene may exert multiple phenotypes through protein multifunctionality. On one hand this allows defects in one gene to have multiple effects. On the other hand, it warns that introduction of a transgene to address one problem, such as an enzyme deficiency, could have unforeseen side-effects if the introduced gene has inappropriate temporal or spatial expression. Consider the familiar glycolytic enzyme lactate dehydrogenase (LDH). As described above, it may also be a structural protein in the eye lens in some species and may have additional unexpected functions. For example, LDH isoforms have

been identified as single-stranded DNA binding proteins associated with actively transcribing regions of chromatin (see Williams, Reddigari and Patel, 1985). Similarly, α-enolase, another glycolytic enzyme which may serve a structural role in lens, has been identified as a cell-surface plasminogen receptor (Miles *et al.*, 1991), while the related γ-enolase has been found associated with the centrosome of HeLa cells (Johnstone, Waisman and Rattner, 1992). In yeast, enolase serves as a heat-shock protein (Iida and Yahara, 1985). These observations and others like them suggest that it might be very important to introduce genes in gene therapy with as much of their normal flanking and intronic DNA as possible to ensure native modes of expression.

References

BAGBY, S., HARVEY, T.S., EAGLE, S.G., INOUYE, S. AND IKURA, M. (1994a). NMR-derived three-dimensional solution structure of protein S complexed with calcium. *Structure* **2**, 107–122.

BAGBY, S., HARVEY, T.S., KAY, L.E., EAGLE, S.G., INOUYE, S. AND IKURA, M. (1994b). Unusual helix-containing greek keys in development- specific Ca^{2+}-binding Protein S. ^{1}H, ^{15}N and ^{13}C assignments and secondary structure determined with the use of multidimensional double and triple resonance heteronuclear NMR spectroscopy. *Biochemistry* **33**, 2409–2421.

BALKAN, W., KLINTWORTH, G.K., BOCK, C.B., AND LINNEY, E. (1992). Transgenic mice expressing a constitutively active retinoic acid receptor in the lens exhibit ocular defects. *Developmental Biology* **151**, 622–625.

BARBOSA, P., CIALKOWSKI, M. AND O'BRIEN, W.E. (1991). Analysis of naturally occurring and site-directed mutations in the argininosuccinate lyase gene. *Journal of Biological Chemistry* **266**, 5286–5290.

BARBOSA, P., WISTOW, G.J., CIALKOWSKI, M., PIATIGORSKY, J. AND O'BRIEN, W.E. (1991). Expression of duck lens δ-crystallin cDNAs in yeast and bacterial hosts: δ2-crystallin is an active argininosuccinate lyase. *Journal of Biological Chemistry* **266**, 22319–22322.

BARNSTABLE, C.J. (1993). Glutamate and GABA in retinal circuitry. *Current Opinion in Neurobiology* **3**, 520–525.

BAX, B., LAPATTO, R., NALINI, V., DRIESSEN, H., LINDLEY, P.F., MAHADEVAN, D., BLUNDELL, T.L. AND SLINGSBY, C. (1990). X-ray analysis of βB2-crystallin and evolution of oligomeric lens proteins. *Nature* **347**, 776–780.

BERBERS, G.A., HOEKMAN, W.A., BLOEMENDAL, H., DE JONG, W.W., KLEINSCHMIDT, T. AND BRAUNITZER, G. (1984). Homology between the primary structures of the major bovine β-crystallin chains. *European Journal of Biochemistry* **139**, 467–479.

BLACKWELL, T.K., KRETZNER, L., BLACKWOOD, E.M., EISENMAN, R.N. AND WEINTRAUB, H. (1990). Sequence-specific DNA binding by the c-Myc protein. *Science* **250**, 1149–1151.

BLOEMENDAL, H., (ED.) (1981). *Molecular and Cellular Biology of the Eye Lens.* Wiley, New York.

BLUNDELL, T., LINDLEY, P., MILLER, L., MOSS, D., SLINGSBY, C., TICKLE, I., TURNELL, B. AND WISTOW, G. (1981). The molecular structure and stability of the eye lens: x-ray analysis of γ-crystallin II. *Nature* **289**, 771–777.

BOHREN, K.M., BULLOCK, B., WERMUTH, B. AND GABBAY, K.H. (1989). The aldo-keto reductase superfamily. cDNAs and deduced amino acid sequences of human aldehyde and aldose reductases. *Journal of Biological Chemistry* **264**, 9547–9551.

BONDY, C.A. AND LIGHTMAN, S.L. (1989). Developmental and physiological pattern of aldose reductase mRNA expression in lens and retina. *Molecular Endocrinology* **3**, 1417–1425.

BORRAS, T., PERSSON, B. AND JORNVALL, H. (1989). Eye lens ζ-crystallin: relationships to the family of long-chain alcohol/polyol dehydrogenases. *Biochemistry* **28**, 6133–6139.

BORRAS, T., PETERSON, C.A. AND PIATIGORSKY, J. (1988). Evidence for positive and negative regulation in the promoter of the chicken δ1-crystallin gene. *Developmental Biology* **127**, 209–219.

BORRAS, T., NICKERSON, J.M., CHEPELINSKY, A.B. AND PIATIGORSKY, J. (1985). Structural and functional evidence for differential promoter activites of the two linked δ-crystallin genes in the chicken. *EMBO Journal* **4**, 445–452.

BRAKENHOFF, R.H., ARTS, H.J., REEK, F.H., LUBSEN, N.H. AND SCHOENMAKERS, J.G. (1990). Human γ-crystallin genes. A gene family on its way to extinction. *Journal of Molecular Biology* **216**, 519–532.

BREITMAN, M.L., LOK, S., WISTOW, G., PIATIGORSKY, J., TRETON, J.A., GOLD, R.J. AND TSUI, L.C. (1984). γ-Crystallin family of the mouse lens: structural and evolution relationships. *Proceedings of the National Academy of Sciences, USA* **81**, 7762–7766.

BREITMAN, M.L., BRYCE, D.M., GIDDENS, E., CLAPOFF, S., GORING, D., TSUI, L.-C., KLINTWORTH, G.K. AND BERNSTEIN, A. (1989). Analysis of lens cell fate and eye morphogenesis in transgenic mice ablated for cells of the lens lineage. *Development* **106**, 457–463.

BRYCE, D.M., LIU, Q., KHOO, W., TSUI, L.C. AND BREITMAN, M.L. (1993). Progressive and regressive fate of lens tumors correlates with subtle differences in transgene expression in γF-crystallin-SV40 T antigen transgenic mice. *Oncogene* **8**, 1611–1620.

BUERSTEDDE, J.M. AND TAKEDA, S. (1991). Increased ratio of targeted to random integration after transfection of chicken B cell lines. *Cell* **67**, 179–188.

CARPER, D., NISHIMURA, C., SHINOHARA, T., DIETZSCHOLD, B., WISTOW, G., CRAFT, C., KADOR, P. AND KINOSHITA, J.H. (1987). Aldose reductase and ρ-crystallin belong to the same protein superfamily as aldehyde reductase. *FEBS Letters* **220**, 209–213.

CARPER, D.A., WISTOW, G., NISHIMURA, C., GRAHAM, C., WATANABE, K., FUJII, Y., HAYASHI, H. AND HAYAISHI, O. (1989). A superfamily of NADPH-dependent reductases in eukaryotes and prokaryotes. *Experimental Eye Research* **49**, 377–388.

CHEPELINSKY, A.B., SOMMER, B. AND PIATIGORSKY, J. (1987). Interaction between two different regulatory elements activates the murine αA-crystallin gene promoter in explanted lens epithelia. *Molecular and Cellular Biology* **7**, 1807–1814.

CHEPELINSKY, A.B., ROBINSON, M.L., OVERBEEK, P.A., PARKER, D.M., CHAN, C.C., JAMIESON, S. AND DICKSON, C. (1994). FGF-3 ectopic expression induces differentiation of central lens epithelia and appearance of secretory epithelia in the eyes of transgenic mice. *Investigative Ophthalmology and Visual Science* **35**, 1998.

CHIESA, R., GAWINOWICZ-KOLKS, M.A. AND SPECTOR, A. (1987). The phosphorylation of the primary gene products of α-crystallin. *Journal of Biological Chemistry* **262**, 1438–1441.

CHIESA, R., GAWINOWICZ-KOLKS, M.A., KLEIMAN, N.J. AND SPECTOR, A. (1987). The phosphorylation sites of the B2 chain of bovine α-crystallin. *Biochemical and Biophysical Research Communications* **144**, 1340–1347.

CHIESI, M., LONGONI, S. AND LIMBRUNO, U. (1990). Cardiac α-crystallin. III. Involvement during heart ischemia. *Molecular and Cellular Biochemistry* **97**, 129–136.

CHIOU, S.H. (1988). A novel crystallin from octopus lens. *FEBS Letters* **241**, 261–264.

CLARKE, F.M., MORTON, D.J., STEPHAN, P. AND WIEDEMANN, J. (1985). The functional duality of glycolytic enzymes: potential integrators of cytoplasmic structure and function. In *Cell Motility: Mechanism and Regulation (10th Yamada Conference)* (H. Ishikawa, S. Hatano and H. Sato, Eds), pp. 235–250. Tokyo University Press, Tokyo.

COOPER, R.L., CONSTABLE, I.J. AND DAVIDSON, L. (1984). Aqueous humor catecholamines. *Current Eye Research* **3**, 809–813.

COX, R.L., GLICK, D.L. AND STRUMWASSER, F. (1991). Isolation and protein sequence identification of *Aplysia californica* lens crystallins. *Biological Bulletin (Woods Hole)* **181**, 333–335.

CVEKL, A., SAX, C.M., BRESNICK, E.H. AND PIATIGORSKY, J. (1994). Complex array of positive and negative elements regulate the chicken αA-crystallin gene: involvement of Pax-6, USF, CREB and/or CREM and AP1 proteins. *Molecular and Cellular Biology* **14**, 7363–7376.

DASGUPTA, S., HOHMAN, T.C. AND CARPER, D. (1992). Hypertonic stress induces αB-crystallin expression. *Experimental Eye Research* **54**, 461–470.

DE JONG, W.W. (1981). Evolution of lens and crystallins. In *Molecular and Cellular Biology of the Eye Lens* (H. Bloemendal, Ed.), pp. 221–278. Wiley-Interscience, New York.

DE JONG, W.W., LEUNISSEN, J.A.M. AND VOORTER, C.E.M. (1993). Evolution of the α-crystallin/small heat-shock protein family. *Molecular Biology and Evolution* **10**, 103–126.

DELAYE, M. AND TARDIEU, A. (1983). Short-range order of crystallin proteins accounts for eye lens transparency. *Nature* **302**, 415–417.

DODEMONT, H., GROENEN, M., JANSEN, L., SCHOENMAKERS, J. AND BLOEMENDAL, H. (1985). Comparison of the crystallin mRNA populations from rat, calf and duck lens. Evidence for a longer αA2-mRNA and two distinct αB2 in the birds. *Biochimica Biophysica Acta* **824**, 284–294.

DONOVAN, D.M., SAX, C.M., KLEMENT, J.F., LI, X., CHEPELINSKY, A.B. AND PIATIGORSKY, J. (1992). Conservation of mouse αA-crystallin promoter activity in chicken lens epithelial cells. *Journal of Molecular Evolution* **35**, 337–345.

DRIESSEN, H.P., HERBRINK, P., BLOEMENDAL, H. AND DE JONG, W.W. (1980). The β-crystallin Bp chain is internally duplicated and homologous γ-crystallin. *Experimental Eye Research* **31**, 243–246.

DUBIN, R.A., WAWROUSEK, E.F. AND PIATIGORSKY, J. (1989). Expression of the murine αB-crystallin gene is not restricted to the lens. *Molecular and Cellular Biology* **9**, 1083–1091.

DUBIN, R.A., ALLY, A.H., CHUNG, S. AND PIATIGORSKY, J. (1990). Human αB-crystallin gene and preferential promoter function in lens. *Genomics* **7**, 594–601.

DUBIN, R.A., GOPAL-SRIVASTAVA, R., WAWROUSEK, E.F. AND PIATIGORSKY, J. (1991). Expression of the murine αB-crystallin gene in lens and skeletal muscle: identification of a muscle-preferred enhancer. *Molecular and Cellular Biology* **11**, 4340–4349.

DUGUID, J.R., ROHWER, R.G. AND SEED, B. (1988). Isolation of cDNAs of scrapie-modulated RNAs by subtractive hybridization of a cDNA library. *Proceedings of the National Academy of Sciences, USA* **85**, 5738–5742.

EGWUAGU, C.E., SZTEIN, J., CHAN, C.C., REID, W., MAHDI, R., NUSSENBLATT, R.B. AND CHEPELINSKY, A.B. (1994). Ectopic expression of γ-interferon in the eyes of transgenic mice induces ocular pathology and MHC class II gene expression. *Investigative Ophthalmology and Visual Science* **35**, 332–341.

EPSTEIN, J., CAI, J., GLASER, T., JEPEAL, L. AND MAAS, R. (1994). Identification of a Pax paired domain recognition sequence and evidence for DNA-dependent conformational changes. *Journal of Biological Chemistry* **269**, 8355–8361.

EVA, A., GRAZIANI, G., ZANNINI, M., MERIN, L.M., KHILLAN, J.S. AND OVERBEEK, P.A. (1991). Dominant dysplasia of the lens in transgenic mice expressing the dbl oncogene. *New Biologist* **3**, 158–168.

FLAHERTY, K.M., MCKAY, D.B., KABSCH, W. AND HOLMES, K.C. (1991). Similarity of the three-dimensional structures of actin and the ATPase fragment of a 70-kDa heat shock cognate protein. *Proceedings of the National Academy of Sciences, USA* **88**, 5041–5045.

FUNAHASHI, J.I., KAMACHI, Y., GOTO, K. AND KONDOH, H. (1991). Identification of nuclear factor δEF1 and its binding site essential for lens-specific activity of the δ1-crystallin enhancer. *Nucleic Acids Research* **19**, 3543–3547.

FUNG-LEUNG, W.P. AND MAK, T.W. (1992). Embryonic stem cells and homologous recombination. *Current Opinion in Immunology* **4**, 189–194.

GAO, C.Y., BASSNETT, S. AND ZELENKA, P.S. Cyclin B, p34cdc2 and H1-kinase activity in terminally differentiating lens fiber cells. Submitted.

GARLAND, D., RAO, P.V., DEL CORSO, A., MURA, U. AND ZIGLER, J.S., JR (1991). ζ-Crystallin is a major protein in the lens of *Camelus dromedarius*. *Archives of Biochemistry and Biophysics* **285**, 134–136.

GIALLONGO, A., OLIVA, D., CALI, L., BARBA, G., BARBIERI, G. AND FEO, S. (1990). Structure of the human gene for α-enolase. *European Journal of Biochemistry* **190**, 567–573.

GLASER, T., WALTON, D.S. AND MAAS, R.L. (1992). Genomic structure, evolutionary conservation and aniridia mutations in the human PAX6 gene. *Nature Genetics* **2**, 232–239.

GONZALEZ, P., HERNANDEZ-CALZADILLA, C., RAO, P.V., RODRIGUEZ, I.R., ZIGLER, J.S., JR AND BORRAS, T. (1994). Comparative analysis of the ζ-crystallin/quinone reductase gene in guinea pig and mouse. *Molecular Biology and Evolution* **11**, 305–315.

GOPAL-SRIVASTAVA, R. AND PIATIGORSKY, J. (1993). The murine αB-crystallin/small heat

shock protein enhancer: identification of αBE-1, αBE-2, αBE- 3, and MRF control elements. *Molecular and Cellular Biology* **13**, 7144–7152.

GOPAL-SRIVASTAVA, R. AND PIATIGORSKY, J. (1994). Identification of a lens-specific regulatory region (LSR) of the murine αB-crystallin gene. *Nucleic Acids Research* **22**, 1281–1286.

GORING, D.R., BREITMAN, M.L. AND TSUI, L.C. (1992). Temporal regulation of six crystallin transcripts during mouse lens development. *Experimental Eye Research* **54**, 785–795.

GORING, D.R., BRYCE, D.M., TSUI, L.C., BREITMAN, M.L. AND LIU, Q. (1993). Developmental regulation and cell type-specific expression of the murine γF-crystallin gene is mediated through a lens-specific element containing the γF-1 binding site. *Developmental Dynamics* **196**, 143–152.

GOTO, K., OKADA, T.S. AND KONDOH, H. (1990). Functional cooperation of lens-specific and nonspecific elements in the δ1-crystallin enhancer. *Molecular and Cellular Biology* **10**, 958–964.

GRAW, J., LIEBSTEIN, A., PIETROWSKI, D., SCHMITT-JOHN, T. AND WERNER, T. (1993). Genomic sequences of murine γB- and γC-crystallin-encoding genes: promoter analysis and complete evolutionary pattern of mouse, rat and human γ-crystallins. *Gene* **136**, 145–156.

GRIEP, A.E., KUWABARA, T., LEE, E.J. AND WESTPHAL, H. (1989). Perturbed development of the mouse lens by polyomavirus large T antigen does not lead to tumor formation. *Genes and Development* **3**, 1075–1085.

GRIEP, A.E., HERBER, R., JEON, S., LOHSE, J.K., DUBIELZIG, R.R. AND LAMBERT, P.F. (1993). Tumorigenicity by human papillomavirus type 16 E6 and E7 in transgenic mice correlates with alterations in epithelial cell growth and differentiation. *Journal of Virology* **67**, 1373–1384.

HALAZONETIS, T.D. AND KANDIL, A.N. (1991). Determination of the c-MYC DNA-binding site. *Proceedings of the National Academy of Sciences, USA* **88**, 6162–6166.

HARDING, J.J. AND CRABBE, M.J.C. (1984). The lens: development, proteins, metabolism and cataract. In *The Eye*, Vol. 1B (H. Davson, Ed.), pp. 207–492. Academic Press, New York.

HARRINGTON, L., KLINTWORTH, G.K., SECOR, T.E. AND BREITMAN, M.L. (1991). Developmental analysis of ocular morphogenesis in αA-crystallin/diphtheria toxin transgenic mice undergoing ablation of the lens. *Developmental Biology* **148**, 508–516.

HARRIS, L.L., TALIAN, J.C. AND ZELENKA, P.S. (1992). Contrasting patterns of c-*myc* and N-*myc* expression in proliferating, quiescent and differentiating cells of the embryonic chicken lens. *Development* **115**, 813–820.

HARTL, F.U., HLODAN, R. AND LANGER, T. (1994). Molecular chaperones in protein folding: the art of avoiding sticky situations. *Trends in Biochemical Sciences* **19**, 20–25.

HAYASHI, S., GOTO, K., OKADA, T.S. AND KONDOH, H. (1987). Lens-specific enhancer in the third intron regulates expression of the chicken δ1-crystallin gene. *Genes and Development* **1**, 818–828.

HEAD, M.W., PETER, A. AND CLAYTON, R.M. (1991). Evidence for the extralenticular expression of members of the β-crystallin gene family in the chick and a comparison with δ-crystallin during differentiation and transdifferentiation. *Differentiation* **48**, 147–156.

HECKER, M., SESSA, W.C., HARRIS, H.J., ANGGARD, E.E. AND VANE, J.R. (1990). The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: cultured endothelial cells recycle L-citrulline to L-arginine. *Proceedings of the National Academy of Sciences, USA* **87**, 8612–8616.

HENDRIKS, W., LEUNISSEN, J., NEVO, E., BLOEMENDAL, H. AND DE JONG, W.W. (1987). The lens protein αA-crystallin of the blind mole rat, *Spalax ehrenbergi*: Evolutionary change and functional constraints. *Proceedings of the National Academy of Sciences, USA* **84**, 5320–5324.

HENDRIKS, W., MULDERS, J.W.M., BIBBY, M.A., SLINGSBY, C., BLOEMENDAL, H. AND DE JONG, W.W. (1988). Duck lens ε-crystallin and lactate dehydrogenase B4 are identical: single-copy gene product with two distinct functions. *Proceedings of the National Academy of Sciences, USA* **85**, 7114–7118.

HILL, R.E. AND HANSON, I.M. (1992). Molecular genetics of the Pax gene family. *Current Opinion in Cell Biology* **4**, 967–972.

HILL, R.E., FAVOR, J., HOGAN, B.L., TON, C.C., SAUNDERS, G.E., HANSON, I.M., PROSSER, J.,

JORDAN, T., HASTIE, N.D. AND VAN HEYNINGEN, V. (1991). Mouse small eye results from mutations in a paired-like homeobox-containing gene. *Nature* **354**, 522–525.

HODIN, J. AND WISTOW, G. (1993). 5'-RACE PCR of mRNA for three taxon-specific crystallins: for each gene one promoter controls both lens and non-lens expression. *Biochemical and Biophysical Research Communications* **190**, 391–396.

HOGG, D., TSUI, L.-C., GORIN, M. AND BREITMAN, M.L. (1986). Characterization of the human β-crystallin gene Hu βA3/A1 reveals ancestral relationships among the βγ-crystallin superfamily. *Journal of Biological Chemistry* **261**, 12420–12427.

HORWITZ, J. (1992). A-Crystallin can function as a molecular chaperone. *Proceedings of the National Academy of Sciences, USA* **89**, 10449–10453.

HUANG, Q.L., RUSSELL, P., STONE, S. AND ZIGLER, J.S. (1987). ζ-Crystallin, a novel lens protein from the guinea pig. *Current Eye Research* **6**, 725–732.

IIDA, H. AND YAHARA, I. (1985). Yeast heat-shock protein of Mr 48,000 is an isoprotein of enolase. *Nature* **315**, 688–690.

INGOLIA, T.D. AND CRAIG, E.A. (1982). Four small *Drosophila* heat shock proteins are related to each other and to mammalian α-crystallin. *Proceedings of the National Academy of Sciences, USA* **79**, 2360–2364.

IWAKI, A., IWAKI, T., GOLDMAN, J.E. AND LIEM, R.K. (1990). Multiple mRNAs of rat brain α-crystallin B chain result from alternative transcriptional initiation. *Journal of Biological Chemistry* **265**, 22197–22203.

IWAKI, T., KUME-IWAKI, A. AND GOLDMAN, J.E. (1990). Cellular distribution of αB-crystallin in non-lenticular tissues. *Journal of Histochemistry and Cytochemistry* **38**, 31–39.

IWAKI, T., KUME-IWAKI, A., LIEM, R.K. AND GOLDMAN, J.E. (1989). αB-crystallin is expressed in non-lenticular tissues and accumulates in Alexander's disease brain. *Cell* **57**, 71–78.

JAHN, D., VERKAMP, E. AND SÖLL, D. (1992). Glutamyl-transfer RNA: a precursor of heme and chlorophyll biosynthesis. *Trends in Biochemical Sciences* **17**, 215–218.

JANNE, J., HYTTINEN, J.M., PEURA, T., TOLVANEN, M., ALHONEN, L. AND HALMEKYTO, M. (1992). Transgenic animals as bioproducers of therapeutic proteins. *Annals of Medicine (Helsinki)* **24**, 273–280.

JAWORSKI, C.J., CHEPELINSKY, A.B. AND PIATIGORSKY, J. (1991). The αA-crystallin gene: conserved features of the 5'-flanking regions in human, mouse, and chicken. *Journal of Molecular Evolution* **33**, 495–505.

JOHNSTONE, S.A., WAISMAN, D.M. AND RATTNER, J.B. (1992). Enolase is present at the centrosome of HeLa cells. *Experimental Cell Research* **202**, 458–463.

KAMACHI, Y. AND KONDOH, H. (1993). Overlapping positive and negative regulatory elements determine lens-specific activity of the δ1-crystallin enhancer. *Molecular and Cellular Biology* **13**, 5206–5215.

KANTOROW, M. AND PIATIGORSKY, J. (1994). α-Crystallin/small heat shock protein has autokinase activity. *Proceedings of the National Academy of Sciences, USA* **91**, 3112–3116.

KANTOROW, M., CVEKL, A., SAX, C. AND PIATIGORSKY, J. (1993). Protein–DNA interactions of the mouse αA-crystallin control regions. *Journal of Molecular Biology* **230**, 425–435.

KATO, K., SHINOHARA, H., KUROBE, N., GOTO, S., INAGUMA, Y. AND OHSHIMA, K. (1991a). Immunoreactive αA-crystallin in rat non-lenticular tissues detected with a sensitive immunoassay method. *Biochemica Biophysica Acta* **1080**, 173–180.

KATO, K., SHINOHARA, H., KUROBE, N., GOTO, S., INAGUMA, Y. AND OHSHIMA, K. (1991b). Tissue distribution and developmental profiles of immunoreactive αB-crystallin in the rat determined with a sensitive immunoassay system. *Biochimica Biophysica Acta* **1074**, 201–208.

KAUR, S., KEY, B., STOCK, J., MCNEISH, J.D., AKESON, R. AND POTTER, S.S. (1989). Targeted ablation of α-crystallin-synthesizing cells produces lens-deficient eyes in transgenic mice. *Development* **105**, 613–619.

KHILLAN, J.S., DEEN, K.C., YU, S.H., SWEET, R.W., ROSENBERG, M. AND WESTPHAL, H. (1988). Gene transactivation mediated by the TAT gene of human immunodeficiency virus in transgenic mice. *Nucleic Acids Research* **16**, 1423–1430.

KIM, R.Y. AND WISTOW, G.J. (1993). Expression of the duck α-enolase/τ-crystallin gene in transgenic mice. *FASEB Journal* **7**, 464–469.

KIM, R.Y., GASSER, R. ABD WISTOW, G.J. (1992). µ-Crystallin is a mammalian homologue of *Agrobacterium* ornithine cyclodeaminase and is expressed in human retina. *Proceedings of the National Academy of Sciences, USA* **89**, 9292–9296.

KIM, R.Y., LIETMAN, T., PIATIGORSKY, J. AND WISTOW, G.J. (1991). Structure and expression of the duck α-enolase/τ-crystallin encoding gene. *Gene* **103**, 193–200.

KLEMENT, J.F., CVEKL, A. AND PIATIGORSKY, J. (1993). Functional elements DE2A, DE2B and DE1A and the TATA box are required for activity of the chicken αA-crystallin gene in transfected lens epithelial cells. *Journal of Biological Chemistry* **268**, 6777–6784.

KLEMENT, J.R., WAWROUSEK, E.F. AND PIATIGORSKY, J. (1989). Tissue-specific expression of the chicken αA-crystallin gene in cultured lens epithelia and transgenic mice. *Journal of Biological Chemistry* **264**, 19837–19844.

KLEMENZ, R., FROHLI, E., AOYAMA, A., HOFFMANN, S., SIMPSON, R.J., MORITZ, R.L. AND SCHAFER, R. (1991a). αB-Crystallin accumulation is a specific response to Ha-*ras* and v-*mos* oncogene expression in mouse NIH 3T3 fibroblasts. *Molecular and Cellular Biology* **11**, 803–812.

KLEMENZ, R., FROHLI, E., STEIGER, R.H., SCHAFTER, R. AND AOYAMA, A. (1991b). αB-Crystallin is a small heat shock protein. *Proceedings of the National Academy of Sciences, USA* **88**, 3652–3656.

KNULL, H.R. AND WALSH, J.L. (1992). Association of glycolytic enzymes with the cytoskeleton. *Current Topics in Cellular Regulation* **33**, 15–30.

KOLLER, B.H. AND SMITHIES, O. (1992). Altering genes in animals by gene targeting. *Annual Review of Immunology* **10**, 705–730.

KOMORI, N., USUKURA, J. AND MATSUMOTO, H. (1992). Drosocrystallin, a major 52 kDa glycoprotein of the *Drosophila melanogaster* corneal lens. Purification, biochemical characterization and subcellular localization. *Journal of Cell Science* **102**, 191–201.

KONDOH, H., YASUDA, K. AND OKADA, T.S. (1983). Tissue-specific expression of a cloned chick δ-crystallin gene in mouse cells. *Nature* **301**, 440–442.

KONDOH, H., KATOH, K., TAKAHASHI, Y., FUJISAWA, H., YOKOYAMA, M., KIMURA, S., KATSUKI, M., SAITO, M., NOMURA, T., HIRAMOTO, Y. AND OKADA, T.S. (1987). Specific expression of the chicken δ-crystallin gene in the lens and the pyramidal neurons of the piriform cortex in transgenic mice. *Developmental Biology* **120**, 177–185.

KONDOH, H., ARAKI, I., YASUDA, K., MATSUBASA, T. AND MORI, M. (1991). Expression of the chicken 'δ2-crystallin' gene in mouse cells: evidence for encoding of argininosuccinate lyase. *Gene* **99**, 267–271.

KRAFT, H.J., HENDRIKS, W., DE JONG, W.W., LUBSEN, N.H. AND SCHOENMAKERS, J.G.G. (1993). Duck lactate dehydrogenase B/ε-crystallin gene: lens recruitment of a GC-promoter. *Journal of Molecular Biology* **229**, 849–859.

LANDEL, C.P., ZHAO, J., BOK, D. AND EVANS, G.A. (1988). Lens-specific expression of recombinant ricin induces developmental defects in the eyes of transgenic mice. *Genes and Development* **2**, 1168–1178.

LEE, D.C., GONZALEZ, P. AND WISTOW, G. (1994). ζ-Crystallin: A lens-specific promoter and the gene recruitment of an enzyme as a crystallin. *Journal of Molecular Biology* **236**, 669–678.

LEE, D.C., KIM, R.Y. AND WISTOW, G.J. (1993). An avian αB-crystallin. Non lens expression and sequence similarities with both small (hsp27) and large (HSP70) heat shock proteins. *Journal of Molecular Biology* **232**, 1221–1226.

LEE, D.C., GONZALEZ, P., RAO, P.V., ZIGLER, J.S., JR AND WISTOW, G.J. (1993). Carbonyl-metabolizing enzymes and their relatives recruited as structural proteins in the eye lens. In *Enzymology and Molecular Biology of Carbonyl Metabolism*, Vol. 4 (H. Weiner, Ed.), pp. 159–168. Plenum Press, New York.

LI, H.S., YANG, J.M., JACOBSON, R.D., PASKO, D. AND SUNDIN, O. (1994). Pax-6 is first expressed in a region of ectoderm anterior to the early neural plate: implications for stepwise determination of the lens. *Developmental Biology* **162**, 181–194.

LI, L.K. AND SPECTOR, A. (1967). The optical rotatory dispersion and circular dichroism of calf lens α-crystallin. *Journal of Biological Chemistry* **242**, 3234–3236.

LI, L.K. AND SPECTOR, A. (1974). Circular dichroism and optical rotatory dispersion of the

aggregates of purified polypeptides of α-crystallin. *Experimental Eye Research* **19**, 49–57.

LI, X., WISTOW, G.J. AND PIATIGORSKY, J. Linkage and expression of the argininosuccinate lyase/δ-crystallin genes of the duck: insertion of a CR1 element in the intragenic spacer. *Biochim. Biophys. Acta*, in press.

LIU, Q.R., TINI, M., TSUI, L.C. AND BREITMAN, M.L. (1991). Interaction of a lens cell transcription factor with the proximal domain of the mouse γF-crystallin promoter. *Molecular and Cellular Biology* **11**, 1531–1537.

LOK, S., TSUI, L.-C., SHINOHARA, T., PIATIGORSKY, J., GOLD, R. AND BREITMAN, M. (1984). Analysis of the mouse r-crystallin gene family: Assignment of multiple cDNAs to discrete genomic sequences and characterization of representative gene. *Nucleic Acids Research* **12**, 4517–4529.

LOK, S., BREITMAN, M.L., CHEPELINSKY, A.B., PIATIGORSKY, J., GOLD, R.J.M. AND TSUI, L.-C. (1985). Lens-specific promoter activity of a mouse γ-crystallin gene. *Molecular and Cellular Biology* **5**, 2221–2230.

LOK, S., STEVENS, W., BREITMAN, M.L. AND TSUI, L.C. (1989). Multiple regulatory elements of the murine r2-crystallin promoter. *Nucleic Acids Research* **17**, 3563–3582.

LOVE, J., GRIBBIN, C., MATHER, C. AND SANG, H. (1994). Transgenic birds by DNA microinjection. *Bio/Technology* **12**, 60–63.

LOWE, J., LANDON, M., PIKE, I., SPENDLOVE, I., MCDERMOTT, H. AND MAYER, R.J. (1990). Dementia with β-amyloid deposition: involvement of αB-crystallin supports two main diseases. *Lancet* **336**, 515–516.

MCDERMOTT, J.B., PETERSON, C.A. AND PIATIGORSKY, J. (1992). Structure and lens expression of the gene encoding chicken βA3/A1-crystallin. *Gene* **117**, 193–200.

MILES, L.A., DAHLBERG, C.M., PLESCIA, J., FELEZ, J. FELEZ, J., KATO, K. AND PLOW, E.F. (1991). Role of cell-surface lysines in plasminogen binding to cells: identification of α-enolase as a candidate plasminogen receptor. *Biochemistry* **30**, 1682–1691.

MIRON, T., VANCOMPERNOLLE, K., VANDEKERCKHOVE, J., WILCHEK, M. AND GEIGER, B. (1991). A 25-kD inhibitor of actin polymerization is a low molecular mass heat shock protein. *Journal of Cell Biology* **114**, 255–261.

MISKIN, R., AXELROD, J.H., GRIEP, A.E., LEE, E., BELIN, D., VASSALLI, J.D. AND WESTPHAL, H. (1990). Human and murine urokinase cDNAs linked to the murine αA-crystallin promoter exhibit lens and non-lens expression in transgenic mice. *Eur. J. Biochem.* **190**, 31–38.

MONTGOMERY, M.K. AND MCFALL-NGAI, M.J. (1992). The muscle-derived lens of a squid bioluminescent organ is biochemically convergent with the ocular lens. Evidence for recruitment of aldehyde dehydrogenase as a predominant structural protein. *Journal of Biological Chemistry* **267**, 20999–21003.

MOORMANN, R.J., DEN DUNNEN, J.T., HEUYERJANS, J., JONGBLOED, R.J., VAN LEEN, R.W., LUBSEN, N.H. AND SCHOENMAKERS, J.G. (1985). Characterization of the rat γ-crystallin gene family and its expression in the eye lens. *Journal of Molecular Biology* **182**, 419–430.

MULDERS, J.W., HENDRIKS, W., BLANKESTEIJN, W.M., BLOEMENDAL, H. AND DE JONG, W.W. (1988). λ-Crystallin, a major rabbit lens protein, is related to hydroxyacyl-coenzyme A dehydrogenases. *Journal of Biological Chemistry* **263**, 15462–15466.

NAKAMURA, T., DONOVAN, D.M., HAMADA, K., SAX, C.M., NORMAN, B., FLANAGAN, J.R., OZATO, K., WESTPHAL, H. AND PIATIGORSKY, J. (1990). Regulation of the mouse αA-crystallin gene: isolation of a cDNA encoding a protein that binds to a *cis* sequence motif shared with the major histocompatibility complex class I gene and other genes. *Molecular and Cellular Biology* **10**, 3700–3708.

NICHOLL, I.D. AND QUINLAN, R.A. (1994). Chaperone activity of α-crystallins modulates intermediate filament assembly. *EMBO Journal* **13**, 945–953.

NICKERSON, J.M., WAWROUSEK, E.F., HAWKINS, J.W., WAKIL, A.S., WISTOW, G.J., THOMAS, G., NORMAN, B.L. AND PIATIGORSKY, J. (1985). The complete sequence of the chicken δ1-crystallin gene and its flanking region. *Journal of Biological Chemistry* **260**, 9100–9105.

NICKERSON, J.M., WAWROUSEK, E.F., BORRAS, T., HAWKINS, J.W., NORMAN, B.L., FILPULA, D.R., NAGLE, J.W., ALLY, A.H. AND PIATIGORSKY, J. (1986). Sequence of the chicken δ2-crystallin gene and its intergenic spacer. Extreme homology with the δ1 crystallin gene. *Journal of Biological Chemistry* **261**, 552–557.

NUMATA, O., TAKEMASA, T., TAKAGI, I., HIRONO, M., HIRANO, H., CHIBA, J. AND WATANABE, Y. (1991). Tetrahymena 14-nm filament-forming protein has citrate synthase activity. *Biochemical and Biophysical Research Communications* **174**, 1028–1034.

OSTRER, H. AND PIATIGORSKY, J. (1980). β-Crystallins of the adult chicken lens: relatedness of the polypeptides and their aggregates. *Experimental Eye Research* **30**, 679–689.

OVERBEEK, P.A., CHEPELINSKY, A.B., KHILLAN, J.S., PIATIGORSKY, J. AND WESTPHAL, H. (1985). Lens-specific expression and developmental regulation of the bacterial chloramphenicol acetyltransferase gene driven by the murine αA-crystallin promoter in transgenic mice. *Proceedings of the National Academy of Sciences, USA* **82**, 7815–7819.

PARKER, D.S., WAWROUSEK, E.F. AND PIATIGORSKY, J. (1988). Expression of the δ-crystallin genes in the embryonic chicken lens. *Developmental Biology* **126**, 375–381.

PEEK, R., VAN DER LOGT, P., LUBSEN, N.H. AND SCHOENMAKERS, J.G.G. (1990). Tissue- and species-specific promoter elements of rat γ-crystallin genes. *Nucleic Acids Research* **18**, 1189–1197.

PEEK, R., KRAFT, H.J., KLOK, E.J., LUBSEN, N.H. AND SCHOENMAKERS, J.G.G. (1992). Activation and repression sequences determine the lens-specific expression of the rat γD-crystallin gene. *Nucleic Acids Research* **20**, 4865–4871.

PEREZ-CASTRO, A.V., TRAN, V.T. AND NGUYEN-HUU, M.C. (1993). Defective lens fiber differentiation and pancreatic tumorigenesis caused by ectopic expression of the cellular retinoic acid-binding protein I. *Development* **119**, 363–375.

PIATIGORSKY, J. (1981). Lens differentiation in vertebrates. A review of cellular and molecular features. *Differentiation* **19**, 134–153.

PIATIGORSKY, J. (1984). δ-Crystallin and their nucleic acids. *Molecular and Cellular Biochemistry* **59**, 33–56.

PIATIGORSKY, J. (1992). Lens crystallins. Innovation associated with changes in gene regulation. *Journal of Biological Chemistry* **267**, 4277–4280.

PIATIGORSKY, J. AND ZELENKA, P.S. (1992). Transcriptional regulation of crystallin genes: *cis* elements, *trans*-factors, and signal transduction systems in the lens. *Advances in Developmental Biochemistry* **1**, 211–256.

PIATIGORSKY, J., O'BRIEN, W.E., NORMAN, B.L., KALUMUK, K., WISTOW, G.J., BORRAS, T., NICKERSON, J.M. AND WAWROUSEK, E.F. (1988). Gene sharing by δ-crystallin and argininosuccinate lyase. *Proceedings of the National Academy of Sciences, USA* **85**, 3479–3483.

PIATIGORSKY, J., HORWITZ, J., KUWABARA, T. AND CUTRESS, C.E. (1989). The cellular eye lens and crystallins of cubomedusan jellyfish. *Journal of Comparative Physiology A* **164**, 577–587.

QUAX-JEUKEN, Y., DRIESSEN, H., LEUNISSEN, J., QUAX, W., DE JONG, W. AND BLOEMENDAL, H. (1985). βs-Crystallin: structure and evolution of a distinct member of the βγ-superfamily. *EMBO Journal* **4**, 2597–2602.

QUIRING, R., WALDORF, U., KLOTER, U. AND GEHRING, W.J. (1994). Homology of the eyeless gene of Drosophila to the small eye gene in mice and Aniridia in humans. *Science* **265**, 785–789.

RAO, G.N. AND COTLIER, E. (1984). Urea cycle enzymes in retina, ciliary body-iris, lens and senile cataracts. *Experimental Eye Research* **39**, 483–495.

RAO, P.V. AND ZIGLER, J.S., JR (1990). Extremely high levels of NADPH in guinea pig lens: correlation with ζ-crystallin concentration. *Biochemical and Biophysical Research Communications* **167**, 1221–1228.

RAO, P.V., KRISHNA, C.M. AND ZIGLER, J.S. (1992). Identification and characterization of the enzymatic activity of ζ-crystallin from guinea pig lens. A novel NADPH:quinone oxidoreductase. *Journal of Biological Chemistry* **267**, 96–102.

RENKAWEK, K., DE JONG, W.W., MERCK, K.B., FRENKEN, C.W., VAN WORKUM, F.P. AND BOSMAN, G.J. (1992). αB-crystallin is present in reactive glia in Creutzfeldt-Jakob disease. *Acta Neuropathologica* **83**, 324–327.

RICHARDSON, J., CVEKL, A. AND WISTOW, G. Pax-6 is essential for lens-specific expression of ζ-crystallin. Submitted.

RODOKANAKI, A., HOLMES, R.K. AND BORRAS, T. (1989). ζ-Crystallin, a novel protein from the guinea pig lens is related to alcohol dehydrogenases. *Gene* **78**, 215–224.

ROQUEMORE, E.P., DELL, A., MORRIS, H.R., PANICO, M., REASON, A.J., SAVOY, L.-A., WISTOW, G.J., ZIGLER, J.S., JR, EARLES, B.J. AND HART, G.W. (1992). Vertebrate lens α-crystallins are modified by *O*-linked *N*-acetylglucosamine. *Journal of Biological Chemistry* **267**, 555–563.

ROTH, H.J., DAS, G.C. AND PIATIGORSKY, J. (1991). Chicken βB1-crystallin gene expression: presence of conserved functional polyomavirus enhancer-like and octamer binding-like promoter elements found in non-lens genes. *Molecular and Cellular Biology* **11**, 1488–1499.

RUDNER, G., KATAR, M. AND MAISEL, H. (1990). Enolase in the avian and turtle lens. *Current Eye Research* **9**, 139–150.

RUSSELL, P., MEAKIN, S.O., HOHMAN, T.C., TSUI, L.C. AND BREITMAN, M.L. (1987). Relationship between proteins encode by three human γ-crystallin genes and distinct polypeptides in the eye lens. *Molecular and Cellular Biology* **7**, 3320–3323.

SANS, N., SCHINDLER, U. AND SCHRÖDER, J. (1988). Ornithine cyclodeaminase from Ti plasmid C58: DNA sequence, enzyme properties and regulation of activity by arginine. *European Journal of Biochemistry* **173**, 123–130.

SAWADA, K., AGATA, K. AND EGUCHI, G. (1992). Crystallin gene expression in the process of lentoidogenesis in cultures of chicken lens epithelial cells. *Experimental Eye Research* **55**, 879–887.

SAX, C. AND PIATIGORSKY, J. (1994). Expression of the α-crystallin/small heat shock proteins/molecular chaperone genes in the lens and other tissues. In *Advances in Enzymology and Related Areas in Molecular Biology* Vol. 69, pp. 155–201. John Wiley, New York.

SAX, C.M., ILAGAN, J.G. AND PIATIGORSKY, J. (1993). Functional redundancy of the DE-1 and αA-CRYBP1 regulatory sites of the mouse αA-crystallin promoter. *Nucleic Acids Research* **21**, 2633–2640.

SAX, C.M., KLEMENT, J.F. AND PIATIGORSKY, J. (1990). Species-specific lens activation of the thymidine kinase promoter by a single copy of the mouse αA-CRYBP1 site and loss of tissue specificity by multimerization. *Molecular and Cellular Biology* **10**, 6813–6816.

SMOLICH, B.D., TARKINGTON, S.K., SAHA, M.S. AND GRAINGER, R.M. (1994). *Xenopus* γ-crystallin gene expression: Evidence that the γ-crystallin gene family is transcribed in lens and non-lens tissues. *Molecular and Cellular Biology* **14**, 1355–1363.

STAPEL, S.O. AND DE JONG, W.W. (1983). Lamprey 48-kDa lens protein represents a novel class of crystallins. *FEBS Letters* **162**, 305–309.

STAPEL, S.O., ZZWEERS, A., DODEMONT, H.J., KAN, J.H. AND DE JONG, W.W. (1985). ε-crystallin, a novel avian and reptilian eye lens protein. *European Journal of Biochemistry* **147**, 129–136.

STOYKOVA, A. AND GRUSS, P. (1994). Roles of Pax-genes in developing and adult brain as suggested by expression patterns. *Journal of Neuroscience* **14**, 1395–1412.

SUMMERS, L., WISTOW, G., NAREBOR, M., MOSS, D., LINDLEY, P., SLINGSBY, C., BLUNDELL, T. AND BARTUNIK, H. (1984). X-ray structure of the lens specific proteins: the crystallins. In *Peptide and Protein Reviews* Vol. 3 (M.T.W. Hearn, Ed.), pp. 147–168. Marcel Dekker, New York.

TAKAHASHI, Y., HANAOKA, K., HAYASAKA, M., KATOH, K., KATO, Y., OKADA, T.S. AND KONDOH, H. (1988). Embryonic stem cell-mediated transfer and correct regulation of the chicken δ-crystallin gene in developing mouse embryos. *Development* **102**, 259–269.

THOMAS, G., ZELENKA, P.S., CUTHBERTSON, R.A., NORMAN, B.L. AND PIATIGORSKY, J. (1990). Differential expression of the two δ-crystallin/argininosuccinate lyase genes in lens, heart and brain of chicken embryos. *New Biologist* **2**, 903–914.

THOMPSON, M.A., HAWKINS, J.W. AND PIATIGORSKY, J. (1987). Complete nucleotide sequence of the chicken αA-crystallin gene and its 5' flanking region. *Gene* **56**, 173–184.

TINI, M., OTULAKOWSKI, G., BREITMAN, M.L., TSUI, L.C. AND GIGUERE, V. (1993). An everted repeat mediates retinoic acid induction of the γF-crystallin gene: evidence of a direct role for retinoids in lens development. *Genes and Development* **7**, 295–307.

TOMAREV, S.I. AND ZINOVIEVA, R.D. (1988). Squid major lens polypeptides are homologous to glutathione S-transferases subunits. *Nature* **336**, 86–88.

TOMAREV, S.I., ZINOVIEVA, R.D. AND PIATIGORSKY, J. (1991). Crystallins of the octopus lens.

Recruitment from detoxification enzymes. *Journal of Biological Chemistry* **266**, 24226–24231.

TOMAREV, S.I., ZINOVIEVA, R.D., DOLGILEVICH, S.M., LUCHIN, S.V., KRAYEV, A.S., SKRYABIN, K.G. AND GAUSE, G.G., JR. (1984). A novel type of crystallin in the frog eye lens. 35-kDa polypeptide is not homologous to any of the major classes of lens crystallins. *FEBS Letters* **171**, 297–302.

TOMAREV, S.I., CHUNG, S., ZINOVIEVA, R.D. AND PIATIGORSKY, J. (1994). Structure and *in vitro* expression of S-crystallins and gluthathione S-transferases of the squid. *Investigative Ophthalmology and Visual Science* **35**, 1997.

TRETON, J.A., JONES, R.E., KING, C.R. AND PIATIGORSKY, J. (1984). Evidence against γ-crystallin DNA or RNA sequences in the chick. *Experimental Eye Research* **39**, 513–522.

VAN DEN HEUVEL, R., HENDRIKS, W., QUAX, W. AND BLOEMENDAL, H. (1985). Complete structure of the hamster αA-crystallin gene. Reflection of an evolutionary history by means of exon shuffling. *Journal of Molecular Biology*, **185**, 273–284.

VAN LEEN, R.W., VAN ROOZENDAAL, K.E.P., LUBSEN, N.H. AND SCHOENMAKERS, J.G.G. (1987). Differential expression of crystallin genes during development of the rat eye lens. *Developmental Biology* **120**, 457–464.

VAN RENS, G.L., DE JONG, W.W. AND BLOEMENDAL, H. (1991). One member of the γ-crystallin gene family, γs, is expressed in birds. *Experimental Eye Research* **53**, 125–138 [letter].

VOORTER, C.E.M., MULDERS, J.W.M., BLOEMENDAL, H. AND DE JONG, W.W. (1986). Some aspects of the phosphorylation of α-crystallin A. *European Journal of Biochemistry* **160**, 203–210.

VOORTER, C.E., DE HAARD-HOEKMAN, W.A., ROERSMA, E.S., MEYER, H.E., BLOEMENDAL, H. AND DE JONG, W.W. (1989). The *in vivo* phosphorylation sites of bovine αB-crystallin. *FEBS Letters* **259**, 50–52.

WARWAR, R.E., KIM, R.Y., WISTOW, G.J. AND ZELENKA, P.S. (1992). The τ-crystallin/α-enolase gene: a candidate for regulation by c-*myc*. *Investigative Ophthalmology and Visual Science* **33**, 794.

WATANABE, K., FUJII, Y., NAKAYAMA, K., OHKUBO, H., KURAMITSU, S., KAGAMIYAMA, H., NAKANISHI, S. AND HAYAISHI, O. (1988). Structural similarity of bovine lung prostaglandin F synthase to lens ε-crystallin [renamed ρ-crystallin] of the European common frog. *Proceedings of the National Academy of Sciences, USA* **85**, 11–15.

WAWROUSEK, E.F., CHEPELINSKY, A.B., MCDERMOTT, J.B. AND PIATIGORSKY, J. (1990). Regulation of the murine αA-crystallin promoter in transgenic mice. *Developmental Biology* **137**, 68–76.

WEINER, H. (Ed.) (1990). *Enzymology and Molecular Biology of Carbonyl Metabolism* (3rd edn). Plenum Press, New York.

WESTPHAL, H. (1989). Transgenic mammals and biotechnology. *FASEB Journal* **3**, 117–120.

WILLIAMS, K.R., REDDIGARI, S. AND PATEL, G.L. (1985). Identification of a nucleic acid helix-destabilizing protein from rat liver as lactate dehydrogenase-5. *Proceedings of the National Academy of Sciences, USA* **82**, 5260–5264.

WILLIAMS, L.A., DING, L., HORWITZ, J. AND PIATIGORSKY, J. (1985). τ-Crystallin from the turtle lens: Purification and partial characterization. *Experimental Eye Research* **40**, 741–749.

WISTOW, G. (1990). Evolution of a protein superfamily: relationships between vertebrate lens crystallins and micro-organism dormancy proteins. *Journal of Molecular Evolution* **30**, 140–145.

WISTOW, G. (1993a). Lens crystallins: a model system for gene recruitment. In *Methods in Enzymology: Molecular Evolution. Producing the Biochemical Data*, Vol. 224 (E.A. Zimmer, T.J. White, R.L. Cann and A.C. Wilson, Eds), pp. 563–575. Academic Press, San Diego, CA.

WISTOW, G. (1993b). Lens crystallins: gene recruitment and evolutionary dynamism. *Trends in Biochemical Science* **18**, 301–306.

WISTOW, G. (1993c). Possible tetramer-based quaternary structures for α-crystallins and small heat-shock proteins. *Experimental Eye Research* **56**, 729–732.

WISTOW, G. AND KIM, H. (1991). Lens protein expression in mammals: taxon-specificity and the recruitment of crystallins. *Journal of Molecular Evolution* **32**, 262–269.

WISTOW, G. AND PIATIGORSKY, J. (1987). Recruitment of enzymes as lens structural proteins. *Science* **236**, 1554–1556.

WISTOW, G. AND PIATIGORSKY, J. (1988). Lens crystallins: evolution and expression of proteins for a highly specialized tissue. *Annual Review of Biochemistry* **57**, 479–504.

WISTOW, G. AND PIATIGORSKY, J. (1990). Gene conversion and splice-site slippage in the argininosuccinate lyases/δ-crystallins of the duck lens: members of an enzyme superfamily. *Gene* **96**, 263–270.

WISTOW, G. AND SEGOVIA, L. (1994). μ-Crystallin: an unexpected marker for photoreceptors. *Investigative Ophthalmology and Visual Science* **35**, 2073.

WISTOW, G., ANDERSON, A. AND PIATIGORSKY, J. (1990). Evidence for neutral and selective processes in the recruitment of enzyme-crystallins in avian lenses. *Proceedings of the National Academy of Sciences, USA* **87**, 6277–6280.

WISTOW, G.J., MULDERS, J.W. AND DE JONG, W.W. (1987). The enzyme lactate dehydrogenase as a structural protein in avian and crocodilian lenses. *Nature* **326**, 622–624.

WISTOW, G., SUMMERS, L. AND BLUNDELL, T. (1985). *Myxococcus xanthus* spore coat protein S may have a similar structure to vertebrate lens βγ-crystallins. *Nature* **315**, 771–773.

WISTOW, G., SLINGSBY, C., BLUNDELL, T., DRIESSEN, H., DE JONG, W. AND BLOEMENDAL, H. (1981). Eye-lens proteins: the three-dimensional structure of β-crystallin predicted from monomeric γ-crystallin. *FEBS Letters* **133**, 9–16.

WISTOW, G., TURNELL, B., SUMMERS, L., SLINGSBY, C., MOSS, D., MILLER, L., LINDLEY, P. AND BLUNDELL, T. (1983). X-ray analysis of the eye lens protein γ-II crystallin at 1.9 Å resolution. *Journal of Molecular Biology* **170**, 175–202.

WISTOW, G.J., LIETMAN, T., WILLIAMS, L.A., STAPEL, S.O., DE JONG, W.W., HORWITZ, J. AND PIATIGORSKY, J. (1988). τ-crystallin/α-enolase: One gene encodes both an enzyme and a lens structural protein. *Journal of Cell Biology* **107**, 2729–2736.

YASUDA, K., KITAMURA, M., OKAZAKI, K. AND TAKEUCHI, M. (1988). Mechanism involved in regulation α-crystallin gene expression. In *UCLA Symposium on the Molecular Biology of the Eye Lens: Genes, Vision, and Ocular Disease*, Vol. 8 (J. Piatigorsky, T. Shinohara and P.S. Zelenka, Eds), pp. 205–213. Alan R. Liss, New York.

YU, C. C.-K., TSUI, L.-C. AND BREITMAN, M.L. (1990). Homologous and heterologous enhancers modulate spatial expression but not cell-type specificity of the murine γF-crystallin promoter. *Development* **110**, 131–139.