CHAPTER 5

CRYSTALLIN GENE EXPRESSION: THE PAX-6 CONNECTION

In spite of their diversity, taxon-specific and ubiquitous crystallin genes of mammals and birds are able to direct specific expression across species in lens cells of other mammals, birds and even amphibians.[1-6] This shows that vertebrates have conserved the ancestral gene cascades required for the development and differentiation of the eye and the lens and that consequently the transcriptional machineries of the lens are held in common among distantly related vertebrate species. Therefore when genes are recruited as crystallins they experience common transcriptional environments no matter in what vertebrate species the recruitment occurs.

However this does not necessarily mean that all crystallin genes use exactly the same mechanisms of expression. The lens maintains several different cell types throughout life and the expression profiles of crystallin genes vary with developmental stage and with the state of differentiation of the lens (see Figs. 1.3 and 1.4). Thus the six γ-crystallin genes γA-F are expressed preferentially in embryonic lens fibers[7-11] while γs-crystallin is expressed later in secondary fibers of the mature lens[12] and αA-crystallin is expressed in both epithelium and fibers throughout life.[13,14] Crystallins such as αA and ζ-crystallin[15] which are expressed in both epithelial cells and in fibers may also experience a boost in gene expression or protein synthesis during fiber cell differentiation to respond to the increased demand for protein in rapidly expanding cell volumes.

Different crystallin genes may therefore respond to different transcriptional environments and one gene may itself experience differences in these environments as it is expressed in different parts of the lens. Furthermore some genes, like αB-crystallin, contain elements for lens and non-lens expression in the same promoter region. This has created complexity and diversity in the expression mechanisms of crystallin genes in spite of all they have in common. For this reason it may be easier to discern some fundamentals of lens-specific expression in a

recently recruited taxon-specific crystallin, like guinea pig ζ-crystallin, than in more ancient ubiquitous crystallins like αA- and αB-crystallin. Nevertheless, recent results have begun to indicate a surprising degree of consensus for a widespread role for Pax-6 as a transcription factor involved in crystallin gene expression.[15-18] Other pattern-forming gene products with expression in lens, such as SOX-2,[19] are also likely to be involved in fine-tuning tissue-specificity and in conferring differential expression patterns within the lens.

PAX-6, EYE DEVELOPMENT AND THE EXPRESSION OF CRYSTALLINS

The differentiation of early embryos into complex, specialized tissues depends upon families of pattern forming "master genes," exemplified by those of the *Hox* families which encode proteins capable of sequence specific DNA-binding through structural motifs known as homeodomains (HD).[20] The homeodomain was first recognized as a conserved DNA-binding motif encoded by homeotic genes of *Drosophila*, such as *antennapedia* and *bithorax*, and was subsequently identified in many other families of DNA-binding proteins.[21-24] Genes for homeodomain-containing proteins are found in conserved clusters throughout the metazoa.[22-24] They are expressed very early in development and have essential roles in establishing segmentation patterns in embryogenesis.[22-24] This is often achieved by establishing overlapping regions of expression of different genes whose products exert positive and negative effects on the expression of their own and other pattern-forming genes as well as a variety of target genes.

Pax genes encode a similar family of proteins which are characterized by another DNA-binding motif, the paired-domain (PD), often, but not always, in conjunction with a homeodomain[25] (Fig. 5.1). The paired-domain too was first identified in *Drosophila*, in the paired gene[25,26] from which its name derives. *Pax* genes are also expressed early in development and play important roles in organogenesis. *Pax-6* is expressed from very early stages in eye and CNS in mouse, chicken and zebrafish[27-30] and its expression in the earliest precursors of chicken lens cells make it a candidate for one of the essential molecular determinants of lens competence.[29] Mutants in *Pax-6* have severe eye defects in mouse (*Small eye*)[31] and humans (aniridia and Peter's anomaly).[32-34] Remarkably it has now been found that homologues of Pax-6 play similar roles in invertebrates.[35] The *Drosophila* gene eyeless has been cloned and shows over 90% identity in predicted amino acid sequence to vertebrate Pax-6 proteins in its PD and HD regions. Mutants in eyeless fail to develop eyes. *Pax-6* homologues have also been detected in cephalopods, flat worms[35] and even in *C.elegans*[36,37] (A. Chisholm, personal communication).

Most dramatically of all, ectopic expression of *Drosophila* or mouse Pax-6 in various parts of *Drosophila* results in the induction of complete

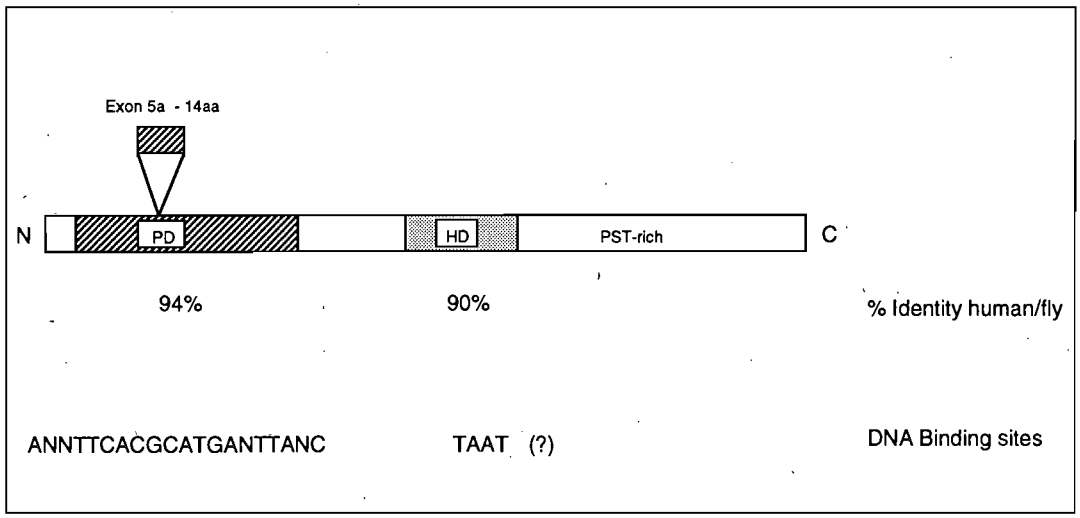

Fig. 5.1. The domain structure of Pax-6. PD: paired domain; HD: homeodomain; PST: the proline, serine, threonine-rich C-terminal region which may have a role in transactivation. Preferred consensus binding sites for PD and HD are shown.

compound eyes.[38] These observations suggest that *Pax-6* has an ancient ancestral role in the establishment of eyes and that all eyes may share a common ancestry in a simple light sensitive organ determined at least in part by *Pax-6* expression (see Fig. 1.1). Even as eyes became more sophisticated and divergent in many lineages the essential role of *Pax-6* was conserved and played a role in the evolutionary elaboration of new structures such as the cellular lens and ciliary body in vertebrates.

An in vitro consensus binding site for the PD of Pax-6 has been derived[39] (Fig. 5.1). This 20 bp sequence is long by the standards of many other transcription factors and reflects the fact that the PD has a bipartite structure. Independent binding of the two sub domains is possible as has been shown by the x-ray structure analysis of the PD itself bound to DNA.[40] The DNA sequence used in this analysis is CGTCACGGTTGA but since it only binds the N-terminal part of the PD it presumably does not represent a full binding site. Most binding studies of PDs from various Pax proteins have used an even shorter sequence, GTTCC.[41] Furthermore, alternative splicing of *Pax-6* transcripts gives rise to a variant which has an insertion in the N-terminal subdomain of the PD (Fig. 5.1) and as a result binds a different consensus sequence from the unspliced form.[42]

Like other HD-containing proteins, members of the Pax family seem to be transcription factors. In vitro experiments have shown that Pax-6 can act as a transcription activator.[43,44] Recently, functional Pax-6 binding sites have been detected in the promoter of the mouse gene

for the neural cell adhesion molecule L1[45] although the expression of this gene is not restricted to Pax-6 expressing cells. These sites conform quite well to the in vitro consensus.[39] However the most proximal site contains a TAAT sequence which also allows binding of Hoxa-1, a HD protein.[45]

Given its essential high-level pattern-forming role in early embryogenesis, Pax-6 was at first sight an improbable candidate for a transcription factor involved in expression of crystallin genes. Crystallins are probably at one of the end points of the molecular cascade in lens development and must maintain expression throughout life. However it is now clear that in at least some cases Pax-6 itself does act as transcription factor in crystallin gene expression.[15-18]

Our present understanding of the transcriptional control of crystallin gene expression is reviewed in the following sections, starting with the taxon-specific crystallins which have undergone more recent recruitment and ending with the more ancient ubiquitous crystallins which seem to have elaborated more complex control mechanisms.

TAXON-SPECFIC CRYSTALLINS

ζ-Crystallin: Pax-6 and the Recruitment of an Enzyme Crystallin

ζ-Crystallin is one of those taxon-specific enzyme crystallins which was first observed as a crystallin and only later proved to be an enzyme, in this case a novel NADPH:quinone oxidoreductase.[46-48] Sequence analysis revealed that this gene uses two separate promoters for lens and non-lens expression in guinea pig tissues[49,50] (Fig. 5.2). The lens promoter is located in what would otherwise be the first intron of the enzyme gene. This intron maps to the untranslated region of the mRNA and transcripts from both promoters splice to the same second exon which contains the initiator methionine codon. Thus the same protein is produced in both cases.

Since this gene makes use of widely separated alternative promoters for lens and non-lens expression, the lens-promoter does not need to accommodate additional binding sites for other functions. Although there is no evidence that other genes have made similar use of alternative promoters, ζ-crystallin illustrates important features of gene recruitment and lens-specific expression which may be generally applicable.

At first sight the lens-specificity of ζ-crystallin could have been achieved by several possible mechanisms. For example, the gene could have acquired a lens-specific enhancer somewhere in the gene which might have activated a TATA sequence already present in the first intron. In this scenario there might be no functional elements in the sequences upstream of the lens-specific alternative first exon. Alternatively the guinea pig could have experienced species-specific modification

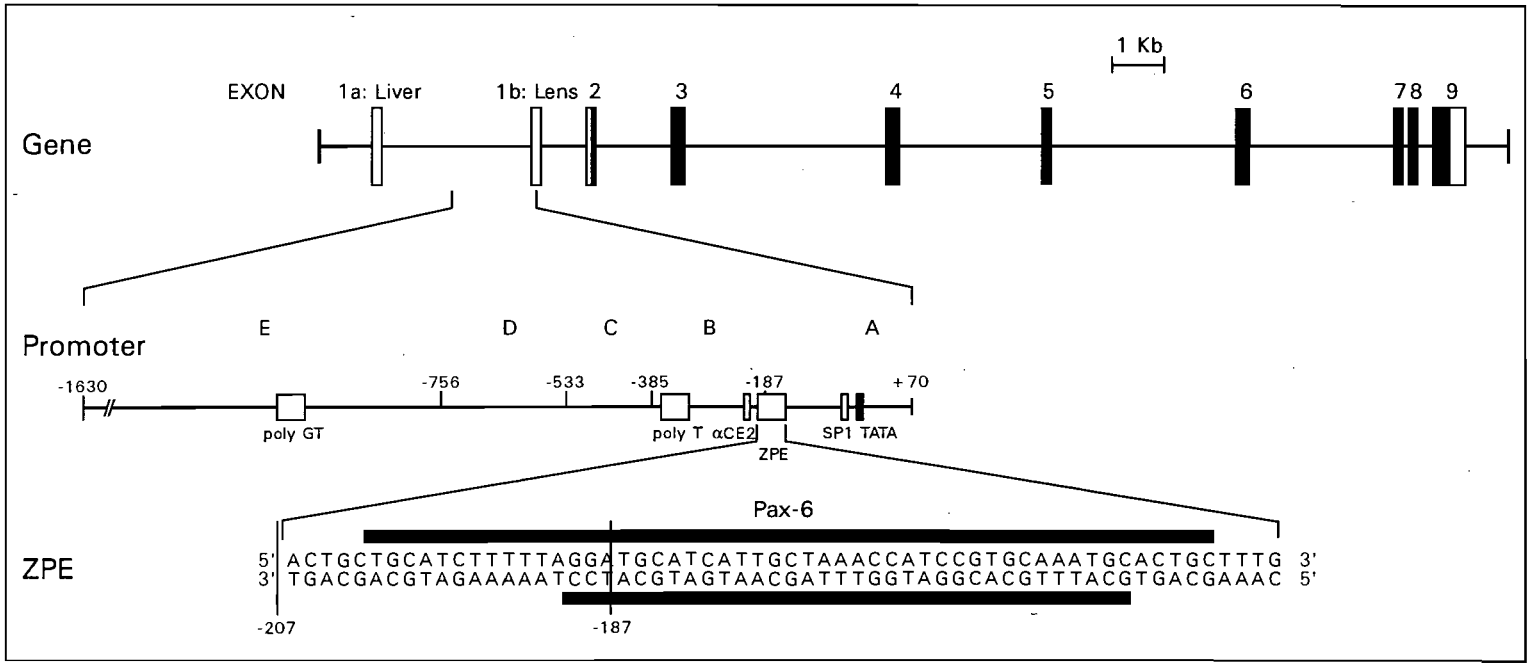

Fig. 5.2. The gene and lens promoter for guinea pig ζ-crystallin. Top: Gene structure. Exons are boxed and coding sequence is shaded. Alternative first exons are marked. Middle: Layout of the lens promoter. αCE2 refers to a sequence identical to one identified as functionally important in the chicken αA-crystallin gene (see Fig. 5.9), Bottom: The ZPE/Pax-6 binding site. This figure is updated from Lee DC, Gonzalez P, Wistow G, J Mol Biol 1994; 236:669-781.

of the complement of transcription factors expressed in its lens. This might have activated a cryptic promoter in the first intron. In such a case the promoter would function only in guinea pig lens and would not exhibit lens-specific expression in other species such as mice.

Fortunately, the mechanism of recruitment proved to be the simplest and most accessible possibility. The recruitment of this enzyme crystallin occurred through acquisition of a lens-specific alternative promoter which does not require host-specific factors[51] (Fig. 5.2). The lens promoter is neatly flanked by 9 bp direct repeats and when the guinea pig promoter is compared to the intron sequence of the homologous but unrecruited gene in mouse no similarity in sequence is observed upstream of the direct repeat in the guinea pig first exon while there is limited sequence similarity downstream of this point. This raises the possibility that the lens promoter may have been inserted into the gene by a transposon-mediated event.[51]

The strong tissue preference of the lens promoter is apparent in both transient transfections of cells in culture and in transgenic mice.[51] While proximal regions of the promoter (-385/+70) have some activity in the brain of transgenic mice this is abolished by the addition of more distal regions (Fig. 5.3). The minimal active lens promoter is differentially footprinted by extracts from lens and non-lens cells. In lens cell extracts a single 50 bp element, the ζ protected element or ZPE, is protected from DNase I digestion (Fig. 5.2). In fibroblast extracts the ZPE is incompletely protected and is flanked by two additional protected elements, the upstream and downstream boxes (UB and DB). This suggests that in lens a tissue-specific (or preferred) factor binds to the promoter at the ZPE to form a transcriptionally active complex while in non-expressing cells, some competing factor(s) occupies the ZPE and the suppression of promoter activity is completed by additional binding of other factors at the UB and DB sites (Fig. 5.4).

The ZPE is a Pax-6 Site

In electrophoretic mobility shift assay (EMSA) the ZPE forms two specific complexes. Complex I is found in extracts of non-lens tissues such as liver and lung. This shows that the competing factor which binds the ZPE in non-expressing cells does not require the cooperation of the UB and DB factors in order to bind. The protein composition of complex I is not yet known. However, the ZPE contains consensus binding sites for several families of general transcription factors[51] including the C/EBP family, octamer family and HLH family.[52]

Complex II is formed in lens extracts while in extracts of lens-derived cells and brain both complexes I and II are present.[15] At this stage it is not known whether both complexes can exist in one cell type or whether they are mutually exclusive. Brain consists of multiple cell types and even the cultured lens-derived cells may contain lens-like and non-lens like populations.

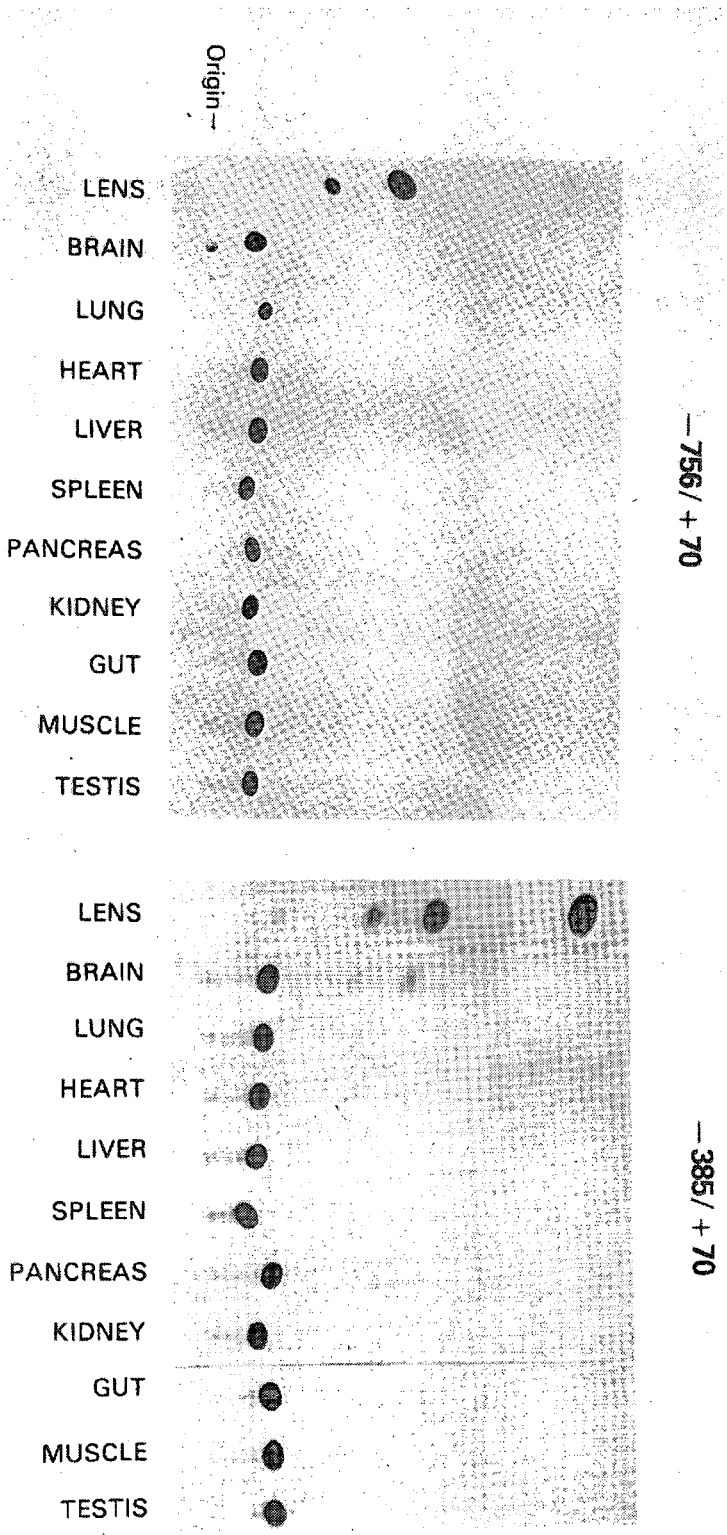

Fig. 5.3. Thin layer chromatography analysis of CAT reporter gene activity in tissues of transgenic mice bearing ζ-crystallin promoter constructs. -756/+70.ZP/CAT (top) is a "full-length" wild type promoter with lens-specificity.[51] -385/+70.ZP/CAT (bottom) exhibits some expression in brain. Reprinted with permission from Lee DC, Gonzalez P, Wistow G, J Mol Biol 1994; 236:669-78

By competitive EMSA a region of the ZPE, designated ZE-1, was defined as essential for formation of complex II. The ZE-1 site represents the core of a consensus Pax-6 binding site in the ZPE.[15,39] Antisera to Pax-6 abolish complex II without affecting complex I. Recombinant human Pax-6 gives an identical footprint on the ζ-crystallin promoter to that formed by mouse lens extract (in preparation). Mutation of the ZPE which abolishes Pax-6 binding in vitro also abolishes promoter activity in vivo[15] (Fig. 5.5). The identification of Pax-6 with complex II is consistent with its expression in both brain and eye. Western blot analysis shows that Pax-6 protein is present in lens and brain and in extracts of αTN4-1 and N/N1003A cells. RT-PCR is also able to detect mRNA for Pax-6 in these tissues and cells.[15] Thus Pax-6 is essential for expression of the ζ-crystallin lens promoter, however other factors which footprint poorly or which rely on protein-protein interactions rather than DNA-binding could also be involved in gene activation (Fig. 5.4).

Pax-6 Expression in Mature Lens

Pax-6 is expressed in mature lens appropriately for a continuing role in tissue-specific gene expression.[15] Pax-6 protein is present in adult guinea pig lens. By immunohistochemical staining Pax-6 is detected most prominently in lens epithelial cells where the nuclei make up a large fraction of cell volume but it is also detectable in the nuclei of the elongating fiber cells in the equatorial region. Immunochemistry for ζ-crystallin in the same system shows that the crystallin is present in the cytoplasm of the epithelial cells and in fiber cells.[15] Its most intense staining is in the equatorial fibers. Thus Pax-6 is present in cells which express ζ-crystallin and the maximum expression of Pax-6 seems to occur prior to the maximum expression of ζ-crystallin protein during lens cell differentiation.

Pax-6 mRNA is present in adult mouse lens and brain but exhibits an interesting tissue-specific pattern of alternative splicing. In mouse brain both of the alternatively spliced mRNAs which correspond to the alternative PD forms of Pax-6 are detected at essentially equal abundance. In contrast, in adult mouse lens and in lens-derived cultured cells the mRNA corresponding to the form of Pax-6 able to bind the ZPE greatly predominates. Thus, while the expression of the *Pax-6* gene is not tissue-specific, lens-specific differences in the abundance of alternatively spliced forms of *Pax-6* mRNA may contribute to tissue discrimination in binding activity.

Pax-6 Binding Sites in Several Genes

In addition to its central importance in the lens-specific expression and gene recruitment of guinea pig ζ-crystallin[15] Pax-6 has been detected in complexes of factors binding to functional elements of the promoters for chicken[16] and mouse αA-crystallins[17] and to the enhancer

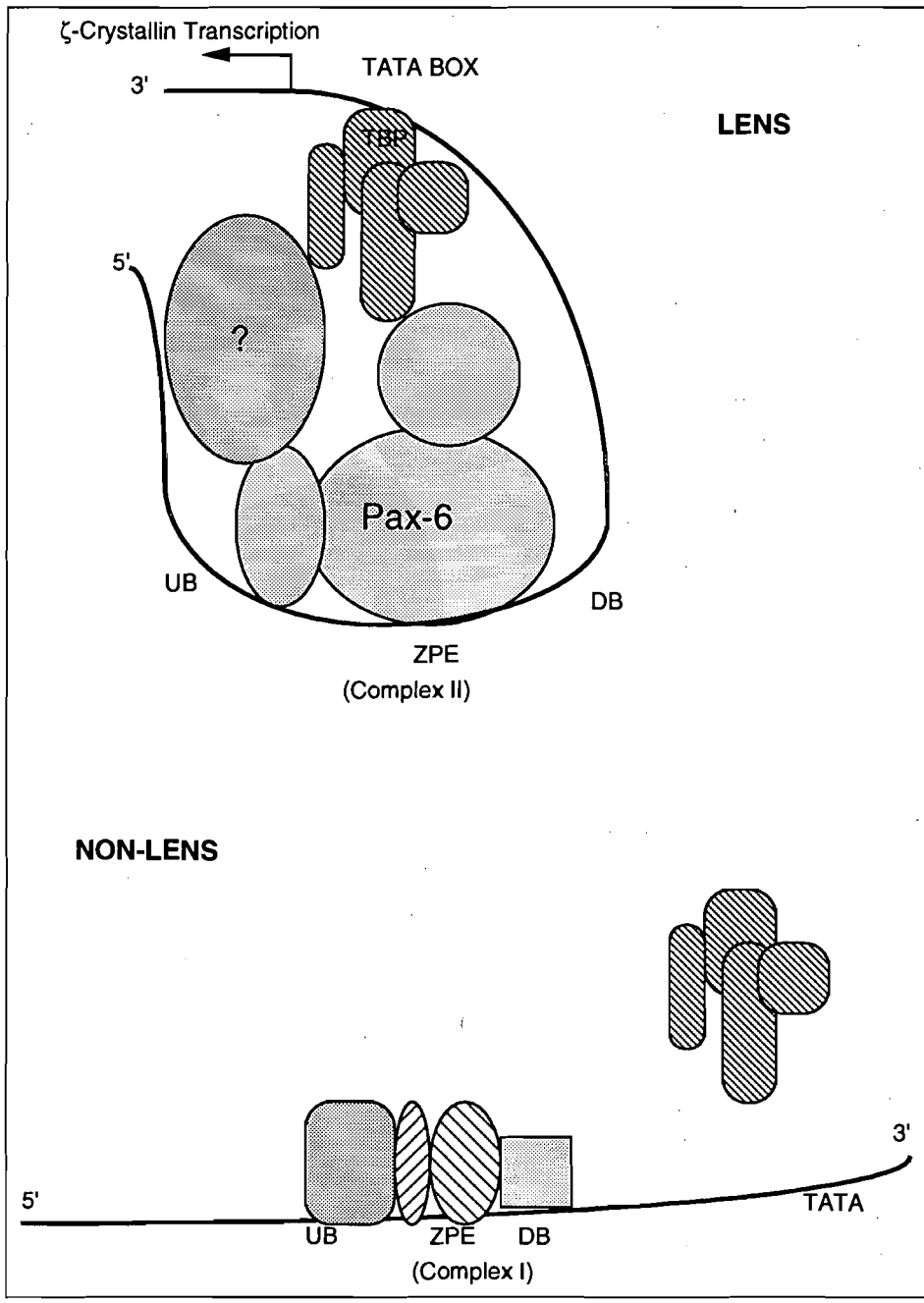

Fig. 5.4. A cartoon to illustrate some features of the ζ-crystallin lens promoter. Top: In lens Pax-6 binds the ZPE and activates the promoter, perhaps in concert with other factors not yet identified which may or may not contact DNA. Bottom: In other tissues a different complex occupies the ZPE together with other factors at the flanking upstream box (UB) and downstream box (DB) and the promoter is inactive.

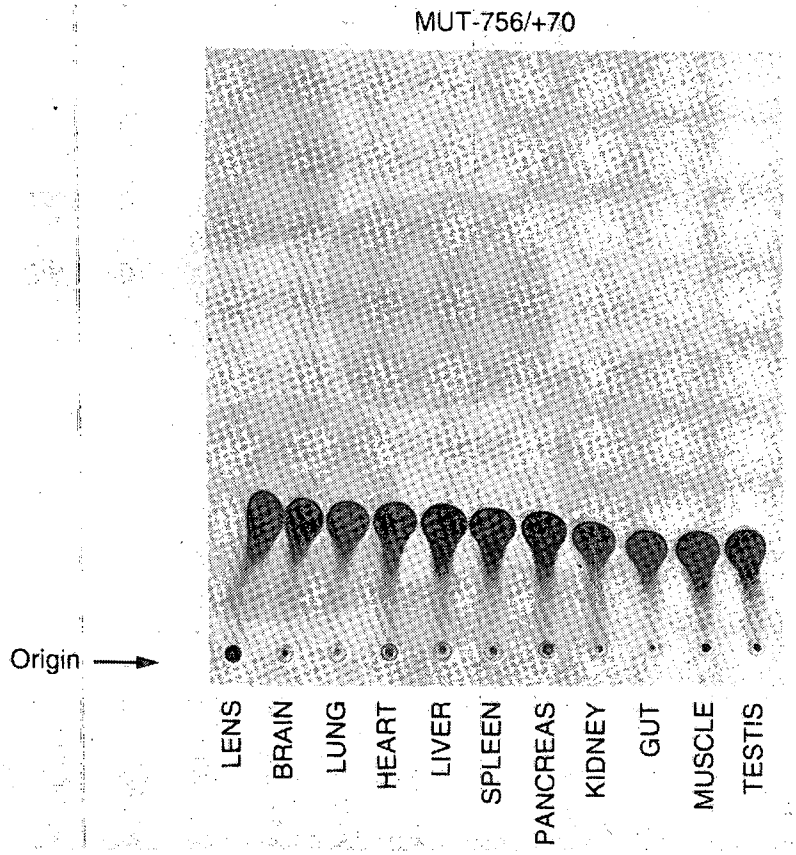

Fig. 5.5. Thin layer chromatography analysis of CAT reporter gene activity in tissues of transgenic mice bearing the -756MUT/+70.ZP/CAT construct which contains specific mutations in the ZPE which abolish Pax-6 binding and promoter activity.

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  t t
A N N T T C A C G C W T S A N T K M N Y   :E
A T T T G C A C G G A T G G T T T A G C   :ζ
T T A T T C A C T A A T G G C T G C A C   :L1
A A T C C C A C T A A T G C C T T C A T   :α
A T T T N C A C K N A T G N N T T M N Y   :con
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Fig. 5.6. Alignment of Pax-6 recognition sequences.
E: The in vitro binding sequence of Epstein et al.[39] Positions of equal preferences are shown by standard nomenclature as follows: W (A or T); S (C or G); K (G or T); M (A or C); Y (C or T). Positions 2 and 3 have a slight preference for T (shown as lower case above the line) which matches other sequences as shown.
ζ: The site from ζ-crystallin in complement.
L1: The proximal site from mouse L1.[45]
α: The -61/-40 site from chicken αA-crystallin.[16]
con: a consensus derived from these sequences.

region of chicken δ1-crystallin,[18] as discussed below. Figure 5.6 shows the consensus Pax-6 PD binding sequence which was determined in vitro[39] compared with the ζ-crystallin ZPE, the most proximal Pax-6 site of mouse neural adhesion protein L1[45] and the chicken αA-crystallin -61/-40 site, which is the closest matching site to the in vitro consensus found so far in other crystallin genes. If positions with no clear preference are omitted from the in vitro consensus, there is 13/16 identity with the ζ-crystallin Pax-6 site (15/18 if positions 2 and 3 of the consensus which have slight preferences for T are included). Interestingly, the L1 and αA-crystallin sites in this alignment share a striking feature absent from both ζ-crystallin and from the in vitro consensus. They contain a consensus HD binding site (TAAT) which in the L1 gene has been shown to bind another HD protein, Hoxa-1.[45]

It seems likely that there is a range of higher and lower affinity sites for Pax-6 binding. The close match of the ζ-crystallin site to the in vitro consensus suggests that it may be a relatively high affinity site and this may explain the very strong footprinting of this site seen in protection analyses using lens cell extracts.[51] "Indeed, in contrast to the partial binding of a PD revealed by the recent x-ray structure,[40] it seems likely that the 50 bp ZPE of ζ-crystallin binds the PD, HD and possibly other C-terminal regions of a single Pax-6 molecule in a contiguous site (in preparation)."

Lens-specificity Through Acquisition of a Pax-6 Binding Site

In the case of ζ-crystallin, which is the result of relatively recent evolutionary events, a simple model of the process of gene recruitment can be envisaged. The initial event may have been the acquisition of a binding site for Pax-6 in an intron of an enzyme gene. Since Pax-6 expression is not limited to lens this could also have conferred expression in other tissues such as iris, retina and brain. However a lens-preferred pattern of expression could have occurred through selective binding of one form of Pax-6 resulting from tissue-specific alternative splicing in lens. Subsequently the expression of the recruited gene could have been further fine-tuned by the addition of other *cis*-elements to the recruited promoter which eliminated expression in other Pax-6 containing tissues. Indeed, the ζ-crystallin promoter does contain upstream sequences which suppress expression in transgenic mouse brain (see ref. 51, unpublished).

It is well known that changes in the expression of pattern-forming genes can produce significantly altered developmental programs. In the same way, acquisition of binding sites for master gene factors like Pax proteins could radically alter the protein composition of a tissue, such as the lens, in one evolutionary step.

PAX-6 AND CATARACT

Defects in Pax-6 expression can have serious effects in the eye. Mice homozygous for small eye have no eye or orbit at all and there is severe facial malformation.[31] Heterozygotes have microphthalmia. Heterozygote humans with aniridia lack a properly formed iris but do not have microphthalmia.[32,33,53] This species difference is interesting in itself. It shows that even such a fundamental gene as *Pax-6* may not function identically in species as closely related as two mammals.

Although the lens is usually unaffected at birth, as patients with aniridia age they also develop cataract, apparently with considerable heterogeneity.[53] This suggests that a single gene dosage of *Pax-6* is sufficient for normal lens development but not for development of the iris in humans. However, the progression of cataract also suggests that a single functional copy of *Pax-6* is not sufficient for the maintenance of a healthy transparent lens. Although analyses of mouse and guinea pig lens show that *Pax-6* expression continues in mature mammalian lens[15] it is also clear from studies in chicken embryos that levels of *Pax-6* mRNA decline during embryogenesis.[29] In neither birds nor mammals is there a complete picture of the level of expression of this gene throughout life. However the following hypothesis is not inconsistent with what is presently known.

Let us suppose that *Pax-6* expression is maximal during embryogenesis during rapid organogenesis and tissue differentiation but that later its expression declines as it adopts a maintenance role. Let us also suppose that in any dependent tissue there is a minimal threshold level of Pax-6 protein required for normal expression of some important target genes. In lens, this level is exceeded even by a single gene dose of *Pax-6* for some time after birth. However as gene expression declines, the single gene cannot maintain the maintenance threshold level. As it falls below this level gene expression is disrupted in lens epithelia and in newly differentiated fibers and opacities form.

Clearly this hypothetical model also has implications for the normal lens with two functioning *Pax-6* genes. Just as in aniridia, the level of *Pax-6* expression would decline. The approach to the critical threshold would be postponed because of the higher gene dosage but eventually, at a later age, the threshold would still be reached. This could be a cause of some cases of senile cataract. Premature senile cataract could occur when some minor difference in gene expression or in mRNA or protein stability led to a more rapid fall in Pax-6 levels in lens.

OTHER TAXON-SPECIFIC CRYSTALLINS

δ-CRYSTALLINS: RECRUITMENT THROUGH A LENS-SPECIFIC ENHANCER

As a result of gene duplication and specialization there are two genes for δ-crystallins in birds. One encodes ASL/δ2-crystallin and in

many birds, including chicken, this gene is not expressed in lens at crystallin-like levels.[5,54-56] The expression of ASL/δ2-crystallin has not been extensively studied. Instead, most work has concentrated on the lens-specialized δ1-crystallin gene which may contribute 90% of the soluble protein in the embryonic chick lens.[57] Initial studies on chicken δ1-crystallin identified a lens-preferred promoter and an upstream negative element.[58,59] However, the key to the high expression of this gene has proved to be an enhancer located in the third intron of the δ1-crystallin gene[60] (Fig. 5.7).

Surprisingly the δ1-enhancer is 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.[61] Even in the duck, the δ-enhancer is highly conserved in the third intron of the ASL/δ2-crystallin gene.[62] Promoter sequences are also highly conserved between chicken and duck δ2-crystallins. Indeed, the only major difference between the sequences separating δ1 and δ2 genes is the insertion of a CR1-type repetitive element in the duck locus.[62] Given this high degree of similarity, what then determines the differences in lens expression seen among different δ-crystallins in different species? The answer is not known although a search is underway for a possible silencer element responsible for suppressing expression of chicken δ2-crystallin in lens.

The δ1-enhancer: SOX and Pax

The δ1-crystallin enhancer contains at least two overlapping binding sites for factors designated δEF1 and δEF2[63-65] (Fig. 5.7). 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.[65] In particular, δEF1 is able to compete with bHLH proteins for binding at a class of E-boxes.[66] One such E-box binding protein is USF which is able to bind the δEF1 site in lens extracts.[18]

Multimers of the δEF2 element can act as a lens-specific enhancer for a heterologous promoter.[65] The lens-preference of the enhancer apparently depends upon binding of multi-component complexes.[65] One binding activity, designated δEF2a, was found to be highly enriched in chicken lens cells and has been cloned revealing identity with SOX-2.[19] The SOX family, whose name derives from SRY-box, is another group involved in pattern-formation and organogenesis during embryonic development.[67-69] Instead of a HD or PD, these proteins use a DNA-binding domain first identified in high mobility group (HMG) proteins. Like other pattern-forming gene products they exhibit restricted and overlapping patterns of expression which contribute to formation of tissue-specificity. Overexpression of SOX-2 increased δ1-crystallin enhancer activity in lens cells but not in fibroblasts.[19] This suggests that lens specificity results from a combination of lens-preferred factors. Indeed, two Pax-6 sites have also been identified in the δ1-crystallin enhancer region.[18] These sites do not correspond to the functionally

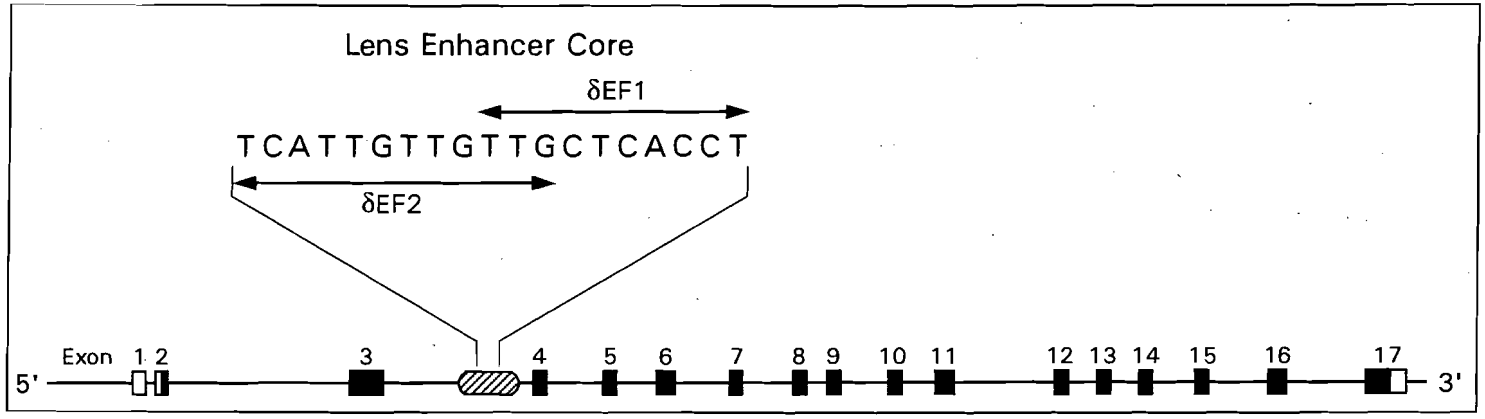

Fig. 5.7. The chicken δ1-crystallin gene and the position of the enhancer. Taken from Wistow G, Richardson J, Jaworski C, Graham C, Sharon-Friling R, Segovia L. In: Tombs MP, ed. Biotechnology and Genetic Engineering Reviews. v. 12. Andover, Hants: Intercept Ltd, 1994; 1-38.

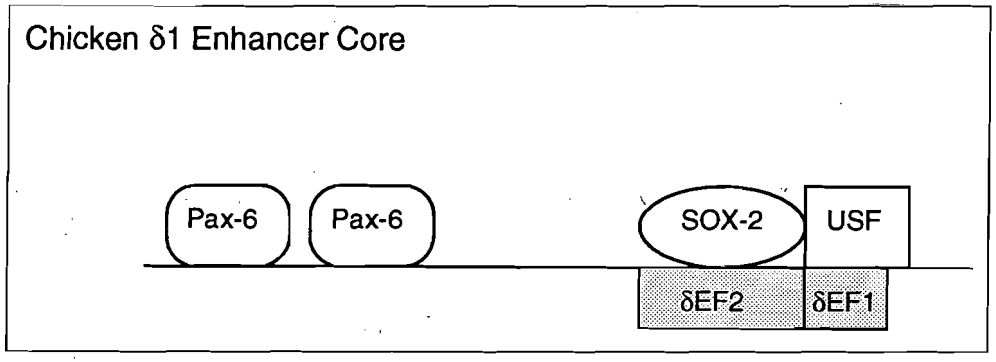

Fig. 5.8. A cartoon showing binding sites identified for the δ1-crystallin enhancer in lens.

defined δEF2 elements but they lie in sequences which are conserved in both δ1 and δ2-crystallin genes in chicken and duck.[18] It thus seems that the δ1-crystallin enhancer may operate through complex binding of different tissue-restricted, pattern-forming gene products involving both Pax-6 and SOX-2 (Fig. 5.8).

α-Enolase/τ-Crystallin

As discussed above, α-enolase/τ-crystallin is not a typical crystallin but it has some interesting features. In the duck lens it is highly abundant in epithelial cells but contributes only a small amount in overall crystallin content to adult lens.[70,71] The duck gene for α-enolase/τ-crystallin has been cloned and some promoter analysis carried out.[70] Although the endogenous gene shows preferential expression in embryonic duck lens epithelia, no lens-preferred expression of the recombinant gene promoter was observed in either transfected cultured cells or in transgenic mice.[70,72] In spite of these unpromising characteristics, this gene does serve to illustrate the way in which different regions of the lens express different complements of proteins. In fact its gene expression appears to be subject to differentiation-specific control and part of this may be due to the myc family of proto-oncogenes.

There is considerable circumstantial evidence associating high levels of expression of α-enolase and c-myc in various cell types.[73] This also applies to the lens in which the ratio of c-myc to N-myc mRNA drops as lens cells differentiate from epithelial cells where α-enolase is abundant to fiber cells in which it is at lower levels.[74] Furthermore, although there is otherwise little sequence similarity between them, the gene promoters of human and duck α-enolase[70,75] both contain an E-box, a potential myc family binding site,[76,77] at equivalent positions about 600 bp upstream of the transcription start site. C-myc can bind to this E-box in the duck gene and co-expression of c-myc induces expression of the duck α-enolase/τ-crystallin gene promoter.[78] This induction is abolished by mutation of the E-box. Clearly, control of α-enolase expression is not the main purpose of c-myc. Instead c-myc and its relatives are part of the essential processes of tissue differentiation. As a possible target of these factors the expression of α-enolase is subservient to that higher function. Its expression in lens may therefore be at least partly a side effect of other processes of differentiation, a lesson that may also be applicable to other crystallins.

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.[79] Identical start sites for transcription were found in both tissues,[79,80] although a second start site 28 bp upstream was also found in heart. High expression of the promoter in both cell types required sequences from the first intron although since it is unable to enhance a heterologous promoter the intron does not contain an enhancer as classically defined.[79] Although

the gene is expressed at higher levels in lens than 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 overexpression.

When levels of endogenous LDHB mRNA were measured in various tissues of chicken and duck it was found that expression in duck exceeded that in chicken in three tissues, lens, retina and pancreas.[81] This is intriguing since these tissues are all sites of Pax-6 expression in birds.[29,82]

UBIQUITOUS CRYSTALLINS

Since their initial recruitment to the lens occurred in a common ancestor of all vertebrates, the genes for the ubiquitous crystallins have had much longer than those of the enzyme crystallins to complicate and elaborate their transcriptional machinery. This increased sophistication may explain the apparent complexity of their promoters compared to that of a recently recruited gene such as ζ-crystallin.

αA-CRYSTALLINS: CONSERVATION AND COMPLEXITY IN UBIQUITOUS CRYSTALLINS

Of the two α-crystallin genes expressed in all vertebrate lenses αA-crystallin is the most tissue-specialized. Presumably a single sHSP/α-crystallin gene was recruited to the lens in a distant common ancestor of all vertebrates. This original single gene would probably have produced both lens and non-lens transcripts from one promoter with a mixture of functional elements. In this respect it would have resembled the modern αB-crystallin. At some point shortly after this initial recruitment there was a gene duplication and specialization of one of the pair of genes for lens. This gene became αA-crystallin. Over time the evolving αA-crystallin gene would have lost its non-lens expression for which there was no selective advantage and would have progressively fine-tuned its expression in lens.

In many species αA-crystallin is the single major provider of protein to the lens.[83] As such it may have been under special pressure to modify its expression during development and differentiation to suit lenses as different as those of mice and chickens. Thus, the promoter of this ancient gene might very well have acquired an unusual degree of complexity with various functional elements overlaying each other to modulate expression in different lineages. Indeed, this is the kind of picture which has emerged from a large number of studies of the gene expression of αA-crystallin.

αA-crystallin gene promoters have been cloned from mouse,[84] hamster,[85] mole rat,[86] chicken[87] and human.[84] Low levels of αA-crystallin have been detected in spleen and thymus in rat,[88] however the expression of this gene is otherwise highly lens-preferred. Both mammalian and chicken αA-crystallin genes share this high expression in the lens

and the chicken αA promoter is expressed in the lens of transgenic mice.[4] This suggests that all vertebrate αA promoters should share conserved functional promoter elements required for lens expression. These elements should be apparent in the approach known as "phylogenetic footprinting"[89] comparing promoter sequences for conserved regions. Alignments of promoter sequences for mouse, human and chicken uncovered four elements clustered close to the transcription start site which were significantly conserved in both sequence and position in all species[84,90] (Fig. 5.9). These elements correspond roughly to the minimal promoter regions of mouse and chicken αA-crystallin genes required for function in cell culture: -111 bp for mouse[91] and -162 bp for chicken.[4,92]

On a larger scale, all three αA-crystallin promoters also share the presence of species-specific repetitive elements at approximately 1 kb upstream of the coding regions.[84] A variety of other short sequence motifs are also common to all three genes but their position and number are not conserved. Whether these are the result of motif shuffling or whether they have no functional significance is not yet known. However, there is evidence that the four major conserved elements are indeed important for gene expression in lens.

In vivo and in vitro footprinting of the mouse αA-crystallin promoter have shown similar protected regions in αTN4-1 cells, mouse lens nuclear extract and in nuclear extracts from L929 fibroblasts.[93] In spite of this similarity, electrophoretic mobility shift assays (EMSA) suggested that different proteins were bound to the same sites in both lens and non-lens environments.

Binding Sites in the Mouse αA-crystallin Promoter

Several elements in the mouse αA-crystallin promoter have been defined by a variety of binding and functional studies. The first of these were the distal (-111/-88) and proximal (-88/-60) elements.[91] Both of these elements were found to be necessary for expression in transient transfection of PLEs. Since then several other studies have led to a more complex picture. The current view of this promoter defines several additional elements (Fig. 5.9).

The most 5' of these, DE1 (-111/-97), is part of the original distal element and contains one of the phylogenetically conserved blocks of sequence. It also corresponds to part of an enhancer which was defined in parallel studies of the closely related hamster αA-crystallin gene. In heterologous promoter experiments, the hamster αA-crystallin enhancer (-180/-85 in that gene) was able to activate the minimal promoter of mouse γF-crystallin in transfections in chicken PLEs.[91,94] DE1 has sequence similarity to a cAMP responsive element (CRE) and recent work has shown that this site can bind general CREB factors thereby activating transcription[17] (Fig. 5.10).

Overlapping the TATA box is the TATA/PE1 (-35/-19) element, while the 5' end of the transcribed region of the gene contains the

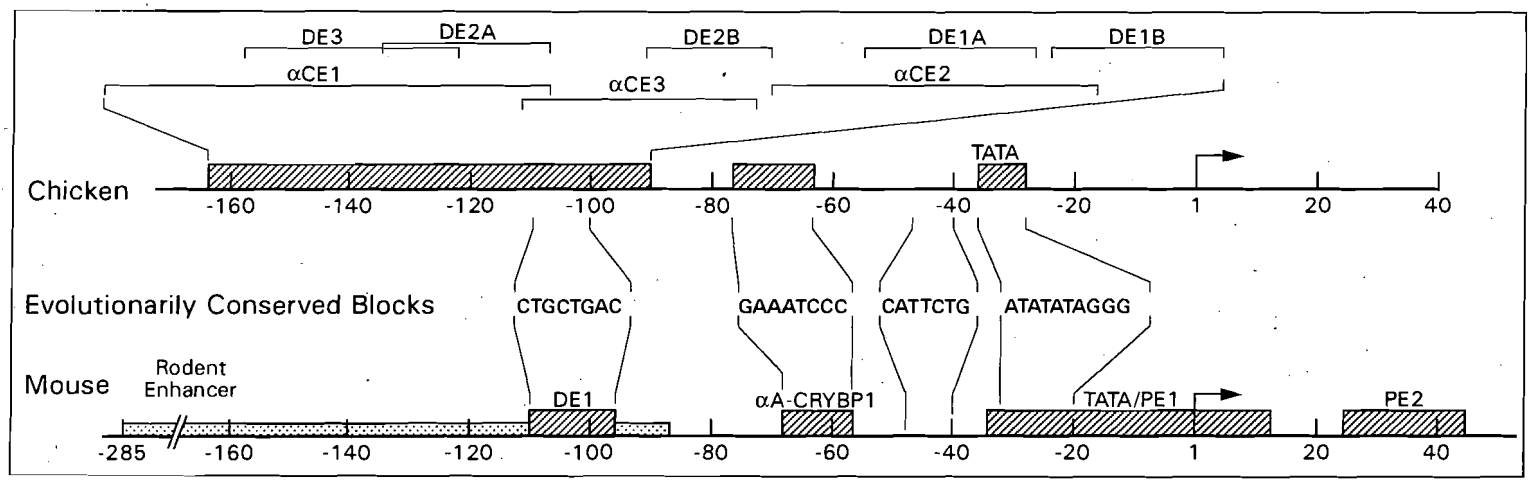

Fig. 5.9. The promoter regions of chicken and mouse αA-crystallin genes. Shaded regions were defined by protection and mutational assays. Sequences evolutionarily conserved among αA-crystallin promoters are also shown. Adapted from Wistow G, Richardson J, Jaworski C, Graham C, Sharon-Friling R, Segovia L. Crystallins, In: Tombs MP, ed. Biotechnology and Genetic Engineering Reviews. v. 12. Andover, Hants: Intercept Ltd, 1994; 1-38.

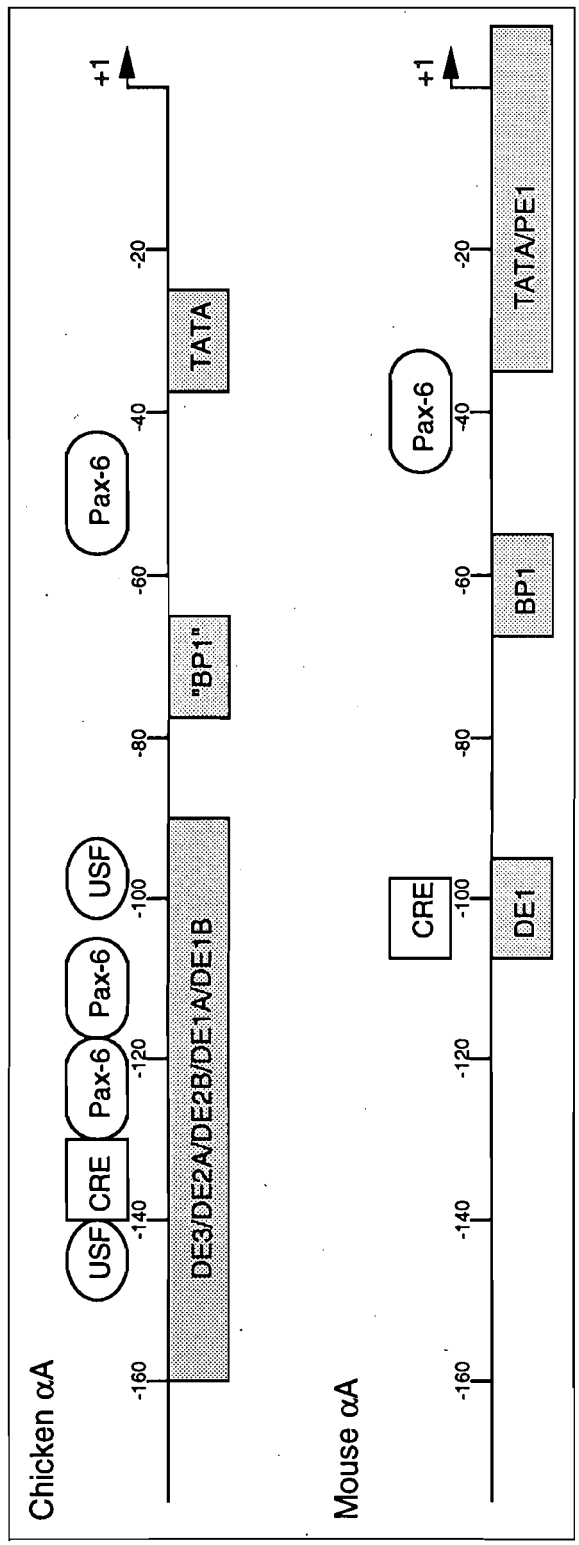

Fig. 5.10. Factor binding sites identified with lens-specific expression of αA-crystallin promoters. BP1 represents the αA-CRYBP1 binding site in mouse. A similar site in chicken is shown as "BP1."

element PE-2 (+24/+43).[90] The PE1 and PE2 sequences are conserved among mammals but not in chicken.[84,90] The PE1 region has been further defined into the TATA box (-31/-26) and the PE1B sequence (-25/-12).[95] Deletions within either element eliminated promoter activity in transient transfections of lens-derived cells but did not eliminate activity in transgenic mice.[95] DNase I footprinting and EMSA analyses showed similar patterns of protein binding in the region in both lens and fibroblast extracts although there was no evidence that TATA-binding protein (TBP) was present in any complex.[95]

αA-CRYBP1

The originally defined proximal element contains an evolutionarily conserved sequence designated motif D[84] which is similar to binding sites of the NFkB family.[52] A factor which binds this site was cloned from αTN4-1 cells and named αA-CRYBP1.[96] This protein, which appears to be the mouse homologue of a human transcription factor called PRDII-BF or MBP-1 which is involved in expression of immune response genes,[97,98] binds the sequence GGGAAATCCC at positions -66/-57 in the mouse promoter. Mutation in the αA-CRYBP1 region of the mouse gene reduces but does not eliminate promoter activity in PLEs and in transfected αTN4-1 cells.[96,99] However the significance of this factor for lens expression in vivo is not clear. αA-CRYBP1 is essentially ubiquitous in its tissue distribution.[96] The enhancer activity of the αA-CRYBP1 site is quite low in transfection studies and is apparent only in αTN4-1 cells in which the αA-CRYBP1 factor is abundant, although multimers of the site can activate expression of a heterologous promoter in a variety of other cells.[100] Furthermore, although αA-CRYBP1 binds a site in the mouse gene promoter it does not bind the equivalent conserved sites in the chicken, mole rat and human promoters which differ from the mouse sequence by only one base.[84,86,100] It thus seems possible that a different factor binds motif D in vivo for normal lens expression and that αA-CRYBP1 has a more general role.

Pax-6

Recently a binding site for Pax-6 has been identified just downstream of the motif D/αA-CRYBP1 site at position -49/-33 in the mouse αA-crystallin promoter[17] (Fig. 5.10). Part, but not all, of this region is well conserved among species and as described below there is evidence that the equivalent region of the chicken αA-crystallin promoter is also able to bind Pax-6.[16] The Pax-6 binding site is flanked by the αA-CRYBP1 and TATA/PE1 elements but mutational and binding analyses of this region had not previously identified this sequence itself as a discrete element in the mouse αA-crystallin promoter.[90]

Cooperativity and Redundancy

One theme which has emerged from studies of the mouse αA-crystallin promoter is that of cooperative binding at multiple elements and functional redundancy in vivo. For example, although both proximal and distal elements were found to be essential for expression in PLEs[91] the -88/+44 promoter construct which lacks the distal element showed lens-specific expression in transgenic mice.[101] This observation has been refined to show that the DE1 and αA-CRYBP1 sites are functionally redundant in transgenic mouse experiments.[101] The reason for the discrepancy between PLE and transgenic mouse studies is not known.

Binding Sites in the Chicken αA-crystallin Promoter

The promoter of the chicken αA-crystallin gene contains evolutionarily conserved sequences which corresponds to the DE1 element of the mouse αA-crystallin promoter (Fig. 5.9). Different laboratories have defined these sequence elements either as DE1A and DE1B[90,102] or as αCE2.[103] Surprisingly, in spite of the conservation of sequence, the mouse DE1 sequence failed to compete with chicken DE1A/B for binding of chicken nuclear extracts in EMSA[102] suggesting that mouse and chicken genes may bind different factors or that there is species specificity in binding of common factors. Upstream of the DE1A,B/αCE2 elements is another set of elements known as DE3 and DE2A[90,102] or as αCE1.[103] The αCE2 element requires the presence of αCE1 for activity[103] suggesting a cooperative interaction. A binding activity specific to the αCE1 element has been identified.[104,105]

Pax-6 and General Factors

Recent work has examined the binding of known factors whose consensus binding sites resemble sites in the chicken αA-crystallin promoter.[16] This has resulted in a picture of a complex array of general and tissue-restricted factors showing differential binding to the functional elements of the chicken αA-crystallin promoter in lens and non-lens extracts (Fig. 5.10). In lens extracts the general factor USF (upstream factor) is able to bind a non-canonical E-box in the DE2A element while cAMP-response element (CRE) binding factors bind immediately downstream. In fibroblast extracts the same sites bind USF and AP1. In both lens and fibroblasts USF complexed with an unknown protein binds to the DE1B site. These studies also showed that another element DE2B together with DE1A and a previously unidentified downstream site at -57/-41 were able to bind Pax-6. Of these, the -57/-41 site shows the closest match to the in vitro binding site for the paired domain (PD) of Pax-6[39] and corresponds to the single Pax-6 binding site identified in the mouse αA-crystallin promoter[17] (Fig. 5.10). Surprisingly, as in the mouse gene, this consensus Pax-6 site was not previously identified as a discrete element in functional or binding studies of the chicken gene.[90]

Both mouse and chicken αA-crystallin promoters bind complex arrays of general factors which may all be important for function. In spite of their similarities in sequence and in patterns of expression there seem to be considerable differences in the detailed molecular mechanisms of the two promoters. These differences probably result from their relative antiquity. Over time these genes have added complexity and redundancy to their transcriptional machinery to fine tune their patterns of expression in different species.

αB-CRYSTALLIN

If αA-crystallin is essentially specific to lens in its expression, the same cannot be said for αB-crystallin.[106-109] This gene seems to retain more of the non-lens expression of the sHSP family ancestor of this family, just as in protein sequence αB-crystallin is closer than αA-crystallin to sHSPs, p20 and the mysterious *C.elegans* sequence as discussed in chapter 3. In many adult tissues of rodents and man αB-crystallin is expressed constitutively.[90,110] It may also be induced by a variety of stresses, such as heat and osmotic shock in cultured mammalian cells and in various disease states.[90,110] In birds too, αB-crystallin mRNA is present in non-lens tissues in hatched ducks, although non-lens expression was undetectable by Northern blot in embryonic tissues.[81,111] However in contrast to similar cultured mammalian cells (NIH 3T3 cells) duck embryonic fibroblasts have no constitutive expression of αB-crystallin mRNA, nor is there any inducibility by either heat or osmotic stress.[112] It has been suggested that these phenomena may be linked and that some or all of the constitutive expression of αB-crystallin mRNA in mammalian cells in culture is due to the stresses of culture.[112]

The combination of several modes of expression contributes to a complex promoter structure in mammalian αB-crystallin genes. The maintenance of clusters of elements for different functions may also be responsible for bestowing much higher conservation of sequence in αB-crystallin promoters of mammals than is found in αA-crystallin genes of the same species. However this high degree of conservation does not extend to the promoter of the duck gene for αB-crystallin, and phylogenetic footprinting of homologous mammalian and avian aB-crystallin genes reveals only a few discrete islands of similarity[112] (Fig. 5.11). This probably reflects the taxon-specific differences in expression patterns of this gene. It seems that in birds αB-crystallin expression has specialized further than in mammals and what may have been the ancestral condition of stress-inducibility has been lost.

Multiple Transcripts

In mouse lens, heart, skeletal muscle and kidney there is a single major transcription start site downstream of a TATA box in the αB-crystallin gene promoter.[108] In addition, heart and skeletal muscle make use of a minor start site between 40 to 50 bp upstream of the major

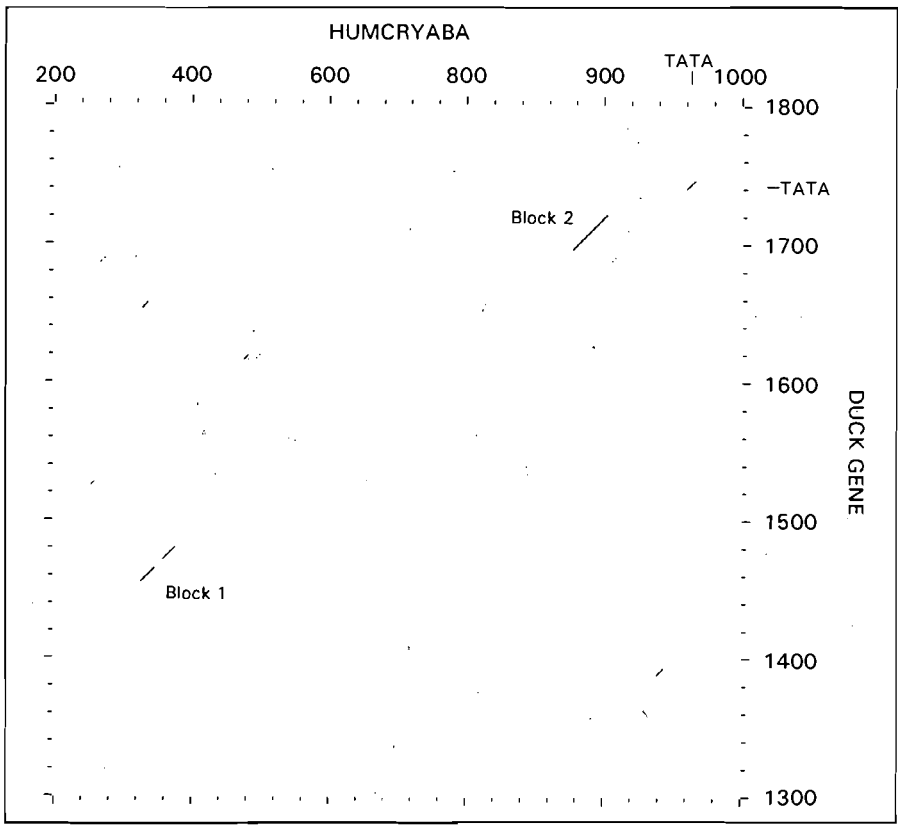

Fig. 5.11. Comparison of promoter regions of duck and human (GenBank entry HUMCRYABA) αB-crystallin genes. Comparison with the equivalent region of the mouse gene gives a very similar result.[112] Block 1, Block 2 and TATA mark the discrete regions of sequence similarity between bird and mammal genes.

site.[108] However, there is also evidence for much longer transcripts in some mammalian tissues which appear to start as much as 474 bp upstream of the major start site.[113] These long transcripts are apparently the predominant form, albeit at low levels, in lung, brain and spleen.[108,113,114] However it has recently been suggested that the far upstream transcripts in mammals are not polyadenylated mRNAs.[115] Furthermore, in duck the major mRNA for αB-crystallin in lung is the same size as in other tissues.[111] Birds do have a long form of αB-crystallin mRNA but this has only been observed in lens, perhaps because of the greater abundance of αB-crystallin in that tissue.[111,116] This longer mRNA arises from use of a downstream alternative site for polyadenylation at the 3' end of the gene.[111,112,117] The major upstream site lies in sequences capable of forming a hairpin loop in RNA transcripts and it has been suggested that this may occasionally hide the site during processing thereby promoting use of the downstream sequence.[111]

Promoter Elements

Although there has been some functional analysis of the human αB-crystallin gene[118] most work has focused on the mouse gene. In transgenic mice the -661/+44 region of the mouse gene promoter was able to direct reporter gene expression in lens and skeletal muscle even when present as a single copy.[119] For detectable expression in heart and other tissues multiple copies of the transgene were required. Transient transfection of deletion mutants showed that -426/-257 contains an enhancer necessary for expression in myotubes while sequences downstream of -115 were essential for lens expression.[119] Binding studies using DNase I protection defined four sites in the enhancer. αBE-1 (-407/-397), αBE-2 (-360/-327) and αBE-3 (-317/-306) were protected by extracts of both myotubes and TN4-1 cells while the fourth region, MRF (-300/-288) which contains a consensus E-box sequence was protected only in myotube extract[120] (Fig. 5.12). In muscle, the enhancer responded to activation by MyoD and myogenin binding through the E-box. In contrast to previous results showing that -115/+44 was essential for lens expression, DNase I protection using αTN4-1 extract, mutagenesis and transient transfection experiments defined -147/-118 as the lens-specific region (LSR).[121]

Most of these sequences are also well conserved in the human gene but in spite of a high degree of general conservation the human gene lacks the E-box of the mouse MRF region (Fig. 5.12). To gain a wider perspective on which elements are well conserved, the mammalian promoters were compared to a more distantly related homologue, the duck αB-crystallin gene[112] (Fig. 5.12). Only the αBE-2 site, which contains a consensus GATA-factor binding sequence[52] was conserved as part of Block 1. However, in contrast to the human gene, the duck gene has an identical E-box in the same general region as the mouse MRF although flanking sequences are not conserved. Other defined elements are much less well conserved. However there is a strikingly well conserved (27/33 identical) block of sequence, Block 2, just upstream of the TATA box in all three genes which lies within the -115/+44 construct originally identified as essential for lens expression. This seems to be a good candidate for an element important in lens preference. Whether Block 2 binds Pax-6 or other lens-preferred factors remains to be seen, although it does not closely resemble known high-affinity Pax-6 sites.

Since αB-crystallin is induced by heat-shock in NIH 3T3 cells it was not surprising that a perfect consensus heat-shock response element (HSRE) (alternative triplet repeats of NGAAN and its complement) is present at positions -53 to -39 in both mouse and human genes[122] (Fig. 5.12). However these sequences and other putative HSREs are absent from the duck gene promoter and accordingly the duck αB-crystallin gene lacks the stress responses exhibited by its mammalian homologues.[112]

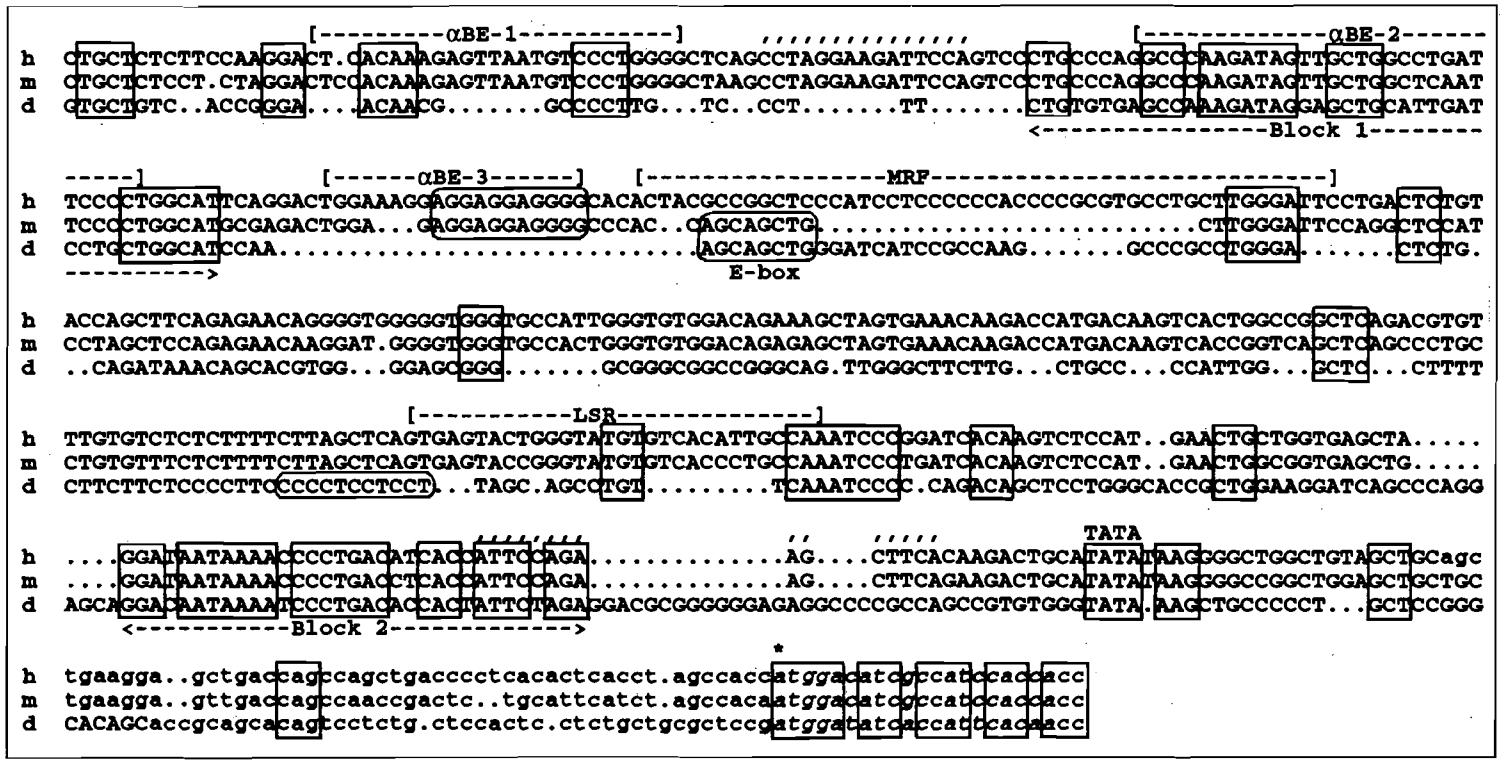

Fig. 5.12. Sequence alignment of promoter regions of human, mouse and duck αB-crystallin genes. αBE-1, αBE-2, αBE-3, MRF, E-box and LSR show functionally defined regions from the mouse promoter. Block 1 and Block 2 show regions of conservation from dot matrix analysis. Putative heat shock response elements are shown by tick marks above the sequences. Transcribed sequences are shown in lower case and protein coding sequence in italics. An asterisk indicates the position of the initiator methionine. Square edged boxes show at least three consecutive bases identical in all three sequences. The E-box which is conserved between mouse and duck and the αBE-3 motif which is present in complement downstream in the duck promoter are shown in round-edged boxes. Reprinted with permission from Wistow G, Graham C. Biochim Biophys Acta 1995: in press.

β-Crystallins

The expression of this multigene family has not been studied with the same intensity as that of other ubiquitous crystallins. β-Crystallins are highly lens-preferred, although it has now been found that detectable levels of various β-crystallin polypeptides are present in a number of non-lens tissues of various developmental origins, including retina, brain, liver and kidney.[123] This means that the promoters for these genes may, like that of αB-crystallin, contain element for expression in different tissues. Members of the family also show differential expression during development and differentiation in the lens adding another level of complexity to studies of their transcriptional regulation.[12,124-126]

Although studies of β-crystallin gene expression are at a relatively early stage, there has been some promoter analysis for two genes from chicken. 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 PLEs than in muscle fibroblasts or HeLa cells.[127] 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. Both -2448/+30 and -434/+30 promoter constructs were lens-specific in transgenic mice[128] providing another example of promoter recognition across species.

Two β-crystallin polypeptides, βA3- and βA1-crystallins, are encoded by a single gene in both mammals and birds.[129,130] A promoter construct containing the fragment -382/+22 of the chicken gene promotes expression of the CAT reporter gene in chicken PLEs but not in dermal fibroblasts.[130] Deletion of sequences between -382 and -143 greatly reduces promoter activity. Other than a putative AP-1 site in chicken βA3/A1-crystallin,[130] no transcription factors have yet been identified for β-crystallin genes.

γ-Crystallins

In contrast to other ubiquitous crystallins, the embryonic γ-crystallins of mammals show no evidence of non-lens expression. Everything about their function and evolutionary history suggests that they have been the most highly specialized lens proteins from a very early stage in vertebrate evolution. Although the same thing has been assumed and proved wrong about one crystallin after another, at this point in our understanding γ-crystallins are lens-specific. Furthermore unlike α-crystallins, for example, γ-crystallins are expressed in only one cell type, differentiated fiber cells. The only variability in their expression is a pattern of developmental regulation.[7-11,131] In view of this, it is not surprising that γ-crystallin gene promoters are generally well conserved both among family members and between species.[132] All have a block of similar sequence extending about 90 bp upstream of the transcription start site.

One challenge in studying these genes is that their natural cellular background, the differentiated fiber cell, is not amenable to transient transfection or other cell culture methods. Instead, researchers have been obliged to make use of other systems. The most apparently heterologous of these is the chicken primary lens epithelial explant (PLE) system. Since γ-crystallins are fiber cell specific and since embryonic γ-crystallins are not even present in the chicken this would appear to be a very different environment for these genes. Nevertheless important data have been garnered from these experiments and confirmed in transgenic mouse studies. Presumably some population of cells derived from chicken PLEs acquires fiber cell-like character in culture and this transcriptional environment is evolutionarily conserved with fiber cells of mice.

In mammals, there are six tightly clustered γ-crystallin genes with standardized names γA-γF.[133-136] 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. In rodents all six γ-crystallins are induced as elongating fiber cells form in the embryonic lens. After birth their expression decreases differentially until only γB-crystallin transcripts are detectable in the adult rat lens.[11,137]

The expression of the mouse γF-crystallin gene (formerly designated γ2-crystallin) has been examined in detail in chicken PLEs.[138] In this system the γF-crystallin gene was shown to have a lens-specific promoter consisting of two upstream enhancer-like elements and a proximal promoter[94] (Fig. 5.13). The enhancer elements were successfully substituted by similar regions of the hamster αA-crystallin gene. When these results were extended to studies in transgenic mice it was found that at early stages in lens development either the enhancers or the proximal promoter could direct gene expression, while later in development cooperation between these elements was required for expression in fiber cells.[139]

The proximal promoter of the γF gene contains a 23 bp element, γF-1, which when multimerized can direct reporter gene expression to lens fiber cells and to hindbrain.[139,140] A factor capable of binding the γF-1 site was cloned from chicken brain and named γFBP.[141] γFBP is a zinc finger protein expressed in the sclerotome during early somitogenesis. Its transcripts undergo alternative splicing and one variant form expressed in lens with developmental regulation acts as a transcriptional repressor. It was suggested that γFBP has a role in regulation of γF-crystallin expression and in sclerotome differentiation.[141]

It was also noticed that the mouse γF-crystallin gene enhancer region contained an "everted repeat" which resembled retinoic acid response elements (RARE) (Fig. 5.13). This was investigated and it was found that expression of the γF-crystallin promoter in PLEs was enhanced by retinoic acid, a potent inducer of cell differentiation. The γF-crystallin element was designated as a novel type of RARE and named

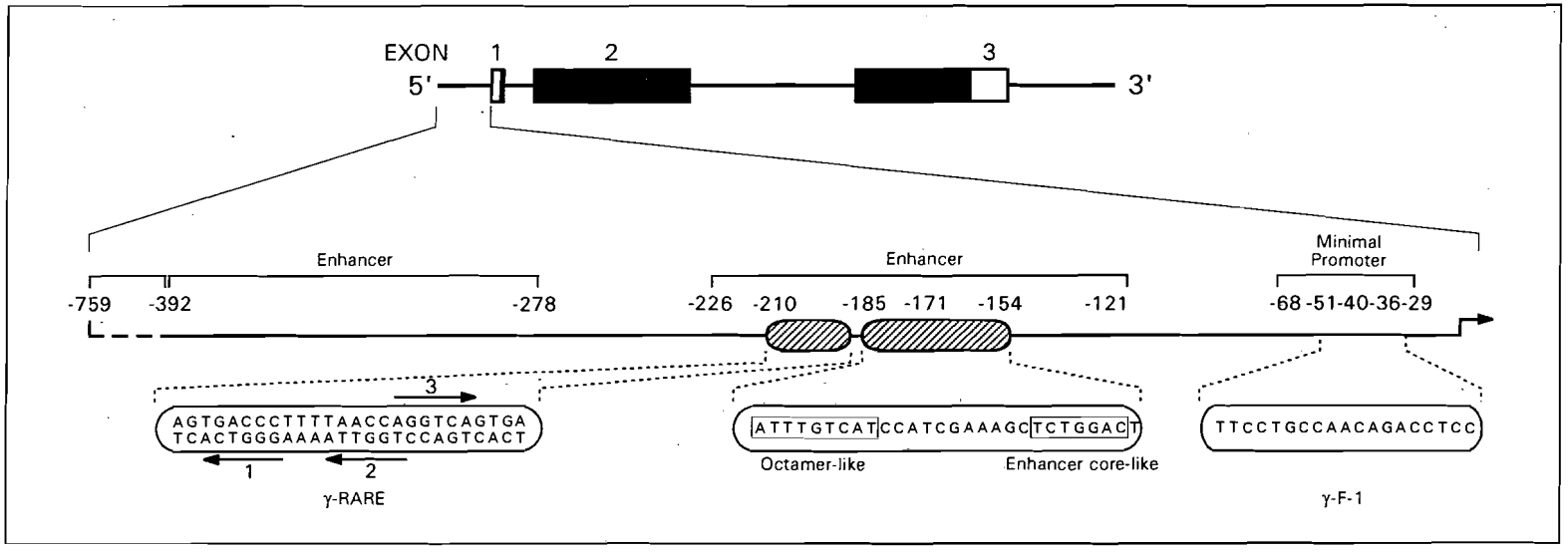

Fig. 5.13. Promoter region of the mouse γF-crystallin gene.

γF-RARE.[142] This element has subsequently been shown to bind heterodimers of the thyroid hormone T3 receptor with either RXR or RAR retinoic acid receptors.[143] Retinoic acid binding proteins seem to have an important role in lens cell differentiation since expression of cellular retinoic acid-binding protein I (CRAB-I)[144] or retinoic acid receptor,[145] under control of the mouse αA-crystallin promoter disrupts fiber cell differentiation in transgenic mice. The CRAB-I transgenic mice also exhibited tumorigenesis in pancreas[144] which is of interest since the important lens transcription factor Pax-6 is also expressed in pancreas.[82]

The other γ-crystallin gene which has been studied intensively is rat γD-crystallin. The promoter was studied by transient transfection into primary mouse lens epithelial cells or into another system, chicken neural retina cells, which can be induced to transdifferentiate into lens-like cells in culture.[146] Functional analyses showed that the γD-crystallin promoter contained at least three important elements but with different requirements for these elements in the two cultured cell systems.[146] The presence of a non-lens silencer was also suggested.[147] Corresponding to the mouse γF-1 site, a 12 nucleotide sequence in the rat γD gene immediately upstream of the TATA box 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.[147] The rat γD- and mouse γF-crystallin promoters were also used to express the CAT reporter gene in transgenic *X.laevis* tadpoles, another impressive illustration of the conservation of lens recognition through evolution.[6]

SOX Proteins and γ-Crystallins

Very recently γF-crystallin has joined the group of crystallins whose expression seems to involve transcriptional activation by the products of pattern forming genes. Just as SOX-2 was found to be able to increase lens-specific activity of the δ1-crystallin enhancer, it was also shown to be essential for lens-specific expression of γF-crystallin.[19] This is an important observation since γ-crystallins are strictly specific to lens fiber cells while Pax-6 is expressed in lens epithelial cells.[15] While genes which are activated in the epithelia may be under the control of Pax-6 itself, fiber-specific genes might be expected to be regulated by other factors which could be downstream of Pax-6 in the eye cascade and in lens differentiation.

TRANSGENICS

Crystallin gene promoters have been used to direct expression of foreign genes to the transgenic mouse lens. As discussed above, some of these experiments were principally concerned with in vivo functional analysis of the promoters themselves, using the bacterial genes chloramphenicol acetyltransferase (CAT) or β-galactosidase (lacZ) as reporters. However crystallin promoters have also been used to express

a variety of oncogenes, viral proteins and toxins in lens to investigate processes of differentiation and development. In many cases this directed expression has made it possible to seriously disrupt the lens without compromising viability.

The most widely used promoter for these experiments has been the extensively characterized and strongly lens-preferred mouse αA-crystallin gene promoter. The -366/+45 fragment of this promoter has been used to transform lens cells with SV40 large T antigen[148] and polyoma virus large T antigen.[149] While SV40 large T was oncogenic in lens polyoma large T was not. The dbl oncogene[150] and human papilloma virus type 16 E6 and E7 oncogenes[151,152] have been used to disrupt lens differentiation. A fusion of retinoic acid receptor-α and lacZ,[145] cellular retinoic acid-binding protein I,[144] human or murine urokinase-type plasminogen activator (uPA)[153] and γ-interferon[154] have all been targeted to mouse lens also resulting in failure of fiber cell differentiation. Human immunodeficiency virus TAT protein has been expressed by the mouse αA-crystallin promoter and has been able to transactivate expression from the HIV long terminal repeat in double transgenics.[155]

The toxins diphtheria toxin A (DT-A)[156] and ricin A[157] under the control of the mouse αA-crystallin promoter have been used to ablate transgenic mouse lens cells resulting in microphthalmia. DT-A expression can completely eliminate lens cells while transgenic mice expressing ricin apparently retain some lens cells. The more complete ablation with DT-A may result from the ability of the toxin to be released from expressing cells and to kill neighboring cells. The FGF (fibroblast growth factor) family members FGF-3/Int-2[158] and aFGF[159] have also been expressed in lens. Lens development was again disrupted. In particular a secreted form of aFGF was able to induce differentiation in lens epithelial cells although a non-secreted form had no effect.[159] These results, together with those comparing DT-A and ricin expression[156,157] suggest that in transgenic animals the -366/+45 mouse αA-crystallin promoter may not be expressed at high levels in epithelial cells.

The -347/+43 fragment of the hamster αA-crystallin promoter has also been used to express CAT in transgenic mouse lens and to ablate cells with DT-A.[94,160] The DT-A lens ablation results are similar to those in mouse, with microphthalmia, loss of lens and several surrounding lens tissues.

The -759/+45 fragment of the mouse γF-crystallin promoter has been used to direct expression of CAT, lacZ, DT-A and SV40 T-antigen to the fiber cells of transgenic mice.[9,94,139,161] A large fragment of the chicken δ1-crystallin promoter (-2200/+51) directed expression of the xanthine-guanine phosphoribosyl transferase gene primarily to the lens of chimeric transgenic mice.[162]

The promoter for a mammalian enzyme crystallin, guinea pig ζ-crystallin, also shows clear lens preference in transgenic mice.[51] Since the endogenous ζ-crystallin gene is expressed at high levels in lens

epithelia[15] this promoter offers the potential to target higher expression of transgenes to epithelial cells than has sometimes been possible with αA-crystallin constructs.

A powerful new technique in transgenic research involves the use of sequence specific prokaryotic DNA recombinases, such as Cre.[163] When directed to a specific tissue the Cre recombinase can be used to splice other transgenic recombinant sequences to activate or inactivate another transgene carrying the recombinase recognition sequence. Already this system has been used to produce targeted activation of the oncogenic SV40 T antigen in lens.[164,165] In the future, homologous recombination could be used to replace a gene with an engineered copy containing recombinase sites which could then be specifically spliced out in the lens by tissue-specific expression of Cre.

POST-TRANSCRIPTIONAL CONTROL

Most studies have concentrated on transcriptional mechanisms for tissue-specific gene expression of crystallins and these have been quite successful. However they may not tell the whole story. For example, although crystallin promoters confer lens-specific expression in transgenic mice, the level of expression of reporter genes does not seem to be comparable to that of the crystallin itself. While this could be due to the absence from the recombinant constructs of enhancers or other positive elements present in the complete gene, it is also possible that post-transcriptional events are also important in high level crystallin expression. These could include enhanced crystallin mRNA stability, specific mechanisms for processing or translation of crystallin mRNA or enhanced crystallin protein stability.

Evidence for such mechanisms comes from observations of a marked discordance between levels of mRNA and protein for αA- and αB-crystallins. In rat lens it was found that the overall level of mRNA for αB was higher than that for αA, the reverse of the relative abundances at the protein level.[12] In the same experiments mRNA for βB2-crystallin was also found to be relatively overrepresented. When rat lens epithelial cells were induced to differentiate in culture using bFGF it was found that crystallin genes were induced at specific time points in the order α, β, γ.[166] This corresponds well to the pattern of crystallin expression in the lens, with γ-crystallins being the most fiber-specific and α-crystallins the least. The mRNAs were stable for several days in culture then all disappeared. It was suggested that bFGF caused a pulse of gene expression for each class of gene. Crystallin mRNAs were then stable until removed by a differentiation specific mechanism.[166] Since crystallin mRNA in the lens is apparently very stable there would be no need for continual gene expression to provide for continuing protein synthesis.

The possibility of a burst of protein synthesis of a taxon-specific crystallin has been suggested by immunochemical localization of ζ-crystallin in adult guinea pig lens.[15] The crystallin was detected in

cytosol in epithelial cells and in cortical fibers, but staining was markedly more intense in the newly elongating fiber cells of the equatorial region. Whether by transcriptional or post-transcriptional mechanisms it makes sense to see an increase in crystallin synthesis as fiber cells elongate and the demand for protein in a single cell increases enormously. Indeed such boosted protein synthesis could contribute to cell elongation in several ways. Increased protein concentrations could contribute to an osmotic pressure gradient increasing the inflow of water and increasing cell volume. Certain crystallins could also participate in elaboration and organization of cytoskeleton for the elongating cell.

SUMMARY

The high expression of crystallins in the lens is mainly the result of tissue-specific transcriptional activation. Specificity results from the interplay of lens-preferred factors including Pax-6 and the products of other pattern-forming genes including SOX-2, together with general factors. The recruitment of crystallin genes occurs through the acquisition of binding sites for these factors.

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