I began to study the transparent eye lens of the chicken as a postdoctoral fellow in the laboratory of Dr. Alfred J. Coulombre at the National Institutes of Health in 1967, because of the many special features making this tissue advantageous for investigating cellular differentiation in eukaryotic cells.\(^1\)\(^2\) The vertebrate lens is an avascular, non-innervated epithelial tissue physically surrounded by a capsule (Fig. 1). Lens cell differentiation involves the cessation of cell division, marked cell elongation during fiber cell formation, and loss of cellular organelles, including the cell nucleus (reviewed elsewhere\(^3\)). Of particular interest to me was that a group of specialized proteins (crystallins) could be recognized as markers for specific gene expression during lens differentiation. Little did I realize that I was embarking on a winding journey that would trace poorly defined lens crystallins through to the regulatory elements controlling the expression of their genes, and that traveling this path would consider, simultaneously, evolutionary and developmental time scales. Jonas Friedenwald predicted such a course for scientific research already in 1922 at the beginning of his career, when he wrote:

> The road of science is a tortuous one, that twists and turns and not infrequently crosses some of the most ancient footpaths.\(^4\)

When I started my postdoctoral work, it was known that crystallins appear already when the presumptive lens cells are induced by the optic vesicle, and that the crystallins accumulate during development until they constitute the major soluble proteins of the lens.\(^5\)\(^8\) Moreover, it was becoming evident that synthesis of different crystallins was temporally and spatially regulated in the developing lens.\(^7\)\(^9\)\(^11\) However, it was also evident that, despite the potential usefulness of the lens for studying the regulation of gene expression during differentiation, we still knew too little about the crystallins to explore the molecular basis for their synthesis. Studies were revealing a growing number of crystallins containing numerous polypeptides,\(^2\)\(^10\)\(^13\)\(^18\) and identification was often based on immunological criteria alone, without distinction between primary gene products and their post-translationally modified forms (reviewed elsewhere\(^19\)\(^20\)). Thus, the crystallins had to be defined at the level of their mRNAs and genes before it would be possible to investigate their regulated expression during development.

Here, I will summarize how combined studies using the techniques of protein sequencing, X-ray crystallography, and recombinant DNA have advanced our understanding of the crystallins. I will then present the progress that has occurred on our understanding of crystallin gene expression during development, and show how these studies have initiated, astonishingly quickly, genetic engineering in the visual system.

**Crystallin Heterogeneity**

It was known as early as 1894 that the lens contains high concentrations of heterogeneous, structural proteins.\(^21\) Numerous studies since then have established that there are four immunologically distinct, major classes of crystallins (\(\alpha\), \(\beta\), \(\gamma\), and \(\delta\)). Reviews covering the \(\alpha\)-, \(\beta\)- and \(\gamma\)-crystallins\(^20\)\(^22\) and \(\delta\)-crystallin\(^23\) have been written. There are also a small number of so-called minor crystallins in vertebrates, such as \(\epsilon\)-crystallin\(^24\)\(^26\) and \(\tau\)-crystallin,\(^27\)\(^30\) among others.\(^31\)

The crystallins are not distributed uniformly throughout the vertebrates (Fig. 2). All vertebrate lenses contain \(\alpha\)- and \(\beta\)-crystallins. \(\delta\)-Crystallin, however, is present only in lenses of birds and reptiles. \(\gamma\)-Crystallin is found in fish, amphibians and mammals, but is absent from birds and reptiles. The physiological significance of having \(\delta\)-crystallin substitute for \(\gamma\)-crystallin in birds and reptiles is not known. It may be related to its greater hydration or \(\alpha\)-helical content, which may aid accommodation in these soft lenses (reviewed elsewhere\(^23\)\(^22\)). There are other differences between \(\gamma\)- and \(\delta\)-crystallin, as described in the reviews cited above.
HETEROGENEITY OF LENS CRYSTALLINS:
SDS-PAGE

Fig. 1. A sagittal paraffin section of a 5-day-old embryonic chicken lens. The cornea is present at the top of the photograph. The cuboidal epithelial cells overlie the elongated fiber cells. The cell nuclei will disintegrate in the central region of fiber mass as the lens matures. This photograph was made from a slide given to me by Dr. A. J. Coulombre in 1968.

which may also satisfy specific physiological needs of the different species.

Each crystallin class has characteristic native molecular weights, and, thus, can be separated by gel filtration. The approximate molecular weights are 800,000 for α-crystallin, 100–200,000 for β-crystallin, 20,000 for γ-crystallin, and 200,000 for δ-crystallin. Subsequent reduction and electrophoresis of the purified crystallins in a denaturing polyacrylamide gel (Fig. 3) demonstrate that each crystallin class is composed of several polypeptides with molecular weights ranging from 20,000 (α and γ) to 50,000 (δ). The γ-crystallins can be further fractionated into a series of extremely similar polypeptides by isoelectric focusing. Thus, a major cause for the native molecular weight differences among crystallins is the aggregation state of their polypeptides.

Extensive post-translational modifications and changes occurring with age further complicate the identification of the different primary crystallin polypeptides. However, the heterogeneity shown in Figure 3 can be achieved by in vitro translation of lens mRNAs. This suggests that there is a separate gene for each crystallin polypeptide. As we shall see below, while this conclusion is essentially correct, there are some surprises.

Crystallin Genes

Chicken δ-crystallin was the first crystallin cDNA to be cloned. This consequently led to our first glimpse of a crystallin gene. cDNA-DNA hybridization
that there are probably only two chicken \( \delta \)-crystallin genes. Examination of cloned fragments of these genes in the electron microscope revealed that they are very complex.41-42 Our first photomicrograph of a heteroduplex showed a minimum of seven introns in one of the cloned \( \delta \)-crystallin gene fragments (Fig. 4). The complicated structure of the \( \delta \)-crystallin gene raised immediate questions concerning the nature of the introns and whether all crystallin genes have numerous introns.

We subsequently cloned and characterized cDNAs for \( \alpha \)-, \( \beta \)-, and \( \gamma \)-crystallins35,49 of the murine lens in order to examine other crystallin genes. In the last few years, other cloned crystallin cDNAs have been constructed.50-64 Many of these have been indispensable for characterizing crystallin genes in different species.65-78 A schematic example of gene structure from each crystallin family is illustrated in Figure 5. Inspection of Figure 5 shows that the representative member of each crystallin class has a different gene structure, with the \( \delta \)-crystallin gene being much more complex than the \( \alpha \)-, \( \beta \)-, or \( \gamma \)-crystallin gene. The references cited above show that all members of a crystallin class have similar structures which have been conserved throughout evolution (see Piatigorsky78 for brief review).

Fig. 4. Electron micrograph showing a heteroduplex generated by hybridizing the cloned chicken \( \delta \)-crystallin genomic fragment gSCr-1 with \( \delta \)-crystallin mRNA.41 The arrows denote the introns which are seen as loops between the hybridized exons. The small stretch of 5' \( \delta \)-crystallin mRNA contains sequences derived from exons 1 and 2 which did not hybridize to the cloned gene fragment; the non-hybridized 3' half of \( \delta \)-crystallin mRNA is seen extending from the cloned DNA since these sequences are not present in gSCr-1. The introns have been labeled C through J to fit our present knowledge of the 61 gene structure.35 The long stretch of double-stranded DNA contiguous with the 5' region of the 61-crystallin gene fragment belongs to a second DNA fragment not containing \( \delta \)-crystallin sequences, that was cloned in the same Charon 4A recombinant bacteriophage.41 This is the first electron photomicrograph taken of a crystallin gene in the project subsequently reported by Bhat and coworkers.41

Relationship Between Protein and Gene Structures For the \( \beta \)- and \( \gamma \)-Crystallins

Investigations from Blundell's, Bloemendal's, and my laboratory have converged to link gene structure with protein structure in the \( \beta \)- and \( \gamma \)-crystallin families. X-ray crystallographic studies from Blundell's laboratory showed that \( \gamma \)-crystallin from the calf lens contains two domains that are joined by a connecting peptide, and that each domain is composed of two very similar structural motifs.79,80 The structural motifs were called "Greek keys," due to their resemblance to the decorations on Greek urns. The \( \gamma \)-crystallin polypeptide showed the highest internal symmetry of any protein examined previously by X-ray crystallography.

Another important finding was from Bloemendal's laboratory. They demonstrated an unexpected sequence similarity between calf \( \gamma \)-II and \( \beta \)-Bp (\( \beta \)-B2) from the bovine lens.81,82 Moreover, both the \( \gamma \)-II and \( \beta \)-Bp sequence have an internal repeat corresponding to the two domains of the tertiary structure of \( \gamma \)-II, and each repeat is itself composed of an internal repeat sequence corresponding to the two structural motifs of each domain. Subsequent studies involving protein83 and cDNA84 sequencing have demonstrated that the similarities between and internal duplications within the primary structures of the \( \beta \)- and \( \gamma \)-crystallins polypeptides extend to the other members of these two families of proteins. It is interesting to note that the greatest variation in sequence among the different \( \gamma \)-crystallins is found in the third motif.85,86 These results indicated that the \( \beta \)- and \( \gamma \)-crystallins are evolutionarily related and form a \( \beta \)-\( \gamma \)-superfamily of proteins. Further discussion of the structure of the \( \beta \)-\( \gamma \)-crystallins can be found elsewhere.80,87
Structures of β- and γ-crystallin polypeptides.

The paths of the polypeptide chains are traced by joining the positions of consecutive Cα atoms. The structure of γII is taken from the coordinates determined by X-ray crystallography at 2.6 Å resolution. The molecule is viewed from a direction perpendicular to the pseudo-dyad which relates the N- and C-terminal domains, emphasizing the intra-molecular symmetry. The model of βBp (βB2) is based on the same coordinate set, modified by interactive computer graphics. The model has been rotated slightly, relative to the view of γII, in order to show the N- and C-terminal "arms." This conformation is that predicted for the "extended" model for βBp dimerization.

I am grateful to Dr. G. J. Wistow for help in preparing this figure.

Since the three-dimensional structure of γII-crystallin was known and a sequence similarity between γII and βBp had been demonstrated, it was possible to predict the structure of the βBp polypeptide by an interactive computer graphics program. As shown in Figure 6, the predicted structure of βBp is extremely similar to that of γII, except that βBp has both an N- and C-terminal peptide extending from the two domain core of the protein. With exception of the βs polypeptide, which could as well be considered a member of the γ-crystallins, all β-crystallin polypeptides appear to have arms extending from their N- and (sometimes) C-terminals. It has been suggested that the terminal extensions of the β-crystallin polypeptides play an important role in the protein-protein interactions of the β-crystallins. The N-terminal arms of the β-crystallins are evolving faster and differ much more from each other than the globular domains of these proteins.

Finally, we showed that the βA3/A1 (formerly β23) polypeptide from the murine lens contains introns separating exons which encode the predicted structural motifs of the protein. This is diagrammed in Figure 7. At that time, we thought that the N-terminal arm was coded for by the same exon as that which encodes the first structural motif. We have shown recently, however, that there are two additional exons which encode this extension. The βB1 gene in the rat and the βA3/A1 gene in the human similarly have separate exons for each structural motif. The N-terminal extension of the rat and chicken (Fig. 5) βB1 gene is encoded entirely on the second exon, while the C-terminal extension is encoded on the last exon along with the fourth structural motif. Analysis of γ-crystallin genes from the rat, mouse, and human showed that, in contrast to the β-crystallin genes, each domain of the protein (rather than each structural motif) is coded for by an exon, although an intron still separates the exons encoding the protein domains (Fig. 7). In Figure 7, the regions coding for motifs 1 and 3 are given similar markings, as are the regions coding for motifs 2 and 4, in both the β- and γ-crystallin gene.

Relationship between crystallin gene and protein structures.
since it appears that these form structurally related pairs\textsuperscript{99,100} and arose by a process of intragenic duplication in this \( \beta\gamma \) superfamily of proteins.\textsuperscript{55}

A Functional Role For Introns

The heterogeneity of primary polypeptides within the \( \beta\gamma \)-crystallins is due largely to several gene duplications followed by separate evolutionary changes within the individual genes. The two primary \( \alpha \)-crystallin polypeptides, \( \alpha A_2 \) and \( \alpha B_2 \), also appear to have arisen by gene duplication.\textsuperscript{91,92} However, in the rodent families Muridae (rat, mouse) and Cricetidae (hamster, gerbil), there is a third \( \alpha \)-crystallin polypeptide, \( \alpha A^m \), which has a 22 (rat) or 23 (mouse, hamster) amino acid sequence inserted between residues 63 and 64 of the \( \alpha A_2 \)-crystallin polypeptide.\textsuperscript{93-96} Interestingly, intron 1 of the \( \alpha A \) gene separates codons 63 and 64 in all species examined, including mice,\textsuperscript{66} hamsters,\textsuperscript{70} chicken (Thompson, Hawkins, and Piatigorsky, in preparation), and humans.\textsuperscript{77} The \( \alpha A_2 \) insert peptide of mice\textsuperscript{66} and hamsters\textsuperscript{70} is neatly encoded within this intron, as illustrated by the open box in Figure 8. The RNA derived from this small (69 base pairs) insert exon is spliced into mature mRNA 10–20% of the time the gene is transcribed; this splicing reaction is not regulated during development.\textsuperscript{97} By contrast, the \( \alpha A_2 \) mRNA is generated by splicing together the RNA sequences derived from exons 1 and 2 80–90% of the time the gene is transcribed, eliminating the sequences derived from the insert exon. We do not know yet the molecular basis for the infrequent inclusion of RNA from the insert exon. One possibility is that its 3' splice junction is GC rather than the more common GT (Fig. 8).

The alternative splicing of the \( \alpha A \)-crystallin gene in rodents may produce a specialized polypeptide tailored to the needs of the lens in these species; another possibility is that the \( \alpha A \)-crystallin splicing variant is an example of evolution testing a modification of this gene without altering the original gene or its product. In any event, alternative RNA splicing of the \( \alpha A \)-crystallin gene shows how an intron can have a functional, as well as a structural role.

Another example of function embedded within an intron is the presence of an enhancer in immunoglobulin genes.\textsuperscript{98-100} Brief reviews on enhancers (regulators of gene activity) can be found elsewhere.\textsuperscript{101,102} So far, no sequences controlling gene expression have been uncovered within introns of crystallin genes.

It is not possible to relate the gene structure for \( \alpha A \)-crystallin to the structure of its encoded protein, as has been done for the \( \beta\gamma \)-crystallins, since the three-dimensional structure of \( \alpha A \)-crystallin is not known.

Nonetheless, interesting speculations have been made, and a two domain structure for the \( \alpha A \)-crystallin polypeptide has been suggested (see Wistow\textsuperscript{103} for further discussion and references).

Surprising Features of \( \delta \)-Crystallin Gene Expression

As shown in Figure 3, there are two chicken \( \delta \)-crystallin polypeptides (50 K and 48 K).\textsuperscript{39} Only two tryptic peptide differences have been noted between these \( \delta \) polypeptides.\textsuperscript{104} Of particular interest is the fact that the ratio of synthesis of the 48 K to the 50 K polypeptides is strongly affected by the intracellular concentration of ions (especially Na\textsuperscript{+}, K\textsuperscript{+} and Cl\textsuperscript{−}) in cultured embryonic lenses.\textsuperscript{105,106} Since both \( \delta \) polypeptides are synthesized in a reticulocyte or a wheat germ lysate supplemented with \( \delta \)-crystallin mRNA\textsuperscript{39,105} or in cultured monkey kidney cells injected with \( \delta \)-crystallin mRNA (see Piatigorsky\textsuperscript{23}), it appeared as if each \( \delta \) polypeptide had its own mRNA. This idea was supported by the observations that the \( \delta 1 \) and \( \delta 2 \) promoters both function in a Hela cell extract,\textsuperscript{108} and by transient expression experiments using cultured embryonic chicken lens epithelia transfected with the pSVO-CAT expression vector containing the \( \delta 1 \) or \( \delta 2 \) promoter.\textsuperscript{109} In addition, the extreme similarity of the polypeptides encoded in the \( \delta 1 \) and \( \delta 2 \) genes (91% identity) suggests strongly that both genes are expressed and subjected to constraints by evolution.\textsuperscript{73} The simplest hypothesis was that each of the two similar \( \delta \)-crystallin genes (Fig. 9) encodes one of the two polypeptides.

Despite the similarity of the two \( \delta \)-crystallin genes, however, all the cDNAs ever reported were derived from the \( \delta 1 \) gene.\textsuperscript{40,44,50,51,54} The EcoR1 site in exon 13 of \( \delta 1 \) (Fig. 9) is absent from the \( \delta 2 \) gene,\textsuperscript{43,75} which has allowed cloned \( \delta \)-crystallin cDNAs to be identified as...
THE δ-CRYSTALLIN GENE LOCUS: STRUCTURE AND EXPRESSION

δ1 products (Nickerson, unpublished). Surprisingly, as shown in Figure 9, mRNA derived from a cloned δ1 cDNA synthesizes both the 48 K and 50 K polypeptides in a reticulocyte lysate. As with authentic δ-crystallin mRNA, the ratio of synthesis of the 48 K to 50 K polypeptides is affected by ions. We do not yet know the mechanism by which the δ1 mRNA produces both polypeptides, but our current experiments indicate that it is not by use of two different AUG initiating codons (Warwrousek, unpublished), as appears to occur with the βA3/A1 mRNA. Future studies must also determine whether the δ2 gene produces a functional mRNA in the lens. An interesting possibility is that the δ2-crystallin gene is expressed in non-lens cells, since some tissues outside of the lens have been reported to contain δ-crystallin RNA sequences. The surprising nature of δ-crystallin synthesis illustrates the variety of mechanisms used to create crystallin heterogeneity and emphasizes the importance of understanding the molecular genetics of each polypeptide.

Temporal and Spatial Regulation of Crystallin Gene Expression

Figure 10 relates schematically the early development of the chicken lens with the initial appearances of α, β, and δ-crystallins. δ-Crystallin is the first crystallin present and can be detected already in the lens placode, before the formation of a lens vesicle. The early appearance of δ-crystallin resulted in its being called FISC (first important soluble crystallin); later, the name was changed to δ-crystallin. We have suggested on the basis of extrapolated cDNA:RNA hybridization data that δ-crystallin mRNA begins to accumulate by 42 hours of development, which is just a few hours after the optic vesicle interacts with the lens ectoderm. It is possible, however, that δ-crystallin mRNA is present before this time.

Fig. 9. Structure and expression of the chicken δ-crystallin gene locus. The gene structures and organization have been published. Exon 13 is represented as a solid box in the δ1 gene to indicate that it contains an EcoRI site lacking in the corresponding exon of the δ2 gene, making it possible to distinguish cDNAs derived from each gene. The autoradiogram of an SDS-polyacrylamide gel shows the two polypeptides synthesized in a reticulocyte lysate supplemented with δ1 mRNA and immunoprecipitated with δ-crystallin antiserum.

The δ1 mRNA was derived from a δ1 cDNA subcloned into an SP6 vector. We are investigating whether the δ2 gene generates an mRNA.

The surprising nature of δ-crystallin synthesis illustrates the variety of mechanisms used to create crystallin heterogeneity and emphasizes the importance of understanding the molecular genetics of each polypeptide.

THE CRYSSTALLINS AND LENS DEVELOPMENT

Fig. 10. Time of appearance of different crystallins during early development of the chicken lens. =, not detectable; +/−, just detectable. This figure was composed with consideration of the immunofluorescence data of Zwaan and Ikeda. I am grateful to Dr. James W. Hawkins for this figure.
onic lens and δ-crystallin accumulates to approximately 70% of the total protein present. After hatching, δ-crystallin synthesis is selectively reduced, and the β-crystallins become the principal proteins in the adult lens. In vitro translations indicate that the mRNA for δ-crystallin is the major mRNA in the embryonic lens, but that it is lost from the chicken lens between 3 and 5 months after hatching. The δ-crystallin mRNAs accumulate in the post-hatched chicken lens.

In addition to the temporal regulation of crystallin gene expression, there is also a marked spatial regulation of crystallin synthesis within the lens. It has been known for some time that γ-crystallin is present only in the lens fiber cells. More recently it has been demonstrated in embryonic rats that β-crystallin, like γ-crystallin, is present only in the fibers. These immunofluorescence experiments show also that the β-crystallins are detected in the peripheral, elongating fiber cells and the deeper cells of the fiber mass, while the γ-crystallins are found only in the more elongated posterior fiber cells.

Spatial regulation occurs within, as well as between, the different families of crystallin genes. Quantitative and qualitative differences in synthesis of α- and β-crystallin polypeptides in the calf lens have been reviewed elsewhere. In chickens, we showed that the mRNA for the βB1 polypeptide (formerly β35) appears as the cells begin to elongate into lens fibers, while the other β-crystallin mRNAs are present both in the epithelial and fiber cells. Figure 11 provides a quantitative estimate for δ-crystallin and for different β-crystallin mRNAs in different regions of the 15-day-old embryonic chicken lens. These data show graphically that each mRNA displays a characteristic distribution in the lens at this stage of development.

Thus, present evidence indicates that the crystallin genes are differentially expressed both temporally and spatially in the developing lens, and that this differential expression results in the uneven distribution of different crystallins within the lens. Although it is possible that the relative amounts of crystallin mRNAs are controlled post-transcriptionally at the level of RNA processing or degradation (for example, see Bower and coworkers), we assume that transcriptional regulation contributes in a major way to the expression of the crystallin genes in the eye lens. Since the levels of the different mRNAs composing a family of crystallin genes appear to be regulated independently, it is likely that each gene contains its own regulatory elements. This does not eliminate the possibility that there are also regulatory elements controlling coordinately more than one crystallin gene. We began, therefore, to investigate the molecular basis for transcription of crystallin genes.

### Strategy For Transcriptional Control Studies

We decided to focus initially on the murine αA-crystallin gene, since we had cloned the 5' region of this gene with its associated flanking sequences and were ready to analyze it in greater detail. There is only a single copy of the αA-crystallin gene, and it is highly conserved throughout evolution. Our strategy was to identify functionally the promoter of the gene by using it to drive a foreign gene in an expression vector; promoters consist of numerous regulatory elements within the 5' flanking sequence of genes. We chose the pSVO-CAT expression vector as a test system, described below. By this type of modular approach, we could add whatever sequences we wished from the αA-crystallin gene or its flanking regions to the expression vector in order to identify putative control elements. In addition, this strategy facilitates later comparisons of regulatory elements from different crystallin genes by allowing us to determine their activity using the same assay.
Fig. 12. A, Schematic drawing of the murine αA-crystallin gene and its 5′ promoter. +1 defines the initiation site of transcription (RNA synthesis). B, Diagram of the pSVO-CAT expression vector. The indicated promoter fragments from the murine αA-crystallin gene were inserted into the Hind III site of the vector. (a) indicates that the promoter was inserted in the proper orientation and (b) indicates that the promoter was inserted in the inverse orientation. These constructions have been described by Chepelinsky and coworkers except that the position numbers have been corrected (Chepelinsky, Sommer, and Piatigorsky, submitted).

Figure 12A shows a diagrammatic representation of the αA-crystallin gene and its promoter. Two different lengths of the putative promoter were examined. In both cases, the 3′ cut was made at nucleotide position +46. This includes 46 base pairs of 5′ untranslated sequence from exon 1. For the longer piece, a cut was made at nucleotide position —346 in the 5′ flanking region, and, for the shorter piece, a cut was made at nucleotide position —88. Minus numbers indicate base pairs upstream from the initiation site of transcription. These two fragments were inserted separately into the pSVO-CAT expression vector in each possible orientation, as shown in Figure 12B. The pSVO-CAT vector contains the bacterial chloramphenicol acetyl transferase (CAT) gene, as well as portions of the simian virus 40 (SV40) genome. The latter donates a polyadenylation site and splicing signals to the CAT primary transcript. This construct makes it possible to test for αA-crystallin promoter function by determining CAT enzyme activity, which is a very sensitive assay. Since animal cells do not contain a CAT gene, any enzymatic activity observed must be generated by the expression of the CAT gene in the vector.

Two methods were used for testing the function of the αA-crystallin promoter, as diagrammed in Figure 13. These consisted of transfecting cultured lens epithelia and creating transgenic mice.

Transfecting Cultured Lens Epithelia From Chicken Embryos

Phillpott and Coulombre discovered that the cells in explanted lens epithelia from 6-day-old chicken embryos elongate when cultured with fetal calf serum. Subsequently, we showed that these elongating lens cells synthesize crystallins and differentiate into fiber-like cells. Since numerous studies had shown that crystallin synthesis arrests in cultured lens cells, we examined the possibility that crystallin promoters could function when introduced into primary explants of the lens epithelia. Multiple copies of the super-coiled expression vector containing the α-CAT fusion gene were introduced into the cells of explanted 14-day-old embryonic chicken lens epithelia. The method for obtaining the lens epithelial explants for the transfection experiments is diagrammed in Figure 14A. Transfections were performed by co-precipitating the plasmids with calcium phosphate 1 day after explantation, and CAT assays were performed 3 days later. Cells remaining in the original explant elongate as they begin to differentiate into lens fibers during the culture period, while the peripheral cells that migrated from the explant remain as an epithelial monolayer. We do not know at present whether all the cells of the explants take up and express the expression vectors, or how many copies of the plasmids are in the cells after transfection. Our finding that foreign genes can be introduced into the cells of explanted lens epithelia has made this system a valuable tool for the study of regulatory sequences of crystallin genes.
**Fig. 14.** A, Explanation of a lens epithelium. The lens is placed on a collagen-coated dish with the anterior capsule facing down (a). The posterior capsule is torn and opened (b). The fiber mass (F) is removed, leaving equatorial (E) and central (C) epithelial cells attached to the lens capsule (c). The epithelium (d) is cut with a scalpel and fixed onto the dish with forceps (e). B, Low-magnification photomicrographs of a 14-day-old embryonic chicken lens explant after 3 days of culture. C, Piece of explant after culture for 1 day. D, Explant and cellular outgrowth after 4 days of culture. Taken from Chepelevsky and coworkers. 132

**Production of Transgenic Mice**

We were fortunate to collaborate with Drs. Heiner Westphal, Paul Overbeek, Jaspal Khillan, and Kathy Mahon (Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, NIH) for the transgenic mice experiments. Initially, in these experiments the α-CAT fusion gene was removed from the pBR322 sequences of the expression vector and microinjected into one of the pronuclei of the fertilized mouse egg. 140 The method is diagrammed in Figure 15. The microinjected eggs were placed into a pseudopregnant foster mother. The litter developing from the microinjected eggs is called the Fo generation. DNA was taken from a snip of tail and tested by hybridization for the presence of the foreign gene (the α-CAT fusion gene). Positive mice are mated to normal mice to test for transmission of the α-CAT gene; their progeny are the F1 generation. If 50% of the F1 progeny carry the foreign gene, one can conclude that it is being carried in the germ line of the Fo mouse. The transgenic experiments contrast with the transfection experiments in that the α-CAT fusion gene becomes integrated into the chromosomes of the host cells. Thus, the conditions...
MOUSE αA PROMOTER FUNCTION IN CHICKEN LENS CELLS

Fig. 16. Thin-layer chromatogram showing CAT activity in lens epithelia transfected with pαA366a-CAT, but not with the other vectors. SVO, pSVO-CAT; 366a, pαA366a-CAT; 88a, pαA88a-CAT; 366b, pαA366b-CAT; 88b, pαA88b-CAT. cm, chloramphenicol; cm-Ac, chloramphenicol 1-acetate; cm-Ac3, chloramphenicol 3-acetate. Taken from Chepelinsky and coworkers,132 except that the position number has been changed from 364 to 366 (Chepelinsky, Sommer, and Piatigorsky, submitted).

for expression of foreign genes in transgenic mice should mimic closely those for gene expression in the normal in vivo environment.

Transient Expression of the α-CAT Fusion Gene

The data shown in Figure 16 show that lens epithelia transfected with the pαA366a-CAT vector (αA promoter inserted in proper orientation) expressed CAT activity, indicating αA-crystallin promoter function.132 By contrast, only a trace amount of CAT activity was present when the epithelia were transfected with pαA366b-CAT (αA promoter inserted in opposite orientation) or with pSVO-CAT (no promoter present). Of particular interest was the fact that neither pαA88a-CAT nor pαA88b-CAT promoted CAT activity in the transfected lens epithelia. Thus, sequences between -88 and -366 appear essential for the proper functioning of the murine αA-crystallin promoter in the explanted embryonic chicken lens epithelia. Additional experiments showed that pαA366a-CAT did not function in cultured embryonic chicken fibroblasts, indicating that this promoter behaves in a tissue-specific fashion.132 Our current experiments have shown that a fragment consisting of nucleotide positions -111 to +46 are sufficient to promote CAT activity in the transfected lens epithelia.141 In addition, we have identified a regulatory element within this region that functions only in the proper orientation or in both orientations, depending on its surrounding sequences (Chapelinsky, Sommer, and Piatigorsky, in preparation).

Transient expression experiments have also been performed using the chicken αA-crystallin gene promoter fused to the δ-crystallin gene.142 These investigators microinjected a hybrid α/δ-crystallin gene into the nuclei of murine lens epithelial cells in primary culture and assayed immunologically for δ-crystallin synthesis. The α/δ hybrid gene was expressed in the murine lens cells, but not in primary cultures of fibroblasts or in L-cells, indicating that the chicken αA-crystallin promoter, like that of the mouse, is tissue-specific. Deletion experiments located 53 base pairs between nucleotide positions -242 and -189 that were critical for promoter function in the lens cells. These sequences displayed enhancer-like properties by being able to function in either orientation and, although less efficiently, when placed approximately 1.7 kilobases downstream in the second intron of the hybrid gene.

Crystallin Promoters Can Function in Foreign Species

Experiments from several laboratories demonstrating that crystallin genes and their promoters can function in lens cells of foreign species are summarized in Figure 17. Kondoh and coworkers143 showed by an immunoperoxidase assay that the cloned 51-crystallin gene from chickens functions well after microinjection into the nucleus of cultured lens epithelial cells from 10-day-old mice. Either very low levels or no δ-crystallin expression was observed when non-lens cells were microinjected with the δ1 gene. Further experiments indicated that the region between positions -51 and -80 is critical for the lens-specific function of the δ1 promoter in the murine lens cells.144 It is important to underscore that mice do not have δ-crystallin genes.31-145146

Figure 17 also shows transfection experiments using various crystallin promoters inserted into the pSVO-CAT expression vector and explanted embryonic chicken lens epithelia. First, Borras and coworkers demonstrated that the chicken δ1 (sequences -344 to +23) and δ2 (sequences -346 to +22) promoters function in the homologous lens epithelial cells.109 Next, as described above, Chepelinsky and coworkers141 showed that the murine αA-crystallin promoter (sequences -366 to +46) is active in explanted chicken lens epithelia. This result complements the microinjection experiments given above, showing that chicken αA-crystallin promoter sequences can function in murine lens epithelial cells, and is not surprising, since both chickens and mice have an αA-crystallin gene. More unexpected are the findings by Lok and coworkers.147 They showed that the murine γ2-crystallin promoter (sequences -392 to +42) functions very well in...
CRYSSTALLIN PROMOTERS FUNCTION IN LENS CELLS
OF FOREIGN SPECIES

Fig. 17. Summary of experiments demonstrating that crystallin promoters can function in lens cells of foreign species. The experiments are discussed in the text. In the experiment by Kondoh and co-workers, the black cell on the left shows a positive immunoperoxidase reaction for \( \delta \)-crystallin while the transparent cells on the right show a negative reaction. The numbers refer to the extent of 5' flanking base pairs on the specified promoters in the pSVO-CAT expression vector. COS cells used in the mouse p72-CAT experiment were derived from monkey cells and contain SV40 T-antigen. The chicken p6-CAT experiment is from Borras and coworkers, and the mouse pA-CAT experiment is from Chepelinsky and coworkers.

Mouse lens epithelial cells

- Microinjection
- Transfection

Chicken lens epithelium

- Microinjection
- Transfection

explanted embryonic chicken lens epithelia, but not in non-lens cells. Deletion of the \( \gamma 2 \) 5' flanking sequences to nucleotide position \(-171\) resulted in loss of promoter activity in the transfected lens epithelia (Fig. 17). Moreover, recent experiments indicate that the \( \gamma 2 \)-crystallin promoter contains an enhancer-like element between positions \(-226\) and \(-31\) and two domains \((-190\) to \(-125\) and \(-105\) to \(+45\)) for optimal promoter function in the chicken lens epithelia. Again, it is important to note that chickens appear to lack \( \gamma \)-crystallin genes. These data suggest that the tissue-specific expression of crystallin genes may have originated very early in the evolution of the lens, and that the different classes of crystallin genes may be regulated by common mechanisms in all species.

Expression of the \( \alpha \)-CAT Fusion Gene in Transgenic Mice

Insertion of the \( \alpha \)-CAT fusion gene into the germ line of a transgenic mouse provides a more stringent test for identifying a region of the \( \alpha A \)-crystallin promoter which functions in a tissue-specific manner. In collaboration with the group of Dr. Heiner Westphal (see above), we obtained two transgenic mice containing the \( \alpha \)-CAT fusion gene in their germ line after microinjection into the pronuclei of 99 fertilized eggs. Hybridization tests indicated that there was probably no more than one \( \alpha \)-CAT fusion gene per haploid genome integrated into the DNA of these mice. Most exciting was the finding that bacterial CAT activity was present in the eyes of these two transgenic mice. No CAT activity was found in the tails of the mice. Moreover, 50% of the F1 progeny of one of the transgenic mice (number 7378) inherited CAT activity in their eyes, indicating Mendelian transmission of the \( \alpha \)-CAT gene.

Figure 18 illustrates the results of CAT assays performed on homogenates of numerous organs of a 6-month-old transgenic mouse heterozygous for the \( \alpha \)-CAT gene. The data show clearly that CAT activity was confined to the eye. We assume that the \( \alpha \)-CAT gene was present in all the organs. Southern-blot analysis showed that the \( \alpha \)-CAT gene was integrated into the DNA of the liver and brain, but the other organs of the transgenic mice were not tested.

Further tests were performed on separated eye tissues of the transgenic mice (derived from mouse 7378). The results are shown in Figure 19. Bacterial CAT activity was found both in the epithelia and fibers of the transgenic mouse lenses. By contrast, no CAT activity was detected in the retina (R) or the remaining (X) eye tissues. In other transgenic mice containing the CAT gene fused to the long terminal repeat of Rous sarcoma virus, CAT activity was preferentially directed to organs rich in tendon, bone, and muscle. This indicates the importance of the \( \alpha A \)-crystallin promoter sequence in directing CAT activity to the lens.
Having established the striking tissue-specificity of the murine αA-crystallin promoter, we investigated whether the α-CAT fusion gene is co-regulated with the endogenous αA-crystallin gene in the transgenic mice. Earlier studies in the rat showed that α-crystallin is the first crystallin synthesized during development of the rodent lens.124 Subsequently, immunofluorescence studies have shown that α-crystallin appears in the developing lens near day 11 in the embryonic mouse.152 We thus compared the time at which αA-crystallin is detectable in an immunoblot assay with that at which CAT activity becomes evident in the F1 progeny from the α-CAT transgenic mouse 7378. In these experiments, both α-crystallin and CAT activity were first detected in the excised eyes of 12.5-day-old embryos.140 CAT activity was not detected in homogenates of the 13.5-day-old embryonic bodies. There was a larger increase in CAT activity than in α-crystallin content between 12.5 and 13.5 days of development, suggesting that the α-CAT and endogenous αA-crystallin promoters may not be activated at precisely the same time in the developing lens. This small time difference, however, may be due to mRNA or protein stability, or other unknown variables in the experimental procedures. In general, the results indicated that the αA-crystallin promoter in the α-CAT fusion gene is properly regulated in the transgenic mice.

It is interesting that lens clarity and crystallin content were not affected in the α-CAT transgenic mice. Thus, the addition of a second αA-crystallin promoter in the mouse genome does not appear to reduce the expression of the authentic αA-crystallin gene. This is consistent with the observation that progeny of our transgenic mice which were homozygous for the α-CAT gene expressed twice as much CAT activity as progeny which were heterozygous for this fusion gene. Together, these findings suggest that the regulatory factors controlling the αA-crystallin promoter are not rate-limiting in the embryonic mouse lens.

The stringent specificity of the αA-crystallin promoter does not appear to be shared by all crystallin genes. Initial experiments using a mixture of cDNAs containing crystallin sequences indicated that embryonic chicken retina express crystallin genes.153,154 Subsequent experiments using cloned cDNAs identified δ-crystallin mRNAs in a number of tissues from chicken embryos.110-112 More recently, δ-crystallin genes have been integrated into PCC3 mouse teratocarcinoma stem cells.155 δ-Crystallin was found in three lines of these cells after they differentiated into skeletal muscle, columnar epithelia, and unidentified spindle-shaped cells. Experiments in which deletion mutants of the
δ1-crystallin gene were microinjected into mouse fibroblasts have provided evidence that the sequence between nucleotide positions $-93$ and $-80$ are responsible for the low level activity of this promoter in the non-lens cells. Identifying the different factors involved in regulating the crystallin genes and understanding how different elements of the crystallin promoters interact with one another to control the expression of their respective genes are among the exciting challenges for future research.

There are a number of examples showing tissue-preference for the expression of foreign genes in transgenic mice (reviewed elsewhere). Many of these examples include the use of extensive 5' flanking sequences, internal gene sequences, and even 3' flanking sequences, leaving the precise regions which are responsible for regulating the expression of the introduced gene unidentified. The most defined sequences for tissue-specific expression in transgenic mice are from the rat elastase I promoter, where 213 base pairs ($-205$ to $+8$) can direct gene expression in the acinar cells of the pancreas, and our experiments, where 412 base pairs ($-366$ to $+46$) of the murine $\alpha$A-cristallin promoter can direct gene expression in the lens.

In addition to the present experiments showing an appropriate developmental control of the $\alpha$-CAT fusion gene, proper developmental regulation of foreign adult $\beta$-globin genes has been demonstrated in transgenic mice. It has even been possible to alter the developmental timing of the $\gamma$-globin gene from its fetal expression in humans to an ancestral embryonic expression by introducing it into the germ line of mice. These data indicate that there are at least some genes, including those expressed in the eye, that can be displaced from their normal chromosomal position, and yet be properly regulated by relatively few sequences at their 5' end. This does not mean that there are not also control sequences located within the genes, or even in their 3' flanking region.

Transformation of Lens Cells by an $\alpha$A-Crystallin-SV40 T Antigen Fusion Gene in Transgenic Mice

The results with the $\alpha$-CAT fusion gene suggested to us that it might be possible to modify the behavior of lens cells by using the $\alpha$A-crystallin promoter to drive other genes in genetically engineered mice. As an initial step in this direction, we attempted, in collaboration with Dr. Heiner Westphal and his colleagues, to neoplastically transform lens cells in the eye in transgenic mice by placing the SV40 T antigen gene under the control of the $\alpha$A-crystallin promoter. This would serve to test further the ability of the $\alpha$A-crystallin promoter to direct the expression of foreign genes in the lens and to examine the possibility that the lens is resistant to malignant transformation.

There are no published reports of lens tumors, suggesting an apparent resistance of the lens to malignancy. Already, in 1948, Sachs and Larsen speculated on possible anticarcinogenic factors concerning the lens. These included physical, metabolic, chemical, and physiological considerations. Numerous reports, however, involving chemical, spontaneous, and viral transformation indicate that lens cells are not intrinsically refractive to uncontrolled proliferation or tumor formation (see Piatigorsky for review). Moreover, cells in the lens epithelium of two strains of chicken and in certain cataracts display multilayering and abnormal growth regulation (see von Sallman and coworkers for further references). It is particularly interesting that thioacetamide-induced cataracts in rainbow trout showed marked proliferation of the lens epithelium and contained a tumor-like cell mass.

We attempted to generate lens tumors in transgenic mice using the T antigen gene of SV40 fused to the $\alpha$A-crystallin promoter, since it has already been demonstrated that cultured lens epithelial cells from hamsters could be transformed with SV40 virus. It has also been shown in transgenic mice that the SV40 early region genes (coding for large and small T antigen) produce tumors in the choroid plexus of the cerebellum. The choroid plexus tumors were found to be due to the preferential expression of the large T antigen gene in this region of the brain directed by the 72 base pair repeat SV40 enhancer sequence. Removal of the SV40 enhancer caused peripheral neuropathies, pancreatic tumors have been induced in transgenic mice containing the SV40 T antigen gene driven by the rat insulin II promoter.

Initially, we obtained seven transgenic mice containing one or more copies of the $\alpha$-T antigen fusion gene. Figure 20 compares the appearance of a normal eye with that of an $\alpha$-T antigen transgenic mouse. The lens of the transgenic mouse is opaque and whitish-yellow. Transgenic mice with the $\alpha$-T antigen fusion gene die between 3 and 4 months of age; however, we have been able to propagate two strains. We do not know why these mice die prematurely.

Analysis of the $\alpha$-T antigen transgenic mice is in progress, and the details will be reported elsewhere (Mahon and coworkers, in preparation). Histological examination revealed that the eyes of 3½-month-old transgenic mice bearing the $\alpha$-T antigen gene were filled...
with dividing cells, which appeared to have been derived from the ruptured lens. This tumor-like mass was vascularized, and contained cells with aberrant shapes.

Immunofluorescence studies indicated that the cells filling the eye contained crystallins in their cytoplasm in 18-day-old α-T antigen transgenic mice. Many cells had α- and β-crystallins, while only a few cells had γ-crystallin. The nuclei of these cells, both in the eye and after cultivation, showed positive immunofluorescence for SV40 T antigen (Fig. 21). This is consistent with the interpretation that T antigen expression caused transformation of the lens cells. Current investigations indicate that lens cell transformation by SV40 T antigen begins early in the embryonic eye. These experiments demonstrate that the αA-crystallin promoter can be used to alter lens phenotype by genetic engineering.

**Summary and a Look Ahead**

In a relatively short period of time, the lens crystallins have been redefined in terms of their genes. Each crystallin gene family has a characteristic pattern of introns reflecting its evolutionary history. Sequencing of genes and cDNAs has accelerated our knowledge of the primary structures of the different crystallins. An important advance coming from sequence comparisons is the newly discovered ancestral relationship linking the β- and γ-crystallins into a βγ-superfamily of proteins. Introns have a structural role by dividing exons which encode individual structural motifs in the β-crystallins and domains in the γ-crystallins. Introns may also have a functional role, as indicated by the generation of αA2 and αA40 polypeptides from the single murine αA-crystallin gene by alternative RNA splicing.

δ-Crystallin never ceases to amaze. Only one (δ1) of two almost identical, linked genes produces two similar polypeptides from the same mRNA by a still unknown mechanism. The crystallin genes are differentially expressed in a time- and space-dependent manner during lens development. Regulation exists both between and within crystallin gene families. Crystallin promoters function with tissue-specificity in foreign species.

Relatively few 5' flanking sequences composing the murine αA-crystallin promoter can be used to express foreign genes selectively in the lens. The developmental regulation of a foreign gene under the control of an αA-crystallin promoter is similar to that of the endogenous αA-crystallin gene in transgenic mice. Use of crystallin promoters has thus opened new possibilities for genetic engineering in the visual system.

As we look to the future and pursue practical solutions to the problems related to genetic engineering, we should not forget that it was curiosity and basic studies that brought us to this point. We are only beginning to understand the mysteries of genes and their expression. Although genetic engineering and gene therapy are very much on the forefront of current thought, germ line applications to humans seem distant (reviewed elsewhere). We must remember that relatively few microinjections of foreign genes into mouse eggs are successful. It is not yet possible to target a foreign gene into a specific, benign site in the host genome or to eliminate a mutant gene from the chromosome. Consequently, gene additions may be very damaging to the individual, depending on the integration site, although recent studies are making progress in this area. Even a successful gene implant results in a heterozygous individual, creating uncertainty as to which offspring would inherit the new gene.
Clearly, much work needs to be accomplished before germ line genetic engineering reaches the clinic. Somatic gene therapy for humans appears closer. It should be noted, however, that significant advances have been accomplished in the ability to genetically engineer the eye of Drosophila.

On a more positive note, recombinant DNA technology has initiated a cascade of experimentally approachable questions which probe genomic structure and function more deeply than ever imaginable. We can now investigate not only how genes are expressed, but what genes control their expression. In addition to studying gene function per se, recombinant DNA methodology allows us to examine structural and functional components of ocular proteins at the molecular level. The production of transgenic mice (and possibly other species) can be used to develop animal models for metabolic disorders and to explore the basis for hereditary and metabolic diseases involving single gene defects in vision. The Philly mouse cataract is an example of a model hereditary eye disorder that may be amenable to experimentation at the level of gene therapy. It is also possible to begin experiments attempting to dissect genetic events during development, such as, for example, the creation of transgenic mice harboring anti-sense gene sequences. A useful by-product of transgenic experiments is the possibility of obtaining insertional mutants. Examples of insertional mutants which identify genetic loci for important biological processes include the recently reported limb deformities in transgenic mice.

The possibilities for leaps in our understanding of gene function seem to be limited principally by imagination, resources, and available time. Thus, we should use our new tools to investigate the richness of the genome and visual system in the spirit of Jonas Friedenwald’s acceptance remarks for the first Proctor Medal Award:

The slogan of our association should not be that basic research is recondite, solemn, austere, but that it is easy, joyous, and exciting. The orchard is full of golden fruit. One can hardly take a step without discovering something new and illuminating.

Let us listen to his words. Thank you for listening to me and thank you for this wonderful award.

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