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Crystallin Genes: Lens Specificity of the Murine αA-Crystallin Gene

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The abundant soluble proteins of the eye lens, the crystallins, are encoded by several gene families which are developmentally regulated in the embryonic lens. We have studied the expression of the murine αA-crystallin gene. Transfection experiments using the pSVO-CAT vector and explanted lens epithelia from embryonic chickens demonstrated proximal (−88 to −60) and distal (−111 to −85) regulatory sequences which interact when the αA-crystallin promoter is activated in the lens cells. Transgenic mouse experiments showed that the sequence between positions −366 to +46 of the αA- crystallin promoter can drive foreign genes selectively in the lens. A fusion gene consisting of this αA-crystallin promoter sequence and the T-antigen gene of SV40 produced a lens tumor in transgenic mice. Thus, crystallin promoters provide a useful model for tissue-specific gene expression and permit targeting the expression of foreign genes to a highly differentiated tissue during development.

Introduction

The function of the transparent lens is to focus the light that enters the eye onto the retina. The clarity of the lens and its appropriate refractive index are attained by accumulation of structural proteins called crystallins (*1,2*). The crystallins comprise approximately 90% of the water-soluble protein of the mature lens. There are four major crystallin gene families, namely, α, β, γ, and δ (*3,4*). α and β are present in all vertebrates; by contrast, γ is absent from birds and reptiles, where it is replaced by δ-crystallins. The crystallin gene families differ in genetic organization and coding information. Each crystallin gene family contains two (α, δ) or more (β, γ) members that code for related polypeptides. The members of the δ and γ crystallin gene family are linked (although one γ is separate), whereas the two α-crystallin genes are on separate chromosomes (*5*); the β genes are either on separate chromosomes or are distantly linked. Both the structures and coding sequences of the crystallin genes have been highly conserved during evolution.

The diagram in Figure 1 illustrates that the crystallin families are distributed differently in the lens. In the rat, α-crystallin is present in the epithelial and fiber cells, β-crystallin is found in the equatorial and central fiber cells, and γ-crystallin is confined to the central fiber cells. The spatial distributions of the lens crystallins reflect the differential expression of the crystallin genes during development (*2,6,7*). Not only are the various families of the crystallin genes expressed differently in the lens, but the individual members of the crystallin gene families are regulated independently during lens cell differentiation. We are interested in understanding the molecular basis for the tissue specificity and developmental regulation of the crystallin genes.

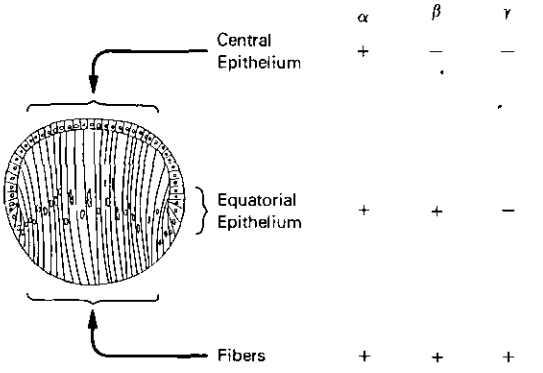

FIGURE 1. Crystallin distribution in the lens. Diagram shows localization of α, β, and γ-crystallins in central and equatorial epithelium and fibers of the rat lens, according to McAvoy (*6*).

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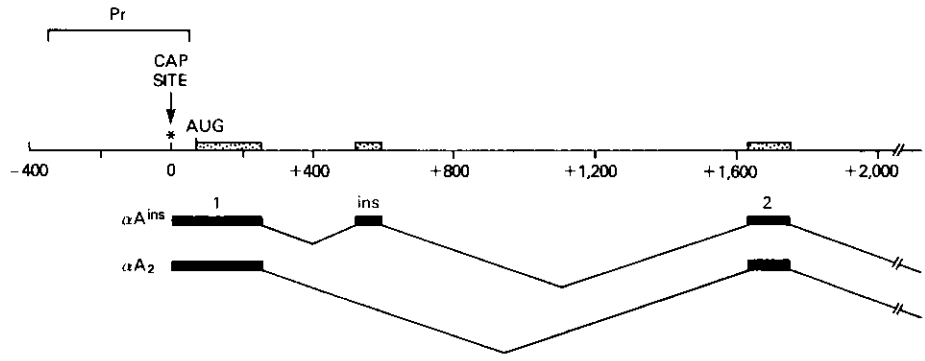

FIGURE 2. Diagram of the murine αA-crystallin gene (*8*). Solid bars: exons. Exon "ins" is present in αA^{ins} mRNA and absent from αA_2 mRNA. Exon 3, present in both mRNAs, is not indicated. Dotted bars: coding sequences. Pr: DNA fragment containing initiation site of transcription but no coding sequences, inserted in expression vectors and tested in explanted lens epithelia and in transgenic mice.

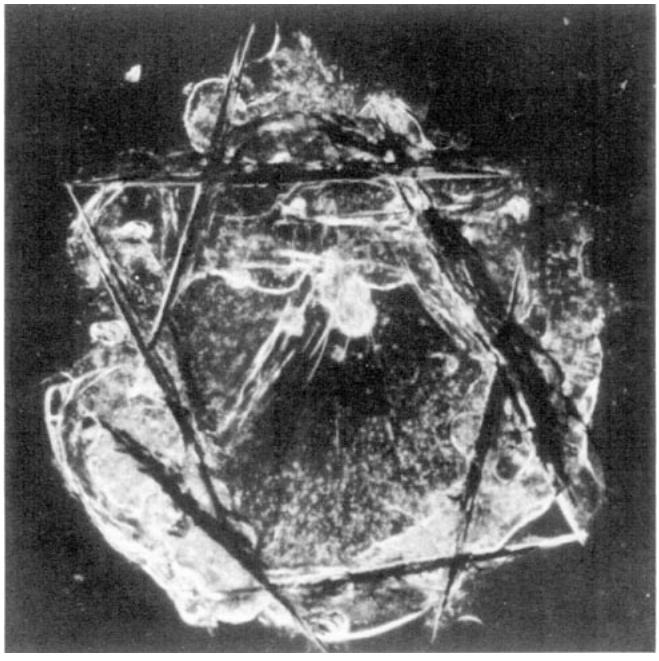

FIGURE 3. Photograph of an explanted lens epithelium from a 14-day-old chicken embryo, used in transient expression assays (*15*). The explant is about 1 mm in diameter.

Activation of the Murine αA-Crystallin Gene Promoter in Explanted Chicken Lens Epithelia

The α-crystallin family contains the αA and αB-crystallin genes (*5,8–10*). These genes are situated on different chromosomes in the human (*5*). The murine αA-crystallin gene is located on chromosome 17 (*11,12*) and codes for two polypeptides, αA_2 and αA^{ins}, that are produced by alternative RNA splicing (*8*). The splicing process that eliminates the insert exon occurs with higher efficiency. This alternative splicing reaction is not developmentally regulated (*13*). We have been studying the 5′ flanking sequences (Pr region) involved in the regulation of transcription of this gene (Fig. 2).

In order to identify DNA sequences that regulate transcription of the murine αA-crystallin gene, DNA fragments containing 5′ flanking sequences of different lengths were fused to the bacterial gene for chloramphenicol acetyltransferase (CAT). The CAT gene is not present in eukaryotic cells and can be tested by a very sensitive enzymatic assay (*14*). Initially, two constructs were made. In the first construct a DNA fragment containing 366 base pairs of 5′ flanking sequence and 46 base pairs of exon 1 (Fig. 2) of the murine αA-crystallin gene was inserted upstream from the CAT gene in the pSVO-CAT vector (*14*). The second construct was similar except that only 88 base pairs of αA-crystallin 5′ flanking sequence were used. The resulting plasmids were called $p\alpha A366_a$-CAT and $p\alpha A88_a$-CAT, respectively. The subscript "a" signifies that the plasmids contain the murine DNA fragment in the same orientation as in the original gene (*15*).

For testing the functional ability of the putative αA-crystallin promoter, we used explanted embryonic chicken lens epithelia that are able to differentiate *in vitro* (*7*). The plasmids were introduced into the explants by the calcium phosphate method (*15*) and CAT assays were performed 3 days later. This system has been very useful for studying crystallin gene regulatory signals (*15,16*).

When explanted lens epithelia (Fig. 3) were transfected with $p\alpha A366_a$-CAT, CAT gene expression was observed (*15*). Negligible CAT gene expression was present when pSVO-CAT (no promoter) (*14*) or $p\alpha A88_a$-CAT was used for transfection (*15*). Neither $p\alpha A366_a$-CAT nor $p\alpha A88_a$-CAT was functional in nonlens cells, indicating that Pr activation of CAT gene expression displays considerable lens specificity (*15*).

A primer extension experiment showed that transcription of the αA-CAT gene starts 46 bp upstream of the αA-crystallin-CAT junction in the hybrid gene in the explanted chicken lens epithelia. This site of initiation of transcription of the hybrid gene was the same

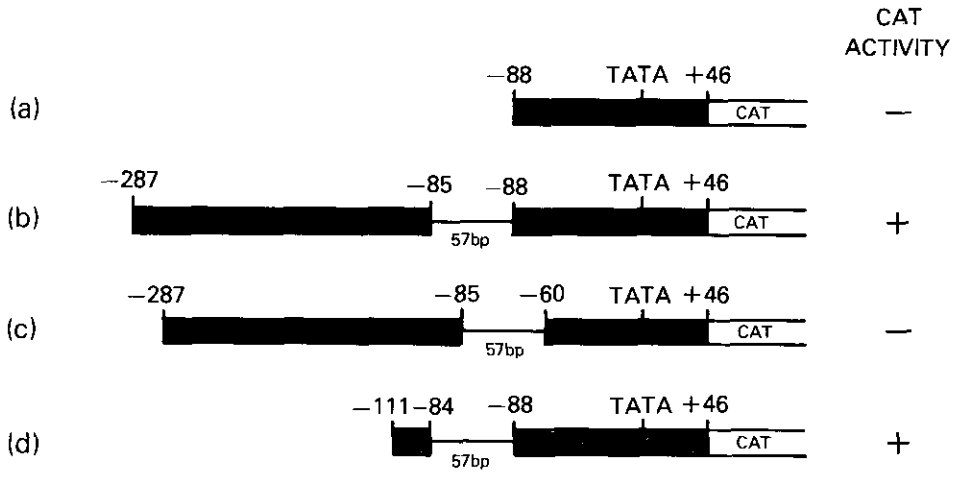

FIGURE 4. Diagrams of constructions containing different murine αA-crystallin 5′ flanking sequences (solid bars) upstream of the CAT gene in the pSVO-CAT vector (*14*). Their ability to activate CAT gene expression in transient assays in lens epithelia (*15*) is indicated by (+) (*16*).

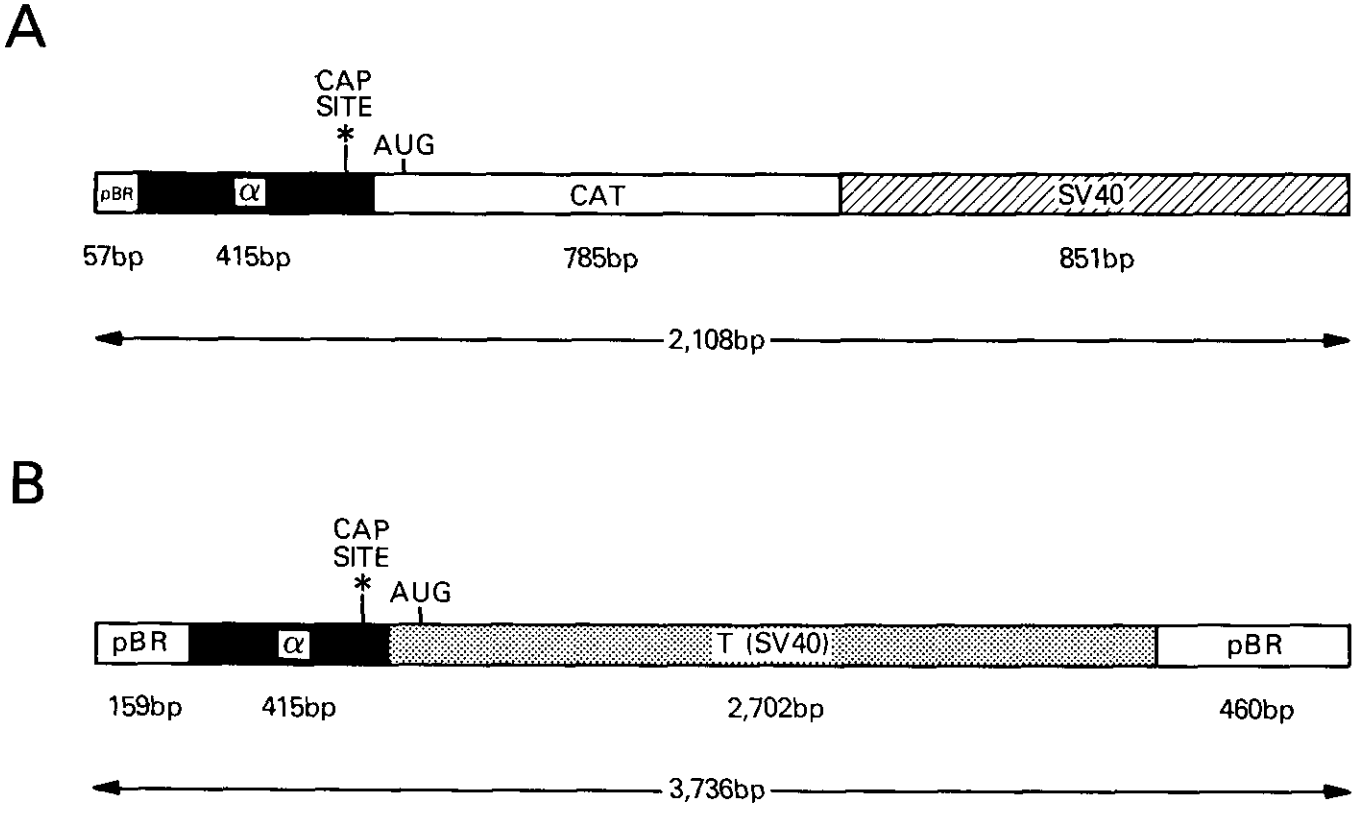

FIGURE 5. Hybrid genes containing the murine αA-crystallin promoter microinjected into the pronuclei of fertilized mouse eggs to generate transgenic mice. (*A*) DNA fragment containing CAT gene coding sequences (empty bar) and SV40 processing signals (hatched bar) downstream from the αA-crystallin promoter (solid bar) injected to produce αA-crystallin-CAT transgenic mice (*36*). (*B*) DNA fragment containing SV40 early region coding and processing sequences (dotted bar) downstream from the αA-crystallin promoter (solid bar) injected to produce αA-crystallin-SV40T transgenic mice. pBR: pBR322 sequences.

as that occurring in the murine αA-crystallin gene *in vivo* (*16*).

Further constructions containing other deletions and insertions in the 5′ flanking region of the murine αA-crystallin gene were tested in the lens explants. The constructs and their ability to generate CAT activity are diagrammed in Figure 4. The experiments revealed two different regulatory elements between positions

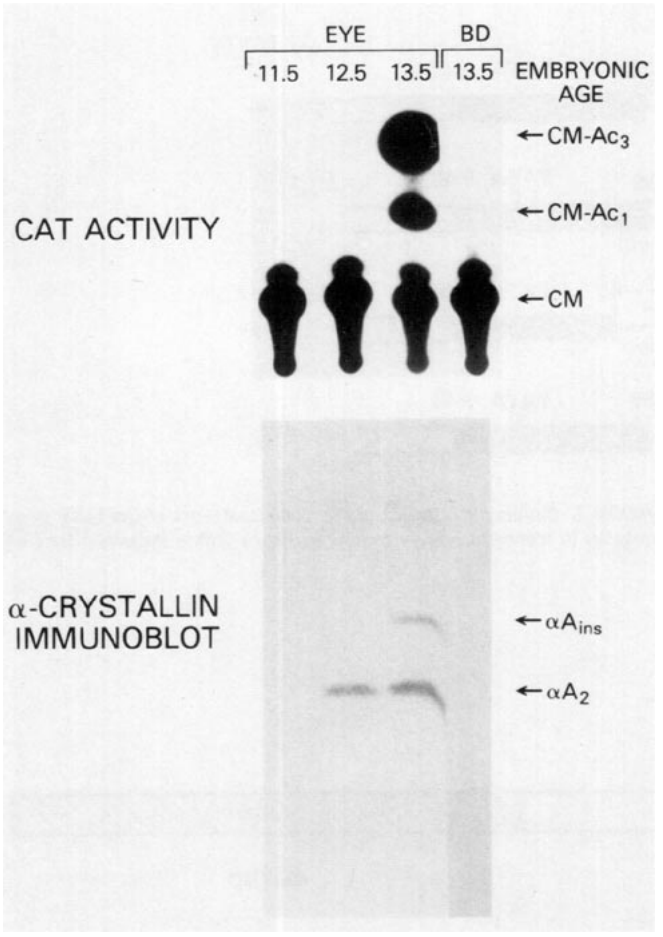

FIGURE 6. CAT activity and α-crystallin appearance during lens development in αA-crystallin-CAT transgenic mice. Data obtained with heterozygous αA-crystallin-CAT transgenic mice strain FVB/N 7378 (*36*). CM: chloramphenicol; $CM\text{-}Ac_1$ and $CM\text{-}Ac_3$: monoacetylated forms of chloramphenicol. BD, headless body; embryonic age in days.

−111 and +46 that appear to interact with one another in order to produce an active αA-crystallin promoter in the hybrid αA-crystallin-CAT gene. The proximal element requires the sequence between positions −88 and −60, and the distal element requires the sequence between positions −85 and −111. The latter can function in either orientation (*16*). These experiments also suggested that the sequence between positions −111 and −366 may contain one or more additional regulatory elements.

Crystallin promoters function in a species-independent fashion (*4*). It is particularly interesting that crystallin promoters can function in lens cells of heterologous species, even when the species from which the lens cells were derived do not contain the crystallin gene being tested. For example, the chicken δ1-crystallin promoter can function in mouse lens cells (*17,18*), which do not contain endogenous δ-crystallin genes, and the mouse γ2-crystallin promoter can function in embryonic chicken γ2 lens cells (*19*), which do not contain endogenous γ-crystallin genes.

To date, no functional consensus regulatory sequence has been detected in the 5′ flanking sequence of different crystallin genes, as has been found for the pancreatic genes (*20*). In the αA-crystallin gene, the 5′ flanking sequence of the mouse (*8*) and hamster (*10*) is highly conserved; however, much of this similarity is lost in the chicken (*21*). In the mouse, rat, and human γ-crystallin gene family there are several sequences upstream from the TATA box that are highly conserved (*19,22–25*). Except for the δ1-crystallin gene (*26,27*), no other crystallin gene contains the 5′CCAAT3′ sequence in their 5′ flanking region (*5,8,10,19,22–25,28–30*). The CCAAT sequence has been found to interact with a specific *trans*-acting factor (*31–33*). At present, we are performing *in vitro* binding experiments with lens nuclear extracts to help us identify *cis*-acting crystallin regulatory sequences that interact with *trans*-acting factors in the lens.

Activation of the Murine αA-Crystallin Gene Promoter in Transgenic Mice

From the experiments described above, we knew that a DNA fragment containing 366 bp upstream and 46 bp downstream from the cap site of the murine αA-crystallin gene contains an active tissue-specific promoter when tested in a transient expression assay in explanted chicken lens epithelia. We were curious to learn whether these sequences would still function with tissue specificity when introduced into the mouse genome. To this end, we created transgenic mice containing the αA-crystallin-CAT hybrid gene. The pronuclei of fertilized FVB/N mouse eggs were microinjected with the αA-crystallin-CAT DNA (*34*) (Fig. 5*A*), and the eggs were transplanted into surrogate mothers (*35–37*).

Two transgenic lines were obtained that contained the αA-crystallin-CAT construct stably integrated into the germline. These mice transmitted the newly acquired gene in a Mendelian fashion. When different tissues of an adult F_1 transgenic mouse from each line were analyzed, CAT activity was found only in the eye (*34*). When the eye was dissected further, CAT activity was confined to the epithelia and fibers of the lens (*34*).

A developmental study showed that the appearance of CAT activity in αA-crystallin-CAT embryonic lenses followed closely the appearance of αA-crystallin (Fig. 6). Thus, this relatively short αA-crystallin 5′ flanking sequence contains sufficient information to direct the expression of a foreign gene to the lens at the same (or closely similar) time as that when the endogenous αA-crystallin gene is expressed. In other experiments using CAT-fusion genes with promoters whose expression is not normally restricted to the lens, CAT activity was found in different tissues of transgenic mice (*38,39*). We conclude therefore that the lens specificity is conveyed by the αA-crystallin promoter.

Our results suggested that we would be able to target the expression of other genes to the lens by fusing them

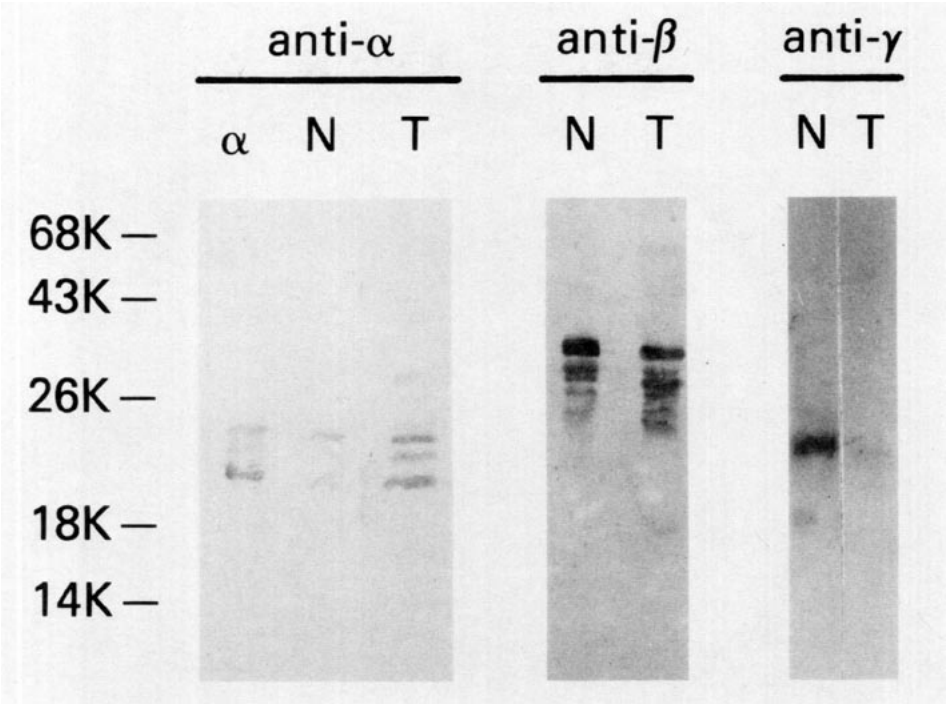

FIGURE 7. Crystallins in αA-crystallin-SV40T transgenic mice. Immunoblots of α, β, and γ-crystallins in the eyes of heterozygous 18-day-old αA-crystallin-SV40T transgenic mice strain FVB/N 7488. Approximately equal amounts of protein were examined from the normal lenses and the transgenic eyes. T: transgenic eyes; N: wild-type FVB/N lenses; α: purified α crystallin. Protein molecular weight markers are indicated on the left side.

to the 415 bp DNA fragment containing the active promoter of the murine αA-crystallin gene, possibly altering lens phenotype. We chose to study the effect of an oncogene on lens differentiation by directing its expression to the lens. The oncogene we used was that for the SV40 large T-antigen (*40–42*). The hybrid gene injected contained the αA- crystallin promoter fused to the SV40 early region lacking its promoter and enhancer (Fig. 5*B*).

Seven F_o transgenic mice were obtained carrying the αA-crystallin-T-antigen fusion gene. All presented the same phenotype when their eyes opened, i.e., white, opaque lenses (*43*). The cellular differentiation of the lens was completely disturbed; the elongation of lens epithelial cells into fibers was prevented and only round mononucleated cells were observed. α and β-crystallins were still present, but γ-crystallin was greatly reduced (Fig. 7). The aberrant lens cells were mitotically active and in 2 to 3 months produced a vascularized lens mass with the growth characteristics of a tumor. This lens tumor was invasive and ultimately broke through the lens capsule and filled the eye cavity (Fig. 8*B*). The presence of SV40 large T-antigen appeared to be the cause of this process, since immunofluorescence experiments demonstrated this antigen was present in the nuclei of the lens cells.

In experiments of others, transgenic mice carrying the SV40 early region containing the SV40 enhancer developed tumors in the choroid plexus (*44–46*). The coding sequences of the SV40 early region fused to the insulin or the elastase promoter of the rat produced tumors in the pancreas of transgenic mice (*47,48*). Although no naturally occurring tumors have been reported in the lens of any vertebrate, we have been able to produce a lens tumor in transgenic mice by using a crystallin promoter to direct the expression of an oncogene to the lens.

Table 1 summarizes the characteristics of the strains of transgenic mice obtained with the αA-crystallin-CAT and αA-crystallin-SV40T fusion genes.

Table 1. Comparison of αA-crystallin-CAT and αA-crystallin-SV40T transgenic mice.

αA-crystallin-CAT	αA-crystallin-SV40T
No noticeable change in eyes	Eyes yellow or white
CAT expression in the lens	SV40 T-antigen expression in the lens
αA-crystallin promoter initiates CAT transcription	αA-crystallin promoter initiates SV40 T-antigen transcription
Does not affect lens differentiation	Interferes with elongation of epithelial cells into fiber cells. Tumor develops in the lens
Does not affect crystallin expression	Affects spatial distribution of α and β crystallins and decreases γ-crystallin
Hereditary transmission (Mendelian fashion)	Hereditary transmission (Mendelian fashion)
Healthy animals	Sick animals; generally die after 4–6 months

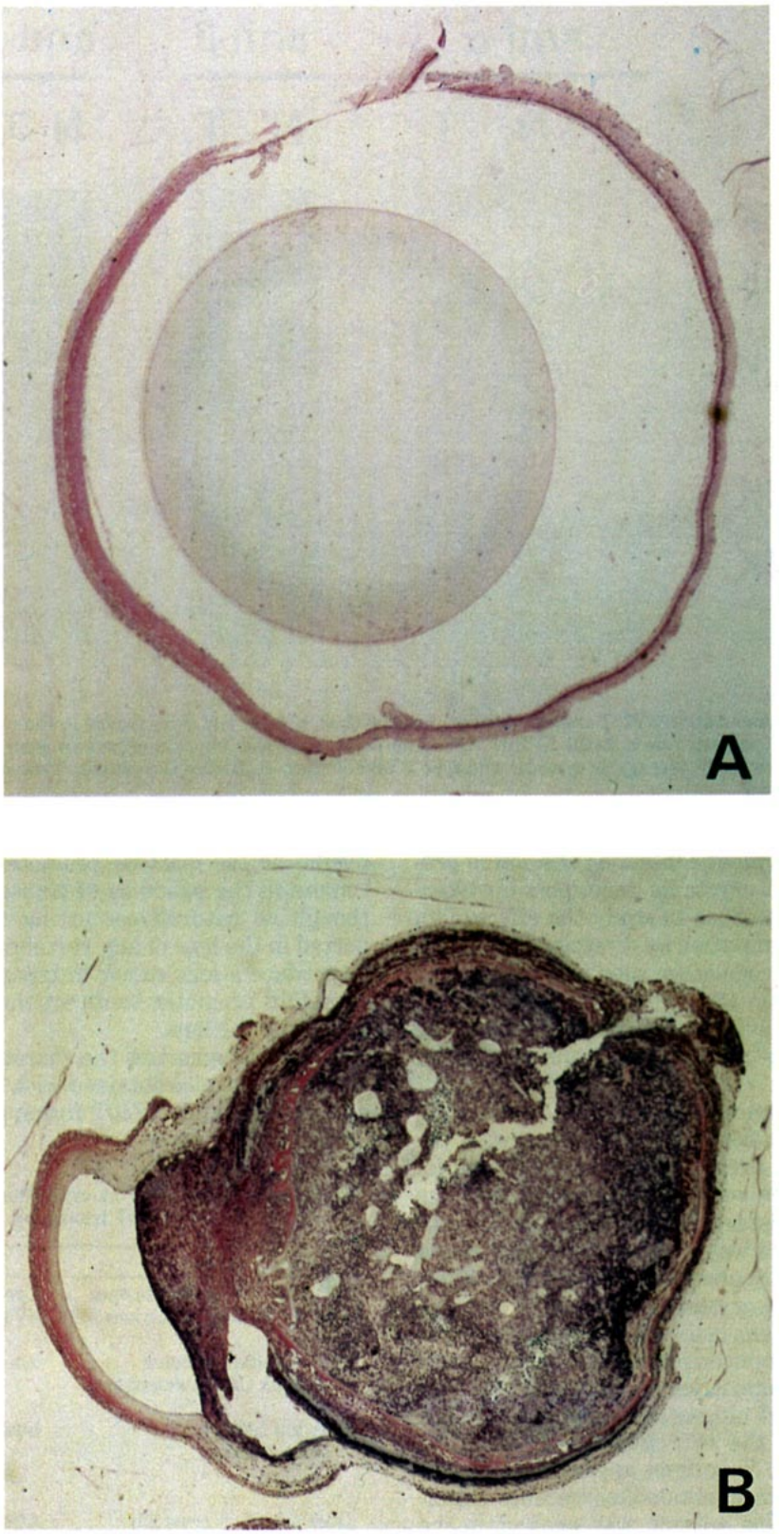

FIGURE 8. Eye morphology in αA-crystallin-SV40T transgenic mice. Giemsa-stained eye sections from: (*A*) wild-type FVB/N mouse and (*B*) heterozygous αA-crystallin-SV40T mouse strain FVB/N 10236. Both mice were 3.5 months old. The cornea of each eye is on the left side and the retina on the right side (*43*).

Future Directions

We have demonstrated that it is possible to target gene expression to the lens with the αA-crystallin promoter by using recombinant DNA techniques. Since the different crystallin gene families are expressed in different parts of the lens (Fig. 1), we presume that the use of regulatory signals from different crystallin genes will permit the expression of foreign genes to be directed to specific regions of the lens. This invaluable tool opens new directions in the study of lens differentiation, with implications for both basic and medical advances in lens research. We are presently using transient expression tests, *in vitro* binding tests, and transgenic mice to investigate further at the molecular level the *cis*- and *trans*-acting regulatory elements responsible for the activation of crystallin promoters in the lens.

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