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Lens-specific expression and developmental regulation of the bacterial chloramphenicol acetyltransferase gene driven by the murine αA-crystallin promoter in transgenic mice

(gene regulation/crystallins/DNA microinjection/development)

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ABSTRACT **Two lines of transgenic mice with one to two copies of a DNA fragment containing nucleotides −364 to +45 of the murine αA-crystallin gene linked to the bacterial chloramphenicol acetyltransferase (CAT) gene expressed the *CAT* gene only in their eye lenses. Both CAT activity and αA-crystallin were first detected in eyes at approximately 12.5 days of embryonic development, suggesting that the αA-*CAT* fusion gene and the endogenous αA-crystallin gene are co-regulated during lens development in the transgenic mice. These experiments show that the murine αA-crystallin gene contains a short, *cis*-acting, tissue-specific regulatory sequence at its 5′ end that can target the expression of the bacterial *CAT* gene, and probably foreign eukaryotic genes, specifically to the ocular lens.**

Crystallins, the major structural proteins of the ocular lens of vertebrates (1, 2), are ideally suited for investigating differential tissue-specific expression of gene families during cellular differentiation (3). Crystallin synthesis is regulated both temporally and spatially during differentiation, resulting in the appearance of a regionally specific pattern of crystallin polypeptides within the lens (4–8). The crystallin genes are preferentially expressed in lens cells, although some δ-crystallin transcripts have been noted in several nonlenticular tissues from embryonic and newborn chickens (9–12).

Recent experiments have indicated that the lens-specific expression of the crystallin genes is regulated by sequences either within or flanking the crystallin genes. A cloned chicken δ-crystallin gene containing approximately 2 kilobases (kb) of 5′ flanking sequences was expressed when microinjected into the nuclei of cultured mouse lens epithelial cells but functioned inefficiently when introduced into nonlens cells (13). We have found in transient expression studies using the pSVO-CAT expression vector (14) that short 5′ flanking sequences of the murine αA- (15) or the chicken δ- (16) crystallin genes can promote gene activity in cultured embryonic chicken lens epithelia. The pSVO-CAT vector contains the gene encoding bacterial chloramphenicol acetyltransferase (CAT; EC 2.3.1.28). Promotion of gene expression by crystallin gene sequences (or any other DNA sequences) inserted into the pSVO-CAT vector can be assayed by CAT activity, a very sensitive enzymatic test that has no background in eukaryotic cells (14).

The production of transgenic mice by microinjecting cloned genes into the nuclei of fertilized mouse eggs presents another powerful method for investigating the control of gene expression (see ref. 17). In the present investigation we have created by microinjection two lines of transgenic mice containing a recombinant αA-crystallin–CAT gene (α–*CAT*) (15) that is expressed selectively in the eye lens and appears to be properly regulated during development.

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MATERIALS AND METHODS

Generation of Transgenic Mice. DNA (1 μg/ml) was microinjected into one of the pronuclei of fertilized NIH FVB/N mouse eggs 12–14 hr after mating, as described by Gordon *et al.* (18). The fertilized eggs were isolated from superovulated FVB/N female mice that had been mated to males of the same strain.

DNA Analysis by Dot-Blot and Southern-Blot Hybridizations. For dot-blot hybridizations, total nucleic acids were isolated from the tails of 3-week-old mice as described by Brinster *et al.* (19). Hybridization was for 20 hr to 2×10^7 cpm of heat-denatured, nick-translated ^{32}P-labeled pRSV-CAT (20) (specific activity $2–4 \times 10^8$ cpm/μg of DNA) at 42°C. The hybridization buffer contained 10% dextran sulfate, 50% (vol/vol) formamide, 5× NaCl/Cit (1× NaCl/Cit is 0.15 M NaCl/0.015 M sodium citrate), 7 mM Tris·HCl at pH 7.5, 1× Denhardt's solution (21), and sheared denatured herring sperm DNA at 20 μg/ml. After hybridization, the filters were washed for 15 min at room temperature with 2× NaCl/Cit containing 0.1% $NaDodSO_4$, followed by 2 hr at 68°C with two changes of 0.1× NaCl/Cit containing 0.5% $NaDodSO_4$. Radioactivity was detected by using Kodak XAR x-ray film and DuPont Cronex intensification screens at −70°C.

For Southern-blot (22) hybridizations, high molecular weight DNA was prepared by homogenization of tail segments in 25 mM Tris·HCl, pH 7.6/10 mM EDTA at 4°C, extracted with phenol, and treated with proteinase K (50 μg/ml) and RNase A (50 μg/ml) in the presence of 0.5% $NaDodSO_4$. For liver and brain tissues, after precipitation of RNA with 3 M sodium acetate (23), the DNA-containing supernate was treated with RNase A, reextracted with phenol, and precipitated with ethanol. Analysis of genomic DNAs by restriction enzyme digestion, agarose gel electrophoresis, transfer to nitrocellulose, and hybridization to ^{32}P-labeled probes were done using standard techniques (24).

CAT Enzyme Assays. Organs were homogenized in 250 mM Tris·HCl, pH 7.6, at 4°C, using a Tekmar Tissumizer, and CAT assays were performed as described elsewhere (14). The homogenates were heated to 65°C for 5 min to inactivate inhibitory factors found in certain tissues (25). Protein concentrations were determined by using a kit from Bio-Rad.

Polyacrylamide Gel Electrophoresis and Immunoblotting. Heat-denatured and reduced water-soluble protein (10,000 × *g* supernatant fraction of a homogenate) was subjected to

Abbreviations: CAT, chloramphenicol acetyltransferase; bp, base pair(s); kb, kilobase(s).

electrophoresis on 15% polyacrylamide/0.1% $NaDodSO_4$ gels (26). Immunoblotting was performed as described in the Transblot Manual from Bio-Rad. The nitrocellulose filter was treated first with an antiserum to mouse α-crystallin made in rabbits (a generous gift of D. Carper, National Eye Institute) and subsequently with anti-rabbit IgG conjugated to horseradish peroxidase (Kirkegaard and Perry, Gaithersburg, MD).

RNA Isolation and Analysis. RNA was extracted from lenses by homogenization in 100 mM Tris·HCl, pH 7.5/12.5 mM EDTA/1% $NaDodSO_4$/150 mM NaCl followed by extraction with phenol and precipitation in 3 M sodium acetate (23) and from other tissues by homogenization in 10 mM Tris·HCl, pH 7.5/20 mM EDTA/0.5% $NaDodSO_4$/75 mM NaCl in the presence of phenol followed by precipitation with 3 M sodium acetate. Formaldehyde-treated RNA was bound to the nitrocellulose filter (27) and hybridization was as for DNA, except that formamide was at 40%, 4× Denhardt's solution and 3–5 × 10^6 cpm of probe were used, and the blots were washed at 52°C.

RESULTS

Generation of Transgenic Mice Containing a Fusion (*α–CAT*) Gene. Ninety-nine embryos of the NIH inbred mouse strain FVB/N were injected with a 2102-base-pair (bp) *Nde* I–*Bam*HI *α–CAT* fragment, which was derived from the pαA364$_a$-CAT plasmid (15). This DNA fragment contains 50 bp of pBR322 sequences (*Acc* I to *Nde* I), a *Hin*dIII linker (14), 364 bp of 5′ flanking sequences, and 45 bp of exon 1 of the murine αA-crystallin gene (15), the bacterial *CAT* gene (785 bp), and 851 bp of the simian virus 40 tumor antigen gene containing splicing and polyadenylylation signals (14). Sixty-five (66%) embryos survived microinjection and gave rise to 11 newborn pups. Two male mice (numbers 7378 and 6290) gave positive dot-blot DNA hybridizations for *CAT* sequences. Mouse 7378 was studied more extensively than mouse 6290. Dot-blot hybridizations utilizing tail and dorsal skin DNAs indicated that there was probably no more than one *α–CAT* fusion gene per haploid genome in these mice (data not shown).

F_1-generation mice were obtained by mating the F_0 positive male mouse 7378 to normal FVB/N females. In the combined progeny of the first two litters, 8 of 16 mice carried *CAT* sequences. The *CAT* sequences were present in a similar copy number as in the F_0 parent, consistent with Mendelian transmission from a single locus of integration.

Genomic Southern-Blot Hybridization Analysis. High molecular weight DNA was isolated from the tails of the 7378 F_1 mice (three positives and one negative for the *α–CAT* gene) and analyzed by Southern-blot hybridization (22). The DNAs were digested with *Bam*HI and *Nde* I and hybridized to a ^{32}P-labeled murine αA-crystallin cDNA (28) (Fig. 1*A*) and a ^{32}P-labeled *α–CAT* DNA (Fig. 1*B*). With the αA-crystallin cDNA probe, the DNAs from the four mice gave the same pattern of hybridization, indicating that the *α–CAT* sequences did not integrate into the endogenous αA-crystallin gene. By contrast, with the *α–CAT* probe the DNAs from the three positive transgenic mice showed a band of 4.7 kb (Fig. 1*B*, lanes 5–7) that was not present in the DNA from the negative sibling (Fig. 1*B*, lane 8). The *α–CAT* DNA fragment mixed with undigested DNA from a normal mouse migrated as expected for a 2.1-kb fragment (Fig. 1*B*, lane 9). The cDNA and the *α–CAT* probes both hybridized to a DNA fragment of 4.0 kb that was present in all the mice. This band presumably contains αA-crystallin sequences that span the transcription initiation site. Southern blots were also made with DNAs from the liver and brain of an F_1 transgenic progeny from mouse 7378, using a *CAT* probe lacking any α-crystallin sequences (Fig. 1*C*). Digestion with *Bgl* II (lanes 10 and 11) or *Xba* I (lanes 12 and 13), neither of which digests the *α–CAT* DNA fragment, gave a single band of *CAT* sequences that was 3 to 4 times larger than the injected *α–CAT* DNA (lane 14). Further tests indicated that the *α/CAT* fusion junction has not been rearranged in the transgenic mice (data not shown). These results indicate that the intact *α–CAT* fusion gene was integrated at one site into the DNA of the transgenic mouse.

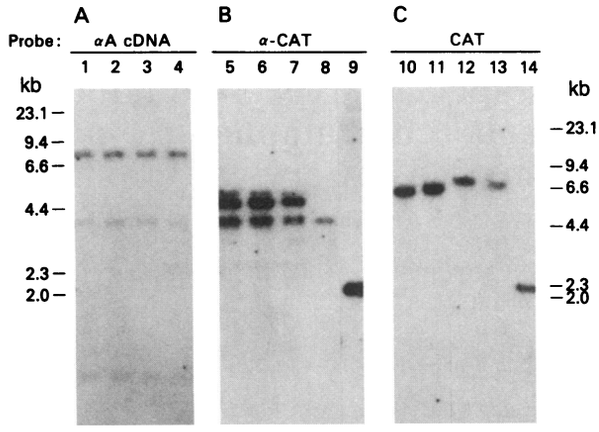

FIG. 1. (*A* and *B*) Southern-blot hybridization of genomic DNA of transgenic mice. High molecular weight DNA was prepared from tail segments of three positive F_1 transgenic mice and one negative F_1 mouse from the same litter. The αA-crystallin cDNA probe (αA cDNA, *Pst* I fragment) was isolated from the pMαACr2 plasmid (28). Ten micrograms of mouse DNA was placed in each lane. The DNAs of lanes 1–8 were digested with *Bam*HI and *Nde* I. Hybridization was at 42°C in 50% formamide. Lanes 1–3, DNAs from three *α–CAT*-positive transgenic mice. Lane 4, DNA from an *α–CAT*-negative F_1 mouse. Lanes 5–8 are duplicates of lanes 1–4, respectively, probed with *α–CAT*. Lane 9: *α–CAT* DNA (5 pg) plus undigested mouse DNA. (*C*) Southern blots of 10 μg of liver (lanes 10 and 12) or brain (lanes 11 and 13) genomic DNA of an F_1 transgenic mouse. The DNAs were digested with *Bgl* II (lanes 10 and 11) or *Xba* I (lanes 12 and 13). A nick-translated *CAT* probe lacking α-crystallin promoter sequences [the *Hin*dIII–*Bam*HI fragment of pSVO-CAT (14)] was used for hybridization. Lane 14 contains the original *α–CAT* DNA fragment that was used for microinjection. Hybridization was at 68°C without formamide.

CAT Activity. CAT assays were conducted on homogenates of 18 different organs of a 6-month-old F_1 transgenic mouse derived from mouse 7378 (Fig. 2). CAT activity was found only in the eye. An identical result was obtained with a 6-month-old F_1 transgenic progeny derived from mouse 6290 (data not shown). Control experiments showed that the different homogenates did not inhibit added commercially obtained CAT enzyme. Additional tests showed Mendelian inheritance of CAT activity in eye homogenates of progeny from the two transgenic mouse strains. Further breeding of 7378 mice resulted in a homozygous strain expressing twice as much CAT activity in the eye. The level of CAT activity in the eye was directly proportional to the amount of ocular protein used in the assay (data not shown). CAT activity progressed linearly for 8 hr at 37°C.

Eyes from a set of 10 heterozygous F_2 transgenic mice (derived from mouse 7378) were pooled and dissected to determine the distribution of the CAT activity within various tissues of the eye (Fig. 3). CAT activity was localized in the epithelium and the fiber cells of the lens, where α-crystallin occurs (6, 29). No activity was found in the retina or in other tissues of the eye. A different set of transgenic mice that carry the *CAT* gene with the α2(I) collagen promoter of the mouse did not show CAT enzymatic activity in their lenses (ref. 30; unpublished data).

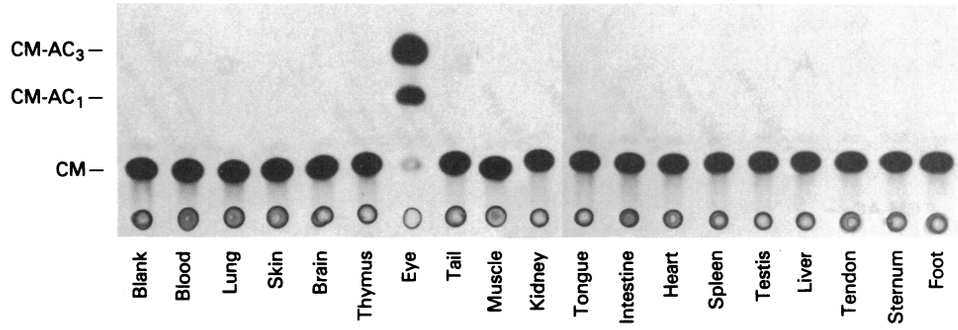

FIG. 2. CAT activity in various organs of a 6-month-old heterozygous transgenic mouse. Samples (10 μg of protein) from the homogenates of the indicated organs were assayed for CAT activity. CM, unacetylated chloramphenicol; CM-AC_1 and CM-AC_3, chloramphenicol acetylated at the 1 hydroxyl and the 3 hydroxyl, respectively.

RNA Assays. Dot-blot hybridization was used to compare the amounts of CAT RNA and αA-crystallin RNA in the lens of a 12-day-old F_1 positive transgenic progeny derived from mouse 7378. The αA-crystallin cDNA and the *CAT* gene both hybridized to the lens RNA of the transgenic mouse, although with greatly different intensities (Fig. 4*A*, lane L). In RNA-blot hybridizations (data not shown), the αA-crystallin cDNA probe hybridized to a single RNA band of about 1400 nucleotides, as expected (28), and the CAT probe hybridized to an RNA band 2000–2500 nucleotides in length. From the relative autoradiographic intensities of the αA-crystallin and CAT RNA bands, we estimate that there was approximately 400 times more αA-crystallin RNA than CAT RNA in the lens.

As a control, we performed another test using lens RNA from a transgenic mouse containing an α2(I) collagen promoter–CAT fusion gene (30). In this case, the αA-crystallin cDNA hybridization was similar to that with the RNA from the *α–CAT* transgenic mouse lens, but the *CAT* probe did not hybridize (Fig. 4*A*, lane Ctr). In addition, the *CAT* probe did not hybridize to 10 μg of RNA from the kidney, spleen, liver, or brain of an F_1 positive transgenic mouse or to ribosomal RNA of yeast (Fig. 4*B*). Similar results were obtained with 20 μg of RNA from these tissues. A β-actin cDNA probe (31) hybridized strongly to all the RNAs of the different tissues but did not hybridize to yeast ribosomal RNA (Fig. 4*B*).

Developmental Regulation of the *α–CAT* Fusion Gene. Since α-crystallin has been first detected by immunofluorescence near 11 days of development (29), we prepared eye extracts from 10.5-, 11.5-, 12.5-, and 13.5-day F_1 embryos of mouse 7378 and analyzed them simultaneously for the presence of CAT activity (Fig. 5*A*) and α-crystallin protein (Fig. 5*B*). Neither signal was detected at 10.5 or 11.5 days of development; no CAT activity was present in homogenates of the embryonic bodies. The appearance of the αA2 and $αA^{ins}$ crystallin polypeptides coincided with low CAT activity in the 12.5-day-old embryonic eye. [The $αA^{ins}$ polypeptide (32) is produced by an alternatively spliced mRNA of the αA-crystallin gene (33).] The specific activity of CAT increased strongly in the interval between days 12.5 and 13.5 of gestation (Fig. 5*A*) and continued to increase until 1 month after birth (not shown).

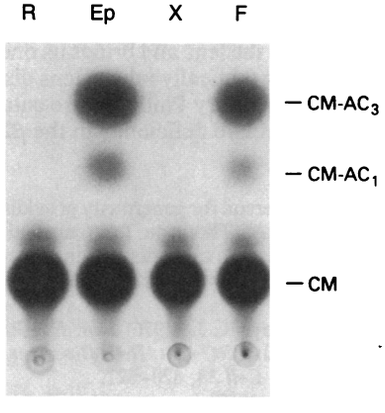

FIG. 3. Localization of CAT activity within the eye. Homogenates assayed were as follows: lane R, retina; lane Ep, lens epithelium; lane X, all eye tissues except lens and retina; lane F, lens fiber cells. Chloramphenicol and acetylated derivatives abbreviated as in Fig. 2.

Crystallins in the Mature Transgenic Mouse Lens. The lenses of the transgenic mice were clear, suggesting that they have a normal complement of crystallins. Accordingly, there was no detectable difference in the lens crystallins in the epithelia or fibers of the normal and F_1 transgenic mice derived from the 7378 mouse (Fig. 5*C*). These electrophoretic patterns correspond to the known pattern of mouse crystallins (34). A similar result was obtained with total lenses from 3-month-old normal and transgenic mice (not shown). Immunoblots of 3-month-old lens protein showed further that there were comparable amounts of α-crystallin in the normal and transgenic mice. Thus, the presence of an additional copy of the αA-crystallin promoter did not reduce the expression of the corresponding endogenous αA-crystallin gene.

DISCUSSION

The present experiments show that a DNA fragment containing only 364 bp of 5′ flanking sequence and 45 bp of exon 1 of the murine αA-crystallin gene can promote tissue-

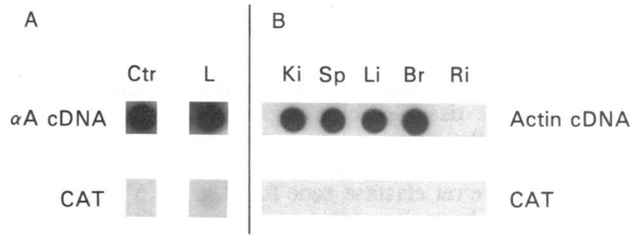

FIG. 4. Dot blots of RNA hybridized to αA-crystallin cDNA [*Pst* I fragment from pMαACr2 (28)], β-actin cDNA [*Pst* I fragment from pA1 (31)], or CAT DNA [*Hin*dIII–*Bam*HI fragment from pSVO-CAT (14)] as indicated on the left and right. The following amounts of RNA were added to the filter: (*A*) αA-cDNA probe, 0.13 μg; CAT probe, 2.25 μg. (*B*) β-Actin and CAT cDNA probes, 10 μg. (*A*) Ctr, lens RNA from a 10-day-old F_1 collagen-CAT transgenic mouse (30); L, lens RNA from a 12-day-old F_1 *α–CAT* transgenic mouse. (*B*) Ki, kidney; Sp, spleen; Li, liver; Br, brain. The tissues were taken from a 12-day-old F_1 *α–CAT* transgenic mouse. Ri, yeast ribosomal RNA.

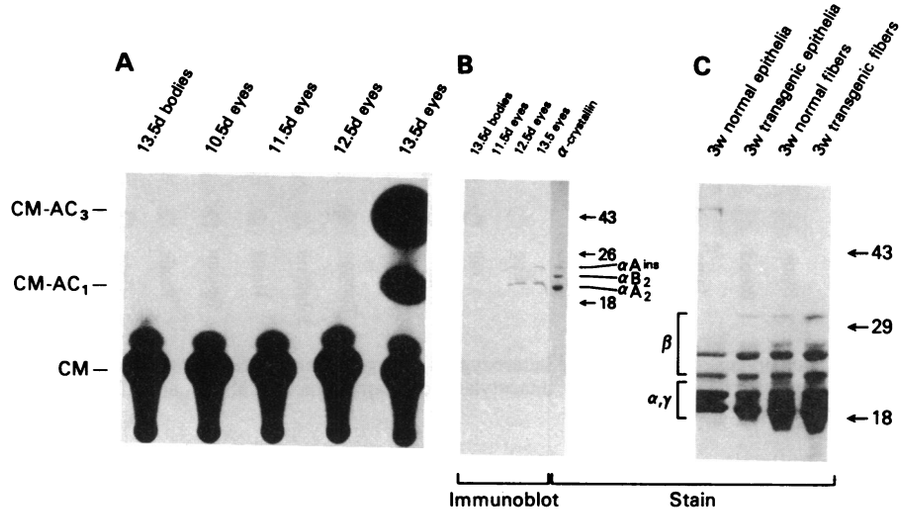

FIG. 5. (*A*) CAT assays of 10 μg of protein from homogenates of eyes of heterozygous transgenic embryos of the indicated ages (d, days). Note the trace of CAT activity (production of 3-acetylchloramphenicol, CM-AC_3) in the 12.5-day-old embryonic eyes. The parents were placed together in cages overnight and the age of the embryos was estimated; they were not precisely staged. (*B*) Immunoblots of the same samples assayed for CAT activity in *A*. The α-crystallin lane contains purified protein. Positions of molecular weight ($\times 10^{-3}$) markers are indicated with arrows. (*C*) Stained $NaDodSO_4$/polyacrylamide gels of water-soluble proteins from isolated lens epithelia and fibers of 3-week-old (3w) normal and heterozygous transgenic mice. α, β, and γ refer to the corresponding crystallin polypeptides. The α and γ polypeptides are not resolved under these conditions.

specific expression of the bacterial *CAT* gene in the lens of a transgenic mouse. This is consistent with earlier transient expression experiments using this *α–CAT* construct (15). Deletion of sequences 5′ to position −87 eliminated the promoter activity of this DNA fragment in the transfection experiments (15). Thus, two independent methods (transient expression of a nonintegrated multicopy plasmid and single copy integration into the genome) both demonstrate lens-specific regulation of *CAT* gene expression by the −364 5′ flanking sequence of the murine αA-crystallin gene. The fact that CAT RNA is confined to the lens of the transgenic mouse suggests a transcriptional control for the tissue-specific CAT activity that we have observed. Other experiments demonstrating CAT activity in other tissues of transgenic mice containing collagen–CAT and Rous sarcoma virus-CAT constructs indicate that the lens is not uniquely suited for CAT gene expression (30). The most likely explanation of our results is that a positive *trans*-acting factor(s) present in the lens activates the αA-crystallin gene regulatory sequence located next to the *CAT* gene.

Tissue-specific expression of foreign genes in transgenic mice has been reported for rearranged κ light chain (35, 36) and μ heavy chain (37) immunoglobulin genes, the chicken transferrin gene (38), the rat pancreatic elastase gene (39), the rat myosin light chain 2 gene (40), and a hybrid mouse/human adult β-globin gene (41). These experiments utilized entire genes plus extensive regions of 5′ flanking sequence, leaving the site of the tissue-specific regulatory sequences unresolved. Recently, experiments in transgenic mice showed that a recombinant gene containing 213 bp of 5′ flanking sequence of the rat elastase gene fused to the 5′ end of the human growth hormone gene was expressed specifically in the acinar cells of the pancreas (42); a hybrid gene containing 660 bp flanking the 5′ end of the rat insulin II gene fused to the large tumor antigen gene of simian virus 40 was expressed selectively in the β cells of the pancreas (43); and a hybrid gene containing 1.2 kb of 5′ flanking sequence from the mouse adult β-globin gene fused to the adult human β-globin gene was regulated properly in the fetal liver (44). Our experiments indicate that nucleotide positions −364 to +45 of the murine αA-crystallin gene are sufficient for both promoting and developmentally regulating gene expression selectively in the lens. This does not mean that other sequences associated with or situated within this gene do not also contribute to the lens-specific functioning of the αA-crystallin gene. The use of the bacterial *CAT* gene in our experiments has the advantage that the bacterial gene presumably does not contain regulatory sequences that may be present within eukaryotic genes.

The low level of *CAT* expression that was promoted by the αA-crystallin regulatory sequences suggests that additional control sequences are used in the natural gene, although other explanations are possible. Full expression of the recombinant *α–CAT* gene may be inhibited by neighboring sequences in the genome; however, the fact that we obtained two transgenic mice with similar levels of CAT activity in the lens argues against this possibility. Second, the CAT RNA may not be as stable in the lens or may not be transported as effectively from the nucleus to the cytoplasm as the αA-crystallin mRNA. Possibly, gene activating factor(s) interact tightly with the αA-crystallin gene (and perhaps other crystallin genes), thereby limiting *α–CAT* gene expression in the transgenic mice. In any event, the high degree of tissue-specific regulation exhibited by the 5′ region of the murine αA-crystallin gene shows that these sequences can be used to express foreign genes in the lens and brings us one step closer to being able to correct genetically inborn lens disorders such as, for example, the hereditary Philly mouse cataract, which is associated with a selective deficiency in the β27-crystallin polypeptide (45).

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