Targeted ablation of α-crystallin-synthesizing cells produces lens-deficient eyes in transgenic mice

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Summary

Genetic ablation techniques were used to study the role of the lens in mammalian eye development. Ablation was accomplished by microinjecting murine eggs with chimeric DNA constructs in which the αA-crystallin gene regulatory sequence (−366 to +46) was fused to the highly cytotoxic diphtheria toxin gene coding sequence. For genetic ablation to be successful the promoter regulating expression should be specific and completely silent in cells necessary for normal mouse development. In this report, we describe the generation and analysis of transgenic mice with this readily discernible phenotype: aphakia or eyes without lens. Of the 109 live-born pups, eight carried the transgene and could be grouped according to the apparent severity of eye malformations. Lines 4, 5 and 6 founder (F₀) mice had the most severe phenotype. Histological analysis revealed: marked reduction in eye size, total absence of lens, increased retinal cell density and extensive whorling of the retinal fibre layers. The line 1 F₀ mouse displayed a distinct lens opacity and lines 2, 3 and 8 F₀ mice were mosaics with a relatively mild, but most unusual phenotype. Their eyes contained a small, highly vacuolated lens. The progeny of these mosaics that inherited the transgene, however, again exhibited the severe phenotype. The aberrant structures of the eyes in which complete genetic ablation of the lens has been achieved suggest that the lens plays a pivotal role in the development of multiple components of the murine eye.

Key words: transgenic mice, genetic ablation, eye development, lens, α-crystallin.

Introduction

During vertebrate development undifferentiated cells are assembled into multicellular tissues and organs. The mechanisms underlying formation of specific cell lineages and the intercellular interactions involved in organogenesis are not well understood, especially in mammalian systems. Traditional approaches to cell lineage analysis include: (1) direct visualization of cells in the intact embryo, (2) tracking the descendants of cells marked with dyes, histochemical tracers or retroviruses, (3) production of genetic mosaics and (4) surgical manipulation of embryos (Herrup, 1987; Rosssant, 1987; Technau, 1987). Although these approaches have been successful in identifying lineage relationships in invertebrates and some lower vertebrate species (Sulston et al. 1983; Weisblat et al. 1978), they have been less successful in studying complex mammalian systems such as the nervous system (Herrup, 1987). An alternative approach to cell lineage analysis is to employ genetic ablation techniques. By combining recombinant DNA and transgenic mouse technologies, it is now possible, in principle, to ablate precisely any specific cell type even in complex mammalian systems (Palmiter et al. 1987; Breitman et al. 1987; Behringer et al. 1988).

Transgenic mice in which specific cellular components have been ablated can be generated by targeted expression of a cytotoxic gene like diphtheria toxin. Precise targeted expression is made possible by microinjecting eggs with fusion vectors in which the coding sequence of diphtheria toxin is placed under the transcriptional control of regulatory elements of genes encoding cell-type-specific proteins. The net result of this manipulation is that the specific cells, in which the regulatory element is active, self destruct at the time when the gene encoding the unique protein first begins to be expressed during normal development. Since germ line transformations are used to induce this toxigenic ablation, a renewable experimental system for analysing cell lineage relationships and intercellular interactions governing organogenesis is obtained.

In this report, we describe generation and analysis of transgenic mice in which the α-crystallin-synthesizing
lens cells of the eye have been ablated by targeted expression of the diphtheria toxin gene. Diphtheria toxin is synthesized as a precursor polypeptide chain (approximately 62×10^3 Mr) by the toxinogenic strains of Corynebacterium diphtheriae. By cleavage of a single peptide bond and a disulphide linkage, the precursor toxin is split into an NH2-terminal fragment (DT-A) and a COOH-terminal fragment (DT-B). DT-A is highly cytotoxic. It encodes an adenosine diphosphate ribosyltransferase that catalyses the ADP ribosylation of a modified histidine residue of elongation factor 2, thus inhibiting protein synthesis and causing cell death.

Although DT-B is not cytotoxic, it promotes the transport of DT-A across the lipid bilayer (Collier, 1975; Pappenheimer, 1977). DT-A has been previously adapted for expression in mammalian cells by providing its structural gene with an initiation codon and promoter from the human metallothionein (MT IIa) gene (Maxwell et al. 1986).

The mature lens is a clear avascular tissue bounded by a collagenous capsule. Immediately beneath the capsule is a layer of undifferentiated, mitotically active, epithelial cells. The terminally differentiated lens fibre cells arise from these epithelial cells (McAvoy, 1981; Piatigorsky, 1981). As the lens grows, the earliest differentiated fibre cells form a nucleus which becomes surrounded by an outer cortex of later-differentiated fibre cells. The cells of the nucleus and cortex are biochemically and structurally distinct and are called nuclear and cortical fibre cells, respectively. α-, β- and γ-crystallins, the major structural proteins of the murine lens, can be used as molecular markers of lens differentiation (McAvoy, 1981; Piatigorsky, 1981). As the lens grows, the earliest differentiated fibre cells form a nucleus which becomes surrounded by an outer cortex of later-differentiated fibre cells. The cells of the nucleus and cortex are biochemically and structurally distinct and are called nuclear and cortical fibre cells, respectively. α-, β- and γ-crystallins, the major structural proteins of the murine lens, can be used as molecular markers of lens differentiation (McAvoy, 1981; Piatigorsky, 1981). α-crystallin is expressed in the lens epithelial cells and the fibre cells of both the lenticular cortex and nucleus; β-crystallin is expressed in only the fibre cells of the lens cortex and nucleus; γ-crystallin expression is restricted to only the lens nuclear fibre cells. In order to study the developmental consequences of complete ablation of all lens cells, we have used the α-crystallin promoter to target DT-A expression to lens epithelial cells and to fibre cells of both the lenticular cortex and nucleus.

Breitman et al. (1987) have previously employed a similar genetic ablation strategy, but using the γ-crystallin promoter which is expressed only in the nuclear fibre cells, to make transgenic mice with microphthalmia and a small lens. Recently Landel et al. (1988) have combined the α-crystallin promoter with the ricin cytotoxic gene to produce a single line of transgenic mice with a somewhat more severe phenotype consisting of a still smaller lens and other interesting eye malformations. In this report we describe the first successful generation of multiple lines of transgenic mice which totally lack all lens structure. The pleiotropic effects of lens absence on the structure of the eye are described, and the potential uses of these lines in studying the role of the lens in eye development are discussed.

### Materials and methods

#### Plasmid constructions

The chimeric vector αC-DT-A-hGH (Fig. 1) was constructed according to standard cloning protocols. The vector p α A366-G-α (Mahon et al. 1987) was digested with NciI to generate the 3.7 kb NciI fragment. This fragment contains the αA-crystallin gene promoter fused to an enhancerless-promoterless SV40 early region. Digestion of the 3.7 kb NciI fragment with BamHI yielded the 571 bp NciI–BamHI fragment. The NciI–BamHI fragment (stippled) contains 159 bp of pBR322 sequence and 412 bp of the mouse αA-crystallin gene sequence; the 412 bp αA-crystallin sequence (–366 to +46) contains the promoter and cap site and can direct lens-specific expression of reporter genes in lens explants (Chepinsky et al. 1985) and transgenic mice (Overbeek et al. 1985). By replacing the 213 bp SalI–BamHI fragment of E. coli hGH (Ornitz et al. 1985) with the 571 bp NciI–BamHI fragment of p α A366-G-α, the recombinant αC-hGH was generated.

The 795 bp DT-A cassette was obtained by digesting the vector 2249-1 (Palmitier et al. 1987) with BglII. The DT-A cassette (vertical lines) contains 33 bp of polylinker and the initial Met-Asp-Pro sequence from the human metallothionein 11 A sequence, 573 bp of DT-A sequence encoding amino acids 3 through 193 and SV40 sequences which provide the carboxy-terminal Ser-Leu, stop codon and the small t intron. The BglII-bound DT-A cassette was inserted into the BamHI site of αC-hGH to generate the vector αC-DT-A-hGH. The 3.5 kb HindIII–EcoRI fragment of αC-DT-A-hGH was used for microinjecting fertilized eggs.

#### Generation and identification of transgenic mice

Transgenic mice were produced by pronuclear microinjection (Gordon & Ruddle, 1983). Briefly, hybrid mice (C57BL/6×C3H/He) were superovulated by injecting with 5 i.u. of follicle-stimulating hormone (FSH) 2-5 days prior to ovulation and 2-5 i.u. of human chorionic gonadotropin (hCG) 0.5 days prior to ovulation. These females were mated overnight with males of the same genotype. The next morning, females showing the presence of a vaginal plug were used as egg donors. Fertilized eggs were removed from the oviducts and methods section.
and microinjected with approximately 2 picolitres of a 1-5 µg ml⁻¹ solution of the 3-5 kb HindIII–EcoRI fragment of α-C-DT-A-hGH. The eggs surviving injection were reimplanted into pseudopregnant albino CD-1 (Charles River) females and the pregnancy allowed to continue to term.

Three-week-old pups were screened for the presence of the transgene by Southern blot analysis (Southern, 1975). Tail DNA was digested with BamHI (BamHI cleaves the injected fragment once, thus generating predictable size fragments of the inserted concatamer). The digested DNAs along with a small amount of the injected fragment (as a positive control) were electrophoresed and Southern blotted. The DNA blot was hybridized at 65°C to 32P-labelled 3-5 kb HindIII–EcoRI fragment of α-C-DT-A-hGH overnight. The 3-5 kb fragment was labelled by the random priming procedure (Feinberg & Vogelstein, 1983). Gene Screen Plus filters were washed as recommended by the manufacturer (NEN). The air-dried filters were exposed overnight to Kodak XAR-5 film at -80°C.

**Histology**

Typically the preservation of lens morphology is difficult when sectioning whole eyes. We tested a number of fixatives and embedding media and found the method described below provided the best results, although folding and tearing in the crystallin lens still sometimes occurred. The eyes from normal and transgenic animals were fixed for 4 h in freshly prepared 4 % paraformaldehyde and 4 % glutaraldehyde in 0.1 M-potassium phosphate buffer, pH 7-4. During this fixation the vitreous chamber in control eyes often partially collapsed. The tissues were then soaked for 12–18 h in 0.1 M-potassium phosphate buffer, pH 7-4, after which they were transferred to 50 % aqueous acetone. Control eyes regained their spheroidal shape. Dehydration was continued through a graded series of acetone before embedding in LX112 (Ladd Research Industries). The resin was polymerized initially at 37°C for 12 h, then at 60°C for three days prior to sectioning on a microtome with glass knives. 1–2 µm serial sections of transgenic eyes were cut and collected and representative sections through control eyes were cut. Sections were stained with toluidine blue.

**Results and discussion**

The chimeric vector α-C-DT-A-hGH was generated by fusing the αA-crystallin gene regulatory sequence −366 to +46 with the DT-A cassette (Fig. 1). It has been shown that the sequence −366 to +46 can direct lens-specific expression of reporter genes in primary lens explants (Chepelinsky et al. 1985) and in transgenic mice (Overbeek et al. 1985). Nevertheless there may be low levels of previously undetected gene expression in non-lens tissues which, when combined with diphtheria toxin, could cause lethality. Thus it was not certain whether viable transgenic mice carrying this chimeric construct could be obtained. Indeed, after these experiments were performed, Landel et al. (1988) reported that the combination of the α-crystallin promoter with the ricin cytotoxic gene gave approximately tenfold fewer transgenics than expected.

We microinjected approximately 400 eggs with the α-C-DT-A-hGH-derived 3-5 kb HindIII–EcoRI gene fragment, and transferred them to pseudopregnant recipients. Southern blot analysis confirmed that 8 of the 109 live-born pups carried the transgene. These numbers compare well with the percentage of transgenic pups generated in other experiments in our laboratory. Thus there was no evidence for general lethality of this construct. At 2 to 3 weeks of age the founder (F0) pups revealed grossly aberrant external eye morphologies (Fig. 2). Three of the F0 pups (lines 4, 5 and 6) had extremely small eyes and will be referred to as the severe phenotype mice. These pups lacked an externally visible eye and therefore could be readily identified in a mixed litter of transgenic and non-transgenic mice. Another founder (line 1) displayed distinct lens opacity in one of its eyes. The remaining founders (lines 2, 3 and 8) had microphthalmia, or eyes smaller than controls. The microphthalmic mice will be referred to as the mild phenotype mice. Line 7 F0 was lost. The transgenic mice were checked for visual function. Mice were held vertically midtail approximately 15 cm above an opaque metal rod and lowered to test the visual placement response (Fox, 1964). Both control and mild phenotype mice responded by reaching out for the rod. The severe phenotype mice had no visual function as determined by their failure to reach out for the rod in this test.

The founder mice were back-crossed with C57BL/6×
Although the retina was still thicker than controls, the approximate diameter of the eyes in the line 5 and 6 of transgenic mice clearly had aberrant morphologies. The animals carrying either the F_0, recombinant, knock-out transgene at a copy number for line 1 (mouse with lens opacity) F_0 type (lines 5 and 6), and one F_0 mouse with mild gene. The approximate transgene copy numbers for the mice in line 8, only 1 of the 28 Fi pups was transgenic. The F_x progeny of mosaic F_0 mice (lines 2, 3 and 8) had readily apparent eye malformations that were characteristic of the severe phenotype mouse. In all lines, Southern blot analysis of the F_1 pups confirmed that the observed phenotype cosegregated with the presence of the transgene. The approximate transgene copy numbers for the lines 4, 5 and 6 (severe phenotype) F_0 mice were 140, >200 and 30, respectively. The approximate transgene copy number for line 1 (mouse with lens opacity) F_0 mouse was 10.

Eyes from two F_0 transgenic mice with severe phenotype (lines 5 and 6), and one F_0 mouse with mild phenotype (line 3) were selected along with weight-matched controls for histologic examination. The eyes of transgenic mice clearly had aberrant morphologies. The approximate diameter of the eyes in the line 5 and 6 mice was 45% of normal and in the line 3 mouse was 75% of normal (Fig. 3A–C). The severe phenotype mice completely lacked a lens (Fig. 3B) and also had structural abnormalities at both the corneal and retinal surfaces which included the following. (1) The iris and cornea were markedly thickened. (2) The iris adhered to and extended completely across the cornea, with no evidence of a pupil. (3) The iris was entirely lined by a cellular layer that in controls was restricted to the ciliary processes. (4) The retinal ganglion cell layer was 2–4 cells thick rather than the normal single cell layer (Fig. 3, marked with solid triangle in D and E). Consequently, ganglion cells were not uniformly spaced towards each other or towards the plexiform layers. The unicellular ganglion cell layer could be due to physical compression of the retina, and/or to a failure of the normal ganglion cell death that occurs in all vertebrate retina including rodents (Potts et al. 1982). Both the optic nerve fibre layer (Fig. 3, marked with dotted line in D and E) and the internal retinal layers were also thickened. (5) The retina extended beyond the normal limits of the ciliary body, folding back onto itself into whorls within the vitreous chamber (Fig. 3B,G and H). This portion of the retina was detached from the sclera and the choroid and was generally quite distorted. Interestingly, a near normal laminar morphology with an outer nuclear layer and photoreceptor-like outer segments was present where the detached retina curled back onto itself to form a closed whorl (Fig. 3H, arrowheads).

The mild phenotype line 3 mouse had a very small lens which was highly distorted and vacuolated (Fig. 3C and I). An amorphous cellular mass was attached to the posterior pole of this distorted lens (Fig. 3, arrow in I). Although the retina was still thicker than controls (Fig. 3D and F) it maintained a normal growth pattern at its periphery and did not form the whorls observed in the severe phenotype mouse (Fig. 3B and C). This mouse also displayed a thickening of its iris together with a narrowing of its pupil.

The line 3 F_0 mouse examined here was clearly a genetic mosaic since only 2 of 32 F_1 pups were transgenic. It is interesting that, even though many of the cells of the developing eye were non-transgenic, the final structure was quite malformed. This suggested that, in this experimental system, the remaining normal cells were unable to compensate fully for the loss of the transgenic cells. Perhaps the unique vacuolated appearance of the lens resulted from the death of that population of lens cells that were transgenic.

To check if lens vacuolation in the line 3 F_0 mouse resulted from its genetic mosaicism, the F_1 transgenic pups were analysed. Both of the transgenic F_1 pups exhibited the severe phenotype as judged by external appearance of the eyes. These pups transmitted the phenotype in the normal Mendelian manner and in each case the presence of the phenotype cosegregated with the presence of the transgene. Histological analysis of the F_2 pups along with littermate controls confirmed absence of the lens in the transgenic progeny. These mice also showed the additional eye malformations typically observed in the severe phenotype line 5 and line 6 mice. These results clearly indicate that total lens ablation occurs in pure transgenic mice and that the aberrant vacuolated lens structure observed in some mice is due to their genetic mosaicism.

In summary, the combination of the α-crystallin promoter directing expression of the diphtheria toxin coding sequences appears precisely to achieve complete
Lens-deficient eyes in transgenic mice
genetic ablation of the lens. An appropriate number of transgenic animals are generated and these animals appear perfectly normal except for their eyes. These results are in marked contrast to those of Landel et al. (1988), who, using the ricin cytotoxic gene, reported a marked reduction in the number of original transgenics born, and these transgenics then exhibited serious infertility problems. Also, in this study, no small ectopic lens-like structures as observed by Landel et al. (1988) were seen. These ectopic structures may be due to the incomplete lethality of the ricin polypeptide construct. Furthermore, unlike in the α-crystallin–ricin mice, we did not observe any toxin-resistant lens epithelial cells in our study. We observed an effective and complete omission of the lens rather than a simple reduction of its size. Therefore, the pattern of eye malformations described herein more accurately reflects the results of eye development in the absence of a lens.

It is interesting to note that Varnum & Stevens (1968) have described a naturally occurring autosomal recessive mutation in the mouse that severely affects lens development. These mice exhibit retinal foldings and abnormalities in the development of the cornea, iris and pupil resembling that of the severe phenotype mice produced by targeted ablation. Our observations strongly suggest that the lens is the primary target of this mutation.

Our results also confirm and extend the elegant studies of Coulombre & Coulombre (1964) on the role of the lens in chick eye development. They reported that, following physical extirpation of the lens, growth of the chick eye was slower than normal but the neural retina continued to increase in area, leading to extensive whorling in the vitreal space. The present cell ablation studies, coupled with these earlier experiments, suggest that growth of the mammalian and chicken neural retina is independent of signals from the lens.

In conclusion, it is clear that the lens plays a key role in normal eye development. Developmental lens ablation does not result simply in a lens-deficient eye, but rather generates a dramatically smaller eye with complex malformations including an aberrant iris and retina. The exact stage at which diphtheria toxin expressing epithelial cells and/or lens fibre cells die must be determined by analysis of transgenic animals at earlier stages of development. The transgenic mice generated in this study may provide a reproducible experimental system for studying development of the multiple components of the eye in total absence of the lens. Since these aphakic mice have been produced by genetic ablation they provide a better system for studying the effects of absence of lens on eye development as opposed to classical approaches in which aphakia is generated by physical extirpation of the pre-existing lens. In addition, unlike the mice made by Landel et al. (1988) using the α-crystallin–ricin construct, the transgenic mice generated with the α-C-DT-A-hGH construct lack the lens completely. Therefore, these α-C-DT-A-hGH transgenic mice represent a potentially powerful system for the analysis of the role of the lens in early eye development.

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References


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