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Genetic Ablation in Transgenic Mice with an Attenuated Diphtheria Toxin A Gene

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We have previously generated microphthalmic mice lacking lens fiber cells by targeting the expression of the diphtheria toxin A (DT-A) gene in transgenic mice with regulatory sequences associated with the mouse γ_2-crystallin gene. Because of the extreme toxicity of DT to animal cells and the potential leakiness of many tissue-specific regulatory regions, we investigated whether there might be an experimental advantage in using a mutant, attenuated form of the DT-A gene (*tox-176*) fused to γ_2-crystallin regulatory sequences to ablate fiber cells in the ocular lens. In contrast to the microphthalmia observed in transgenic animals carrying the native DT-A gene, independent lines of mice transgenic for the $\gamma_2$tox176 construct displayed predominantly cataracts or clinical anophthalmia. These contrasting phenotypes were transmitted within each pedigree, although for some lines some phenotypic heterogeneity among offspring was noted. The difference in phenotype between cataractous and clinically anophthalmic transgenic lines could not be ascribed to differences in the transgene copy number. Instead, the results suggest that transgene expression and hence the extent of genetic ablation are modulated by the site of chromosomal integration and, to a lesser extent, by epigenetic events. They also suggest that the attenuated $\gamma_2$tox176 construct can integrate into chromosomal regions that are particularly favorable for expression without compromising embryological development and therefore that the *tox-176* gene may be more versatile and effective than the wild-type DT-A gene for achieving genetic ablation with a broad range of cell- or tissue-specific regulatory sequences.

The mechanisms governing organogenesis and other morphogenetic processes in higher organisms involve complex interactions among different cell types. Both intrinsic and extrinsic factors may act to determine cell fate, rendering it difficult to discern the role that individual processes play in effecting cell commitment. The ability to ablate specific cell types during development provides an experimental approach for investigating these processes and assessing the specific contributions that cellular interactions play in determining cell fate. Recently, we and others have shown that selective ablation of specific cell types can be achieved by introducing into the mouse germ line genes encoding potent cytotoxins, such as the catalytic subunits of diphtheria toxin or ricin, driven by promoter-enhancer elements derived from genes known to be expressed in a single cell type (1, 3, 6, 9). One limitation of this approach, however, is that many promoter-toxin gene constructs are likely to be lethal to the developing embryo. This would be true for promoters that are active in a broad spectrum of cell types or in cell types that are essential for embryo viability. In addition, embryo lethality might result if the use of a so-called tissue- or cell type-specific promoter resulted in ectopic toxin gene expression outside the intended lineage. Ectopic patterns of expression of a transgene could result from the intrinsic leakiness of the transcriptional regulatory sequences or from chromosomal-position effects at the site of transgene integration in the host genome (10). Because of the extreme cytotoxicity of presently employed toxin genes, such ectopic expression, even if it represented only a small fraction of the levels of expression in the target tissue, could present a significant experimental problem.

In view of the considerations mentioned above, we attempted to enhance the target cell specificity of genetic ablations by mitigating the problems of promoter leakiness and chromosomal-position effects. One method by which this might be achieved involves the use of attenuated toxin genes which require a substantially higher level of expression than their nonattenuated or wild-type counterparts to produce a lethal event.

In this paper, we report on the use of an attenuated form of the diphtheria toxin A (DT-A) gene driven by mouse γ_2-crystallin regulatory sequences to mediate ablation events in the ocular lens. We show that, in contrast to the microphthalmia observed in transgenic animals hemizygous for the native DT-A gene (2, 3), independent lines of transgenic mice carrying the attenuated DT-A gene fall into two groups with disparate phenotypes. One group of animals displays cataracts predominantly or exclusively, with occasional slight microphthalmia, whereas the other group shows primarily clinical anophthalmia. These distinct ocular phenotypes were generally transmitted by founder animals to their transgenic offspring, although for some lines some variability in phenotypic transmission was noted. The phenotypic differences between the cataractous and anophthalmic transgenic lines could not be ascribed to differences in transgene copy number. Instead, the results are consistent with the idea that chromosomal-position effects modulate the extent of transgene expression and that, in contrast to the γ_2-crystallin–DT-A (γ_2DT-A) gene, the attenuated γ_2DT-A construct is free to integrate into chromosomal locations that are

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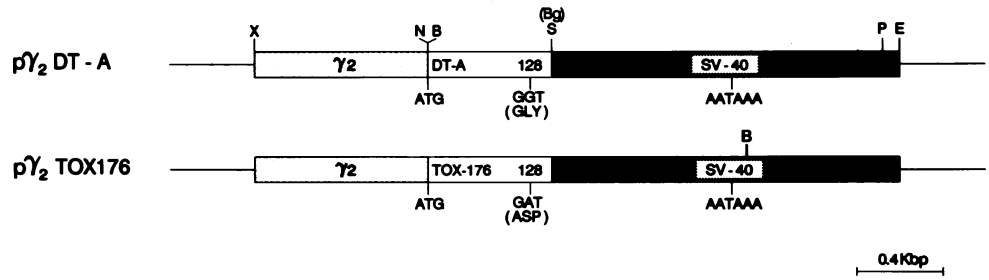

FIG. 1. Diagram of the microinjected mouse $\gamma_2$tox176 construct. The wild-type γ_2DT-A gene is shown at the top for comparison. $\gamma_2$tox176 contains a *Bam*HI site in the simian virus 40 (SV-40) segment that is absent in γ_2DT-A. The point mutation in *tox-176* results in a Gly-to-Asp substitution at residue 128 (7), as indicated. Abbreviations: X, *Xba*I; N, *Nco*I; B, *Bam*HI; S, *Sau*3A; Bg, *Bgl*II; P, *Pst*I; E, *Eco*RI.

"hot spots" for expression without resulting in embryo lethality.

MATERIALS AND METHODS

DNA constructs. The 2.98-kilobase-pair (kbp) *Xba*I-*Eco*RI fragment from p$\gamma_2$tox176 (Fig. 1) was used to generate transgenic mice. p$\gamma_2$tox176 was derived from pγ_2DT-A (3) by replacing the 2.2-kbp *Nco*I-*Eco*RI fragment of pγ_2DT-A with the corresponding fragment of pTH1-176 (7).

Generation and genotyping of transgenic animals. Transgenic mice carrying the γ_2-crystallin–tox176 ($\gamma_2$tox176) construct were produced on an outbred CD1 background essentially as described by Hogan et al. (5). To identify animals transgenic for the $\gamma_2$tox176 construct, DNA was prepared from tail biopsies by conventional procedures (5) and the presence of the transgene was detected by Southern blot analysis (12) with the *tox-176*-specific 1.46-kbp *Bam*HI fragment labeled by nick translation (11) or random priming (4) as a probe. Homozygous $\gamma_2$tox176/$\gamma_2$tox176 animals were identified by Southern blot (12) hybridization of genomic DNA with the intact 2.98-kbp *Xba*I-*Eco*RI $\gamma_2$tox176 fragment, which includes the *tox-176* gene, simian virus 40 splicing and polyadenylation signals, and mouse γ_2-crystallin sequences spanning −759 to +45 relative to the transcriptional start site. The mouse γ_2-crystallin sequences in the probe allowed standardization of the transgene copy number relative to the endogenous γ_2-crystallin gene.

RNA analysis. Total RNA was extracted from the eyes of 1-day-old normal and transgenic mice hemizygous for the $\gamma_2$tox176 construct, electrophoresed through 1.5% formaldehyde-agarose gels, and blotted to nitrocellulose. Conditions for prehybridization and hybridization were as described previously (3). Probes for Northern (RNA) blot analysis included a 166-bp *Xho*II-*Stu*I fragment derived from mouse γ_2-crystallin cDNA (8) and the *tox-176*-specific 1.46-kbp *Bam*HI fragment.

Histologic studies. Eyes were fixed in Zenker fixative, dehydrated through graded alcohols, and embedded in paraplast as previously described (2). Sections (6 μm thick) were stained with hematoxylin and eosin.

RESULTS

Transgenic mice with a γ_2-crystallin–*tox-176* gene. To evaluate the potential of an attenuated toxin gene for genetic ablation studies, we used the mouse γ_2-crystallin promoter to target the expression of the *tox-176* mutant (see Fig. 1) to the ocular lens. The *tox-176* gene has been estimated to be 30-fold less cytotoxic than the wild-type DT-A gene as the result of a point mutation that results in a Gly-to-Asp amino acid substitution at residue 128 (7). We had shown previously that transgenic mice hemizygous for the native DT-A gene driven by the mouse γ_2-crystallin promoter manifest microphthalmia as the result of the ablation of central nuclear lens fiber cells (3). In the experiments described below, we used the same promoter to target the expression of the *tox-176* gene. This permitted us to compare directly the ocular phenotypes induced by both types of toxin gene constructs.

Fertilized CD1 mouse eggs were microinjected with the 2.98-kbp $\gamma_2$tox176 *Xba*I-*Eco*RI fragment and implanted into pseudopregnant foster mothers. Out of 121 offspring recovered, we obtained 11 transgenic mice displaying a marked ocular phenotype. Significantly, none of these animals showed the microphthalmia that was characteristic of all five transgenic mice that were hemizygous for the wild-type γ_2DT-A gene (3; unpublished data). Instead, all showed an ocular phenotype that was either less or more severe than that produced by the wild-type construct. Seven of the founder animals had cataracts with occasional slight microphthalmia, whereas the other four showed clinical anophthalmia, which was found on histological analysis to be severe microphthalmia (Fig. 2 and 3). Backcrosses to CD1 mice established that both phenotypes could be transmitted by the founder animals to their transgenic F_1 offspring. For some lines the phenotype of the founder was faithfully transmitted to transgenic offspring, whereas for others some variability in phenotypic transmission was observed. For example, all transgenic offspring of Tg10B and Tg9 showed clinical anophthalmia and cataracts, respectively, just as the founders did. In contrast, 17 of 114 transgenic progeny derived from the cataractous founder Tg3 showed microphthalmia or clinical anophthalmia and, in an extreme case, 24 of 46 transgenic offspring derived from the clinically anophthalmic founder Tg1 showed the milder phenotypes of microphthalmia or cataracts. Thus, for these lines, the

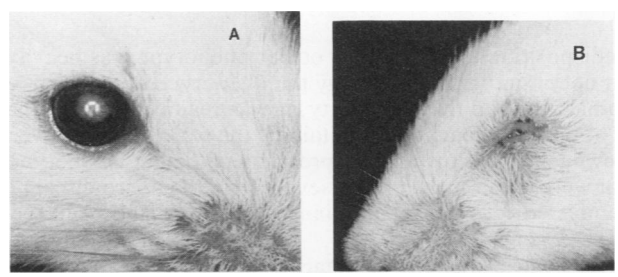

FIG. 2. Ocular phenotypes of $\gamma_2$tox176 transgenic mice showing a cataract (A) or clinical anophthalmos (B).

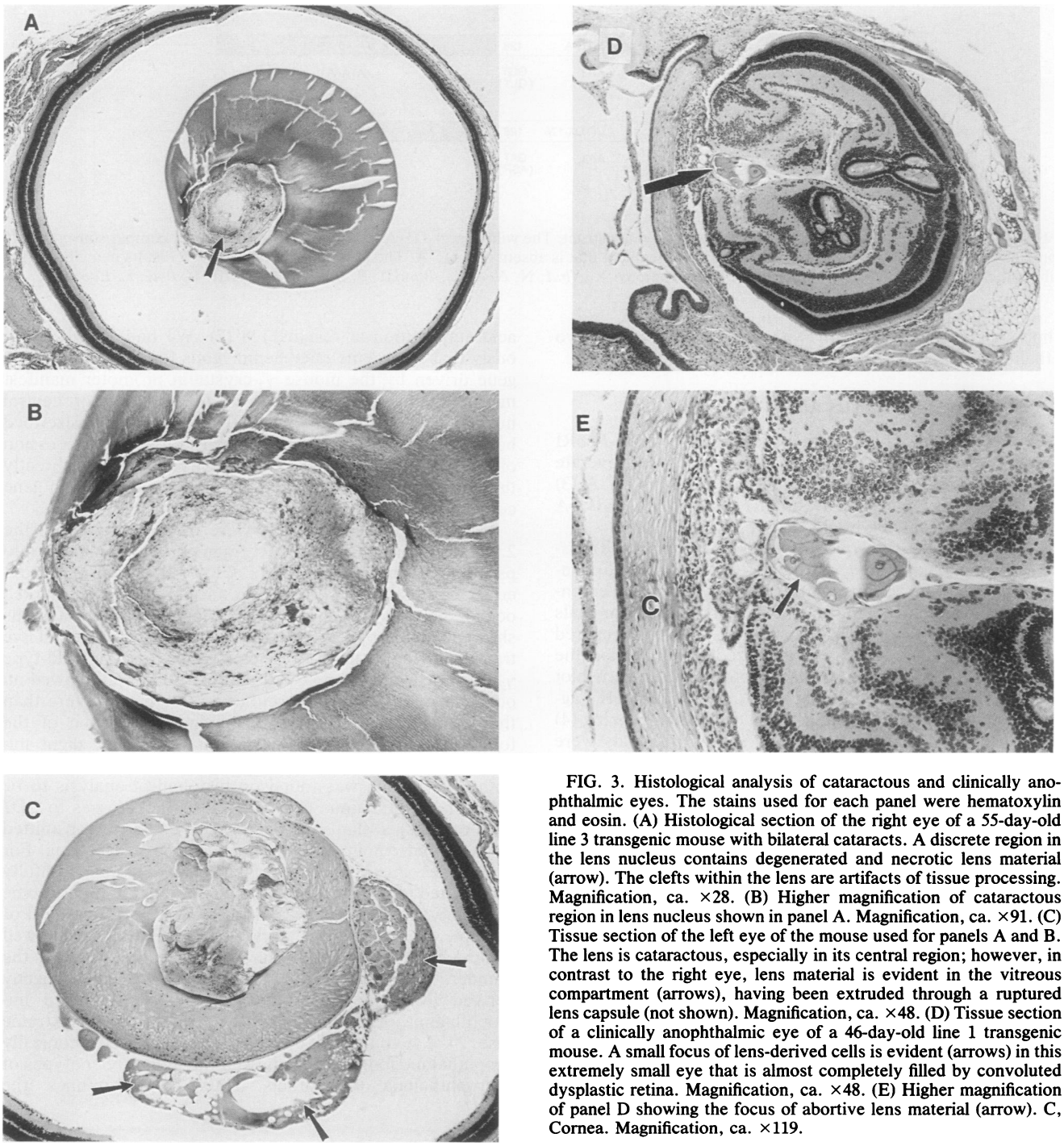

FIG. 3. Histological analysis of cataractous and clinically anophthalmic eyes. The stains used for each panel were hematoxylin and eosin. (A) Histological section of the right eye of a 55-day-old line 3 transgenic mouse with bilateral cataracts. A discrete region in the lens nucleus contains degenerated and necrotic lens material (arrow). The clefts within the lens are artifacts of tissue processing. Magnification, ca. ×28. (B) Higher magnification of cataractous region in lens nucleus shown in panel A. Magnification, ca. ×91. (C) Tissue section of the left eye of the mouse used for panels A and B. The lens is cataractous, especially in its central region; however, in contrast to the right eye, lens material is evident in the vitreous compartment (arrows), having been extruded through a ruptured lens capsule (not shown). Magnification, ca. ×48. (D) Tissue section of a clinically anophthalmic eye of a 46-day-old line 1 transgenic mouse. A small focus of lens-derived cells is evident (arrows) in this extremely small eye that is almost completely filled by convoluted dysplastic retina. Magnification, ca. ×48. (E) Higher magnification of panel D showing the focus of abortive lens material (arrow). C, Cornea. Magnification, ca. ×119.

predisposition to a particular ocular phenotype was heavily, but not entirely, influenced by the phenotype of the founder animal. Despite this variability, it was readily apparent that all of the transgenic lines containing the γ_2*tox-176* gene were skewed heavily towards expression of ocular phenotypes that were either more or less severe than the microphthalmia manifested by transgenic lines containing the wild-type γ_2DT-A gene (2, 3).

Histological analysis of cataractous and clinically anophthalmic eyes. To understand the cellular basis for the marked difference in ocular phenotype amongst the $\gamma_2$tox176 cataractous and clinically anophthalmic transgenic mice and to study the effects of the *tox-176* gene on lens organogenesis, we carried out histological analyses of the eyes of transgenic F_1 animals. Figure 3A shows a section through the right eye of an animal with bilateral cataracts sacrificed at 55 days. The lens appeared normal in size; however, a dense, well-demarcated cataract was apparent in the central nuclear region of the lens, comprising those fiber cells that are laid down early during organogenesis and that are specifically ablated in transgenic mice carrying the wild-type DT-A gene driven by the mouse γ_2-crystallin promoter (2). Higher

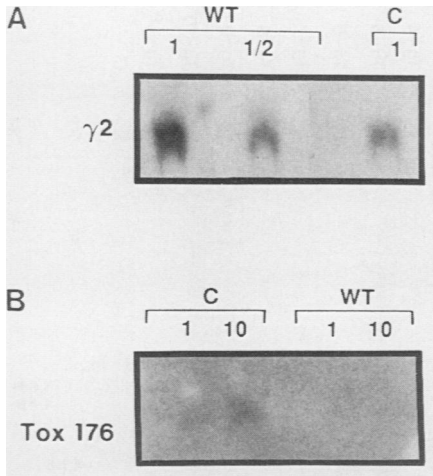

FIG. 4. Northern blot analysis of γ_2-crystallin and $\gamma_2$tox176 transcripts in the eyes of wild-type and cataractous transgenic mice. Total eye RNAs prepared from newborn wild-type (WT) and line 11 cataractous (C) transgenic mice were electrophoresed through 1.5% formaldehyde-agarose gels, blotted to nitrocellulose, and hybridized to a γ_2-specific subprobe derived from mouse γ_2-crystallin cDNA (A) and the *tox-176* 1.46-kbp *Bam*HI fragment (B). The relative eye equivalents of total RNA loaded in each lane are indicated at the top. Filters were exposed for 1 (A) and 3 (B) days.

magnification revealed that the cataract consisted of an amorphous cellular mass, most likely representing necrotic or degenerating fiber cells (Fig. 3B). The other eye of this animal contained an abnormal lens and an abnormally thin lens capsule through which extensively vacuolated lens material had herniated into the vitreous body (Fig. 3C). When we examined the eye of a transgenic animal with clinical anophthalmia, we found that it contained only a small rudiment of a lens (Fig. 3D and E). In addition, the small eye lacked a discernible anterior chamber and vitreous body and showed the typical convolution of dysplastic sensory retina that is characteristic of transgenic mice in which cells of the lens lineage have been extensively ablated (2, 6).

Gene expression in cataractous lenses. The observations described above are consistent with the idea that the lenses of transgenic mice bearing cataracts contain a significant fraction of nuclear fiber cells that express the *tox-176* gene and yet are able to persist for prolonged periods before dying. Hence, the levels of attenuated toxin that accumulate in the central fiber cells are probably insufficient to effect rapid cell death. In this case, the effects of the *tox-176* gene on crystallin gene expression would be expected to be only marginal in very young animals. Northern blot analyses revealed that the level of γ_2-crystallin transcripts in lenses derived from newborn mice of one of the cataractous lines (Tg11) was reduced only twofold (Fig. 4A) compared with the >30-fold reduction for microphthalmic mice carrying the wild-type γ_2DT-A gene (2). More important, it was possible to detect a low level of tox176 transcripts in these cataractous lenses (Fig. 4B), consistent with the view that subthreshold or nonlethal levels of tox176 protein are synthesized in the central nuclear fiber cells during maturation of the embryonic lens.

Ocular phenotype and transgene copy number. Two possible explanations could account for our observation that independent lines of $\gamma_2$tox176 transgenic mice display predominantly either cataracts or clinical anophthalmia. First, the difference between lines could be due to variation in the number of copies of the $\gamma_2$tox176 construct in the germ line. In this case, transgenic lines with clinical anophthalmia would be expected to have a significantly higher transgene copy number than transgenic lines showing predominantly cataracts. Second, the difference in ocular phenotypes could be due to chromosomal-position effects that modulate the extent of transgene expression. To distinguish between these possibilities, genomic DNA samples from the different transgenic lines were prepared from tail biopsies and analyzed by Southern blotting.

Figure 5A shows the number of discrete insertions of the transgene in the different transgenic lines detected after digestion with *Sac*I, a restriction endonuclease which does not cut within $\gamma_2$tox176, and hybridization with a tox176 probe. As is usually found to be the case, the microinjected DNA had integrated at a single chromosomal site in most of the transgenic lines. However, two of the lines with clinical anophthalmia, Tg7 and Tg10, contained two discrete insertions of the transgene. The two inserts in Tg10 segregated independently during subsequent mating, yielding two sublines, Tg10A and Tg10B, both of which showed the clinical anophthalmia characteristic of the founder. Tg7, on the other hand, proved to be a sterile male, prohibiting subsequent analysis of phenotype transmission. The cause of the sterility of this animal was not investigated.

To estimate the transgene copy number in the different transgenic lines, the genomic DNAs were digested with *Bam*HI, which cuts twice within the transgene to liberate an internal 1.46-kbp fragment, and hybridized with the intact $\gamma_2$tox176 construct (Fig. 5B). The transgene copy number was then estimated by comparing the intensity of the 1.46-kbp *tox-176*-specific band with that of the endogenous γ-crystallin bands. Overall, there was no significant difference in the range of copy numbers observed for the transgenic lines predisposed to clinical anophthalmia and the lines showing predominantly or exclusively cataracts (Table 1). On average, the lines with clinical anophthalmia predominant contained 2.4 copies of the transgene, which was not significantly different from the average of 1.9 copies found for the lines with predominantly or exclusively cataracts. More importantly, only a single copy of the $\gamma_2$tox176 construct was present in one of the lines showing exclusively the severe ocular phenotype (line Tg10A). On the basis of these observations, we conclude that the differences in ocular phenotype manifested by the cataractous and clinically anophthalmic transgenic lines are not due to differences in gene dosage and hence are more likely to be consequences of chromosomal-position effects that influence the extent of transgene expression.

DISCUSSION

The present study was undertaken to evaluate the use of an attenuated version of the DT-A gene for genetic ablation studies, as it seemed likely that the extreme cytotoxicity of the native DT-A gene (one molecule of the DT-A chain is cytotoxic [13]) in conjunction with chromosomal position effects might sometimes lead to embryo lethality. Using the mouse γ_2-crystallin promoter to target the expression of the mutant *tox-176* gene, we generated transgenic mouse lines that could be classified into two major groups: one group which comprised lines that exhibited predominantly or exclusively cataracts and a second group which comprised lines that displayed predominantly or exclusively clinical anophthalmia. These disparate ocular phenotypes consti-

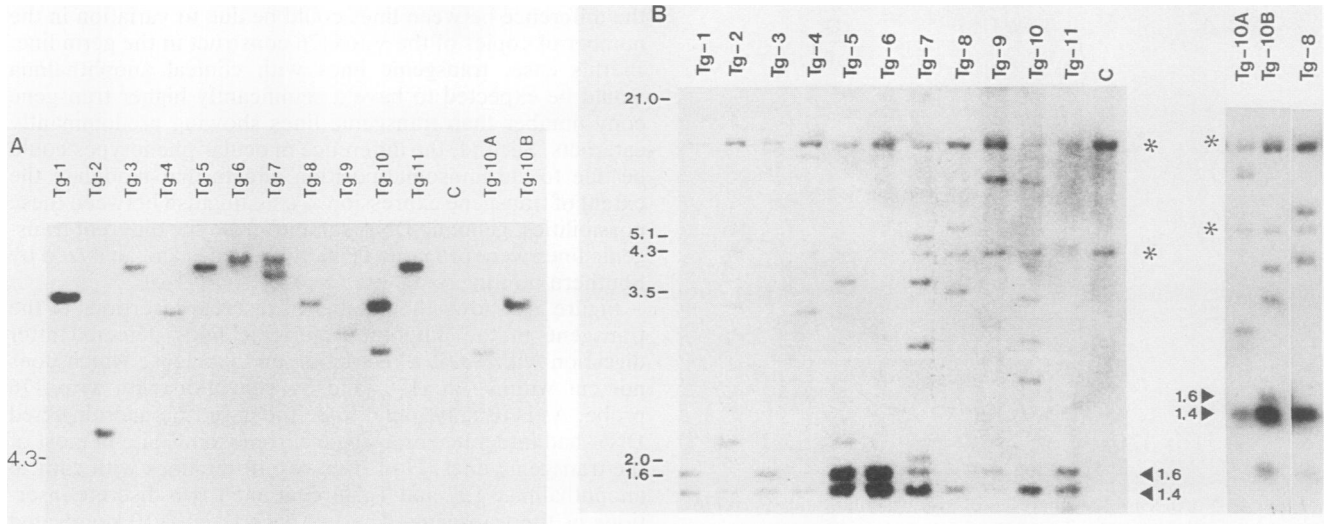

FIG. 5. Southern blot analysis of $\gamma_2$tox176 sequences in transgenic mouse lines displaying predominantly cataracts or clinical anophthalmia. (A) Mouse genomic DNAs were digested with *Sac*I, which does not cut within the $\gamma_2$tox176 sequence, and hybridized to a *tox-176*-specific probe. (B) Mouse DNAs were digested with *Bam*HI and hybridized to the $\gamma_2$tox176 construct. Asterisks mark endogenous γ-crystallin bands hybridizing to the $\gamma_2$tox176 probe. Arrowheads denote the internal *tox-176* 1.46-kbp *Bam*HI fragment and the 1.6-kbp *Bam*HI fragment which is generated upon head-to-tail integrations of the $\gamma_2$tox176 transgene. The transgene copy number was estimated from the intensity of the *tox-176*-specific 1.46-kbp band relative to that of the endogenous γ-crystallin bands. The relative intensities of these bands for a given transgenic line showed slight variation in independent hybridizations, presumably reflecting differences in the labeled sequence complexity of independently prepared $\gamma_2$tox176 probes (compare Tg8 in panels A and B). All results shown in panel B are for transgenic F_1 animals, except for Tg7, Tg9, and Tg10, which were the founders. Tg7 was a sterile founder. Tg10 contained two insertions which segregated from each other, yielding the sublines Tg10A and Tg10B. Tg9 transmitted only the 9.6-kbp *Bam*HI fragment at detectable frequency, indicating that a noninactivating deletion or rearrangement had occurred in the $\gamma_2$tox176 construct. Lanes C, DNA from a wild-type control mouse.

tuted an apparent paradox, since they were at the same time both less severe and more severe than the microphthalmia that was displayed by all five previously generated transgenic lines carrying the wild-type DT-A gene. The difference between the two groups of $\gamma_2$tox176 mice could not be explained solely on the basis of differences in gene dosage and hence must be at least in part due to chromosomal-position effects. Such effects have previously been described for randomly integrated transgenes, resulting in ectopic or variable levels of expression, or both, among independent transgenic lines (10).

TABLE 1. Number of inserts and dosage of transgene in $\gamma_2$tox176 transgenic mouse lines

Transgenic line	Phenotype	No. of:	
		Inserts[a]	Copies[b]
Tg-1	Clinical anophthalmia	1	2
Tg-5	Clinical anophthalmia	1	4
Tg-7	Clinical anophthalmia	2	3
Tg-10	Clinical anophthalmia	2	3
Tg-10A	Clinical anophthalmia	1	1
Tg-10B	Clinical anophthalmia	1	2
Tg-2	Cataract	1	1
Tg-3	Cataract	1	2
Tg-4	Cataract	1	1
Tg-6	Cataract	1	4
Tg-8	Cataract	1	1
Tg-9	Cataract	1	2
Tg-11	Cataract	1	2

[a] Determined by digesting cellular DNA with *Sac*I, a restriction enzyme which does not cut with the transgene.

[b] Determined by digesting cellular DNA with *Bam*HI, a restriction enzyme which cuts twice within the transgene.

The disparate ocular phenotypes manifested by the two groups of $\gamma_2$tox176 mice can be explained most simply if there exist two major classes of integration sites: one class which is particularly favorable to the expression of the $\gamma_2$tox176 transgene that predisposes to clinical anophthalmia and a second class which is considerably less favorable to transgene expression that predisposes to the less severe phenotype of cataracts. On the basis of the relative incidence of obtaining these two phenotypes, it appears that there is not a large difference in the frequencies of the two classes of site in the germ line. However, we cannot rule out the possibility that there is an experimental bias which precludes detection of a fraction of the sites that are favorable to expression. This would be true, for example, if some of these sites were to evoke ectopic expression leading to embryo lethality.

The results obtained with the tox176 construct can be contrasted with our previous observations with transgenic lines hemizygous for the native DT-A gene (2, 3). These animals were almost always microphthalmic; all five founder animals generated were microphthalmic, and after extensive mating only 5 of 162 transgenic progeny exhibited bilateral severe microphthalmos (clinical anophthalmia) in the hemizygous state. The most likely explanation for this finding is that the more potent γ_2DT-A construct, unlike the γ_2*tox-176* gene, is lethal after integration into sites that are favorable to expression. This would occur if such sites conferred a low level of ectopic expression or if the mouse γ_2-crystallin promoter contained a low level of intrinsic leakiness. In either case, the extreme cytotoxicity of the DT-A gene would lead to embryo lethality. According to this model, microphthalmic animals carrying the native DT-A gene would be analogous to cataractous lines carrying the *tox-176*

gene in that both phenotypes result from integration events at chromosomal sites that are not conducive to transgene expression. The potential lethality of the γ_2DT-A construct suggests that the frequency of obtaining transgenic animals with the γ_2DT-A gene should be lower than that with the γ_2*tox-176* gene. While we did obtain a higher percentage of transgenic animals with the tox176 construct (10%) than with the DT-A gene (5%), the number of transgenic animals produced in our study was insufficient for this difference to be statistically significant.

Although the site of transgene integration can account for the phenotypic difference between the cataractous and clinically anophthalmic lines, it does not explain the phenotypic heterogeneity observed within some of the transgenic pedigrees. In an earlier paper (2), we reported a more subtle degree of phenotypic heterogeneity among lenses of microphthalmic animals carrying the wild-type γ_2DT-A transgene that was evident only upon histological analysis. In that paper, we show that this variability correlated with the extent of ablation of γ_2-crystallin-producing cells and was unlikely to be due to somatic mutation. This led us to propose that the variability in the extent of ablation within a transgenic pedigree could be explained by epigenetic mechanisms that modulate transcriptional activation of the transgene. In the work reported here, the more striking phenotypic heterogeneity observed within some of the $\gamma_2$tox176 pedigrees presumably stems from the fact that the $\gamma_2$tox176 construct is less cytotoxic than the wild-type DT-A gene and therefore that somatic expression of the transgene may not always occur at levels sufficient to result in cell killing. Consequently, quantitative as well as qualitative differences in transgene expression, resulting from epigenetic mechanisms, would be expected to contribute to phenotypic heterogeneity, producing an even greater variability in the extent of genetic ablation. We therefore conclude that there are three parameters which can influence ocular phenotype: (i) expression of the *tox-176* gene as opposed to the wild-type DT-A gene, (ii) the site of transgene integration, and (iii) epigenetic mechanisms affecting transgene expressivity.

Our studies with the $\gamma_2$tox176 construct also showed that it is possible to generate ocular phenotypes in which the levels of toxin that accumulate in central nuclear lens fiber cells are not acutely lethal but rather lead to eventual cellular death over a considerable period. Thus, it should be possible to use attenuated toxin genes in conjunction with appropriate promoter elements to create transgenic models of a variety of degenerative disorders. Such technology might be particularly useful when applied to cellular components of the nervous system.

Finally, our experience with the *tox-176* gene suggests that the use of attenuated toxin genes should expand the range of promoter elements that might be used to effect ablation. One example would be those promoter elements that are highly active in only one or a few nonessential cell types but are otherwise constitutively active at a very low level. In this connection, we have recently observed that for the promoter of the myelin basic protein gene, it has been possible to obtain viable transgenic animals only after targeting the less-virulent *tox-176* gene (unpublished observations). Thus, the utility of genetic ablation to address questions concerning cell fate and organogenesis in the developing mammalian embryo should expand as the result of further definition of tissue-specific promoter elements together with the use of attenuated forms of the DT-A gene.

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