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Enlargement of the Globe With Ocular Malformations in c-*Myc* Transgenic Mice

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Purpose: To study the ocular development in transgenic mice carrying the mouse c-*myc* gene under the control of the Mx gene promoter (Mx-c-myc).

Methods: Transgenic mice were generated by standard techniques. For histological studies, the tissues were fixed with 10% buffered formalin, embedded in paraffin according to the standard procedure and sliced in 4-μm sections. c-*Myc* expression was investigated by reverse transcriptase–polymerase chain reaction and Southern blot analysis.

Results: A line of the Mx-c-myc mice displayed progressive enlargement of the globe with other ocular malformations. Histologically, the enlarged eyes exhibited closed cornea-iris angle, microphakia, corneal epithelial disorders, and attenuation of the inner retinal layers. Developmental analysis of eyes from these Mx-c-myc mice revealed irregular development of the iris and ciliary body at embryonic day 15.5 and the closed angle at 1 week of age. Leaky exogenous c-*myc* expression was detected in cornea, iris, lens, and retina from the Mx-c-myc mice by reverse transcriptase–polymerase chain reaction and Southern blot analysis. No other developmental abnormalities were observed in the Mx-c-myc mice. The anterior segment of the enlarged eyes showed the closed angle with elongation of the iris and ciliary body. There was no attenuation in the outer retinal layers from the outer plexiform layer to the retinal pigment epithelium.

Conclusions: We conclude that the buphthalmos and accompanying changes were not due to expression of the exogenous c-*myc* in cornea and retina but may be the secondary changes of elevated intraocular pressure. We suggest that Mx-c-myc mice can serve as a useful model for investigating the development of the anterior segment and the genesis of buphthalmos. **Jpn J Ophthalmol 1999;43:201–208**

Key Words: Buphthalmos, c-*myc*, microphakia, ocular malformation, transgenic mouse.

Introduction

The technical progress in manipulating the mouse genome including transgenic and knockout technologies has generated many types of mutant mice with various phenotypes. Some mice have been regarded as models of human disease and proved to have great potential for investigating the etiology, pathogenesis, and possible therapy of various human diseases.[1]

Ocular malformation is one of the frequent phenotypes in mutant mice. More specifically, anterior segment anomalies have been reported in several lines of mutant mice,[2–6] suggesting that the development of the anterior segment is strictly controlled by many biological molecules.

The c-*myc* gene was identified as the cellular homologue of the oncogene of avian retroviruses.[7] c-*Myc* functions in part as a sequence-specific transcription factor to transactivate gene expression.[8,9] During ontogeny, c-*myc* transcripts have been detected in a wide variety of developing tissues,[10] and c-*myc* is well correlated with active proliferation[11] in midgestational mouse embryos. Furthermore, study

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of c-*myc* null mutant mice revealed that c-*myc* plays an essential role in embryogenesis.[12] During the maturation of the lens cells, arrest in growth and differentiation of the epithelial cells are associated with downregulation of c-*myc* expression.[13] Because the constitutive expression of c-*myc* in the lens fiber cells of transgenic mice resulted in microphakia,[14] downregulation of c-*myc* expression is necessary for proper development of the lens tissue. Although many lines of c-*myc* transgenic mice with various promoters have been reported, none of these transgenic mice display ocular malformations that result in buphthalmos.

To investigate the role of c-*myc* in mouse development, we have generated transgenic mice carrying the mouse c-*myc* gene. In this study, we present a line of c-*myc* transgenic (Mx-c-myc) mice that exhibited progressive enlargement of the globe with other ocular malformations.

Materials and Methods

Construction of the Mx-c-myc Transgene

The Mx-c-myc transgene was constructed using a 1.7-kb BamHI fragment containing the Mx gene promoter[15] in the pMxhGH[16] and a 2.8-kb BamHI–HindIII fragment of the mouse c-*myc* genomic gene inserted into the multiple cloning sites of the pBluescriptIISK$^+$ (Promega Corp., Madison, WI, USA). Because exon 1 of the c-*myc* gene encodes a nontranslated region with a negative regulatory element, the region including exons 2 and 3 of the gene was used for the construction. A 160-bp HindIII–KpnI fragment of the SV40 polyadenylation signal sequence was inserted into the 3′ end of the c-*myc* fragment in the pBluescriptIISK$^+$ (Figure 1).

Generation and Screening of Transgenic Mice

Transgenic mice were generated by the method described by Hogan et al[17] Briefly, a 4.7-kb EcoRI–KpnI fragment of the Mx-c-myc gene was microinjected into the pronucleus of fertilized eggs from C57BL/6CrSlc mice (Japan SLC, Hamamatsu). The injected eggs were returned to the oviducts of pseudopregnant females from the ICR strain (Japan CLEA, Tokyo). The day after the plugging was defined as embryonic day 0.5. All experimental procedures conformed to the ARVO Resolution on the Use of Animals in Research.

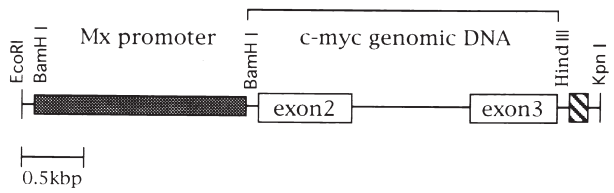

Figure 1. Map of Mx-c-myc transgene. Hatched bar represents SV40 polyadenylation signal sequence.

Screening of transgenic mice was performed by Southern blot analysis.[18] Briefly, 10 μg of genomic DNA extracted from the tail of each mouse was transferred onto a nylon membrane (Hybond N; Amersham International, Buckinghamshire, UK) and fixed by ultraviolet irradiation with Spectrolinker (Schleicher & Schuell, Dassel, Germany) and by baking at 80°C for 30 minutes. The filter was prehybridized for 1 hour then hybridized with a digoxigenin-labeled probe (10 ng/mL) overnight at 42°C in 50% formamide hybridization buffer. After hybridization, the filter was washed twice for 5 minutes with 2× SSC and 0.1% SDS at room temperature and twice for 15 minutes with 0.1× SSC and 0.1% SDS at 42°C. The digoxigenin-labeled probe was detected with sheep antidigoxigenin antibody conjugated with alkaline phosphatase. The antibody detection reaction was performed using the enhanced chemiluminescent detection system (Boehringer Mannheim, Mannheim, Germany). A 230-bp BamHI–HindIII fragment of the SV40 polyadenylation signal was subcloned into PGEM vectors (Promega) and labeled with digoxigenin (Boehringer Mannheim) by polymerase chain reaction with T7 and SP6 primers as a probe.

Reverse Transcriptase–Polymerase Chain Reaction

The level of c-*myc* mRNA in various eye tissues was determined by reverse transcriptase–polymerase chain reaction and Southern blot analysis. Briefly, total RNA was extracted from each tissue using Trizol Reagent (Gibco BRL, Gaithersburg, MD, USA). cDNA was made from 100 ng of the total RNA by reverse transcribing the RNA with oligo(dT) as the primer at 42°C for 15 minutes in a volume of 20 μL. Polymerase chain reaction was performed on 1 μL of the cDNA with 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim), 2 mmol/L $MgCl_2$, 0.2 mmol/L dNTP solution, and primers in a volume of 25 mL. The polymerase chain reaction consisted of 30 cycles with denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1.5 minutes. The exogenous and the endogenous c-*myc* cDNA were amplified with a set of primers specific for the sequences of the exon 2 (P2) and the SV40 polyadenylation signal (P4), and for the se-

quences of exon 1 (P1) and exon 3 (P3) of the c-*myc* gene, respectively. Sequences of the primers were: P1, 5′-TAAGAAGGCAGCAGCTGGAGT-3′; P2, 5′-TATCACCAGCAACAGCAGAG-3′; P3, 5′-CCTCTTCTCCACAGACACCA-3′; and P4, 5′-TAGAGTCGACCTGCAGGCAT-3′.

After 30 cycles of the chain reaction, 10 μL of the products were separated on a 1% agarose gel and transferred on to a nylon membrane (Amersham International). The c-*myc* cDNA was detected by Southern blot as described above. The digoxigenin-labeled probe was made by polymerase chain reaction on a 400-bp PstI fragment in exon 2 of the genomic c-*myc* gene. Polymerase chain reaction for the glyceraldehyde-3-phosphate dehydrogenase cDNA was performed as control for the amount of the cDNA as described previously.[19] The glyceraldehyde-3-phosphate dehydrogenase cDNA in 10 μL of the polymerase chain reaction products was detected by gel electrophoresis with ethidium bromide.

Histological Analysis

The samples were fixed in 10% buffered formalin, embedded in paraffin according to standard procedures and cut in 4-μm sections. The sections were dewaxed in xylene, redehydrated through an ethanol series, and stained with hematoxylin and eosin.

Results

Ocular Abnormality in Mx-c-myc Mice

To investigate the role of the c-*myc* protooncogene in each step of the development of the mouse eye, we generated transgenic mice carrying the exogenous c-*myc* gene under the control of the mouse Mx gene promoter (Figure 1) that was reported to be inducible by interferon α/β in most tissues.[15] Unexpectedly, the line of Mx-c-myc mice displayed progressive enlargement of the eyes with development of corneal opacity even without induction by interferon α/β. At 6 weeks of age, unilateral or bilateral enlargement of eyes was evident in about 30% (10 of 32) of the Mx-c-myc mice as shown in Figure 2A. Comparison of eyes from the Mx-c-myc mice with those from control littermates (nontransgenic littermates) revealed enlargement of the globe with microphakia (Figure 2B). No developmental abnormality other than the eyes was observed in the Mx-c-myc mice.

We examined the enlarged eyes histologically and compared them with the control eyes at 6 weeks of age. The anterior segment of the enlarged eyes showed that the corneo-iris angle was closed with elongation of the iris and ciliary body (Figures 3A, 3C). The opaque corneas displayed thinning of the corneal epithelium with vesicular changes on the external surface (Figure 3E) indicating an edematous state.

In the posterior segment of the enlarged eyes, the retina was thinner (Figure 3A). Higher magnification revealed attenuation of the inner retinal layers from the nerve fiber layer to the inner nuclear layer (Figure 4A). A dramatic decrease in the cell number was also observed in the ganglion cell layer. However, there was no attenuation in the outer retinal layers from the outer plexiform layer to the retinal pigment epithelium.

These alterations resembled the ocular lesions caused by long-standing glaucoma in humans.[20] The corneal and retinal lesions were observed in the enlarged eyes from 4 weeks of age, but not in nonenlarged eyes from the Mx-c-myc mice (data not shown).

Developmental Stages of Mx-c-myc Eyes

To determine the pathogenesis of the ocular malformations in the Mx-c-myc eyes, we examined the anterior segment histologically at different developmental stages. No apparent abnormality was detected in eyes from the Mx-c-myc embryos until embryonic day 14.5. From embryonic day 15.5, eyes from the Mx-c-myc embryos could be distinguished from those of the control littermates by external observation. As shown in Figure 5A, the developing pupil

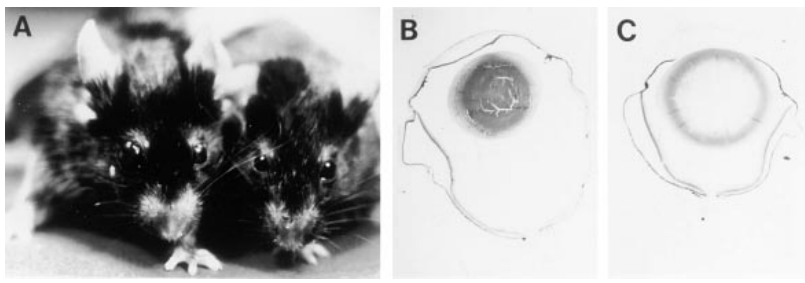

Figure 2. Comparison of Mx-c-myc and control mice eyes at 6 weeks of age. (**A**) Eyes of Mx-c-myc mouse (left) are clearly larger than eyes of control littermate (right). Histological specimen of enlarged eye (**B**) and control littermate eye (**C**) (×5, hematoxylin-eosin stain).

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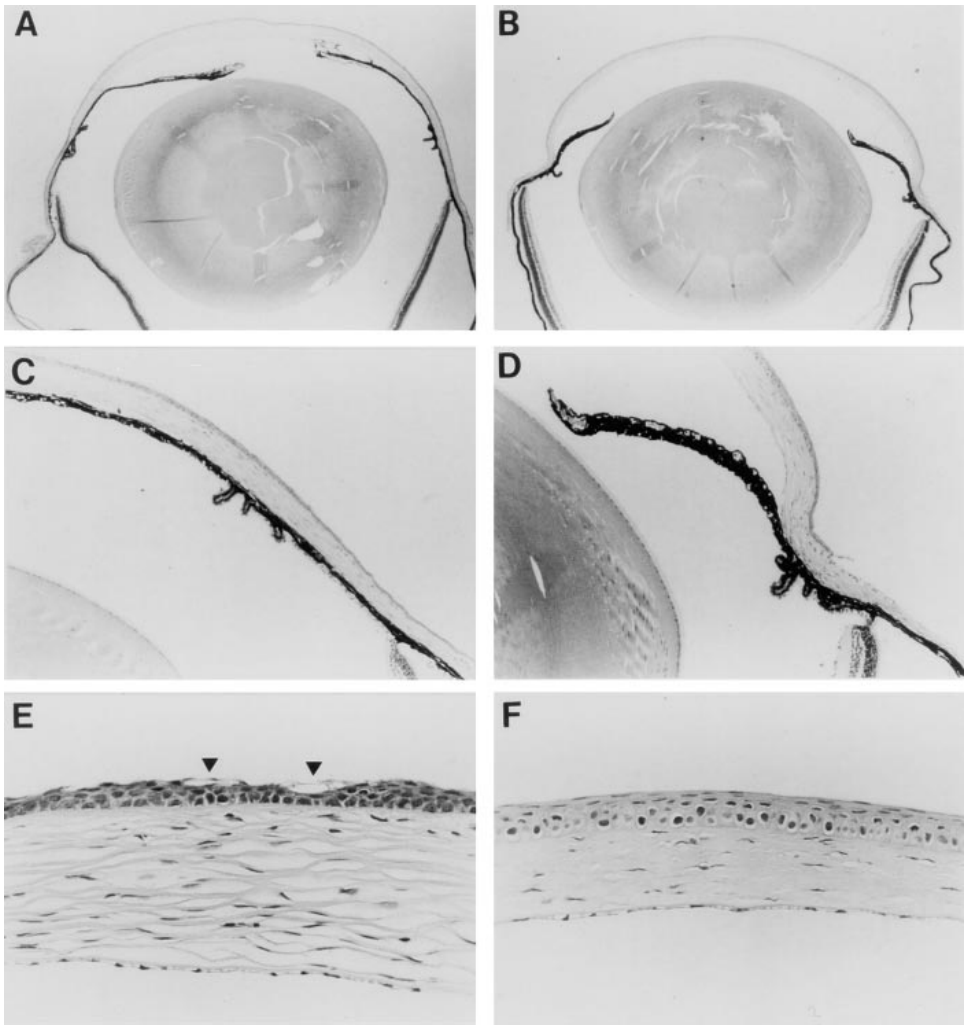

Figure 3. Histological analysis of eyes from Mx-c-myc mice and control littermate at 6 weeks of age. Anterior segment of Mx-c-myc eye (**A**) and control littermate eye (**B**) (×10, hematoxylin-eosin stain). Angle of Mx-c-myc eye (**C**) and control eye (**D**) (×25 hematoxylin-eosin stain). Cornea of Mx-c-myc eye (**E**) and control eye (**F**) (×100, hematoxylin-eosin stain). Many vesicular formations (arrowheads) are observed on the corneal surface of Mx-c-myc eye.

of Mx-c-myc embryos was narrow and irregularly shaped, whereas the pupil of control littermates was wide and rounded. Histological analysis (Figures 5B, D) revealed that the irregularity in the shape of the developing pupil of the Mx-c-myc eyes could be attributed to abnormal elongation of the tip of the optic cup, which is believed to be the rudiment of the iris and ciliary body.[21] Microphakia was also observed in the Mx-c-myc eyes (Figure 5B).

At 1 week of age, the closed angle with elongation of the iris and ciliary body was observed in eyes from the Mx-c-myc mice (Figure 6A). The angle of all eyes from control littermates at this stage was open (Figure 6B). Because microphakia was also observed in eyes from the Mx-c-myc embryos at 1 week of age, the diameter of the lens was measured. The mean diameter (1.39 ± 0.07 mm) of the lens from the Mx-c-myc mice (22 mice) was smaller than that (1.65 ± 0.06 mm) of the control littermates (14 mice) ($P < .01$).

*Exogenous c-*myc *Expression in Mx-c-myc Mice Ocular Structures*

Expression of the exogenous and the endogenous c-*myc* was analyzed in total RNA extracted from each ocular structure of the Mx-c-myc mice by reverse transcriptase–polymerase chain reaction and Southern blot method. Leaky exogenous c-*myc* ex-

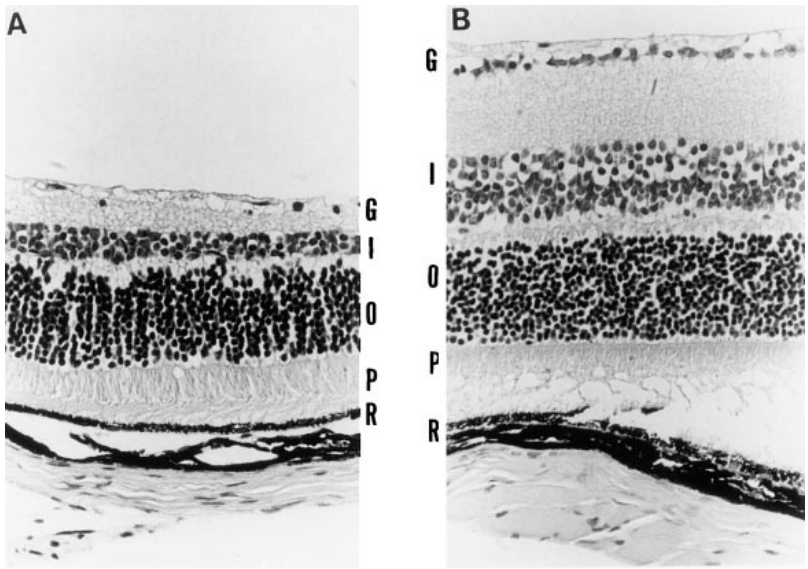

Figure 4. Histological analysis of retina in enlarged Mx-c-myc eye (**A**) and control eye (**B**) at 6 weeks of age. (×50, hematoxylin-eosin stain). G: ganglion cell layer, I: inner nuclear layer, O: outer nuclear layer, P: photoreceptor layer, R: retinal pigment epithelium. Attenuation of inner retinal layers is observed in enlarged eye.

pression was detected in the cornea, iris, lens, and retina of Mx-c-myc mice at 4 weeks of age (Figure 7). Endogenous c-*myc* expression was also detected in the same ocular structure from both the Mx-c-myc mice and the control littermates. Exogenous c-*myc* expression was also detected in total RNA from the whole eyeball of the Mx-c-myc mice at embryonic day 15.5, 17.5, and at birth (data not shown).

Discussion

We have presented a line of Mx-c-myc mice that exhibited novel ocular malformations. These mice displayed progressive enlargement of the globe with development of corneal opacity. Similar changes are associated with buphthalmos in congenital glaucoma. Indeed, some of the histological findings of the enlarged eyes, including the closed angle, corneal lesions, and the retinal attenuation, support the suggestion that the ocular changes resulted from elevated intraocular pressure. The closed angle that obstructs outflow of aqueous humor from trabecular and uveoscleral pathways could be explained as the primary pathogenesis of elevated intraocular pressure. Because only the enlarged eyes exhibited the corneal and retinal lesions, these lesions may not be due to expression of the exogenous c-*myc* in cornea and retina but may be secondary changes of elevated intraocular pressure. However, it cannot be concluded that enlargement of the eyes is due to elevated intraocular pressure because we did not measure the intraocular pressure of the mouse eye due to its relatively small size. Many cases of glaucomatous phenotypes exhibiting buphthalmic eyes have been reported in other species.[22]

Although no significant change in the shape of the optic disc, such as the wide cupping seen in human buphthalmos, was observed in the Mx-c-myc mice, probably because of a difference in species, we suggest the possibility that the Mx-c-myc mice could be the first mouse model for buphthalmos research.

Mesenchymal cells derived from the neural crests migrate to ocular rudiments and develop into most of the anterior ocular structures.[23] Therefore, abnormal migration and differentiation of the neural crest cells are associated with anterior segment anomalies.[23] Mechanisms of the angle formation can be explained as the result of atrophy,[24,25] cleavage,[26,27] or reorganization[28] of the mesenchymal cells. However, the precise mechanism has not yet been established. The Mx-c-myc eyes at the embryonic stage exhibited elongated rudiments of the iris and ciliary body. Overgrowth of these tissues may modify the structure of the angle and result in the closed angle. We suggest that further study of the closed angle in the Mx-c-myc eyes can provide useful information for understanding the mechanism of the angle formation.

There are two possibilities to explain the etiology of the ocular lesions in the Mx-c-myc mice. One is the exogenous c-*myc* expression by the transgene. During ontogeny, c-*myc* transcripts are detected in a wide variety of developing tissues.[10,11] This expression is known to correlate well with active proliferation[7–9] and its downregulation accompanies mitotic arrest and the onset of differentiation. Therefore, overexpression of c-*myc* may induce overgrowth of

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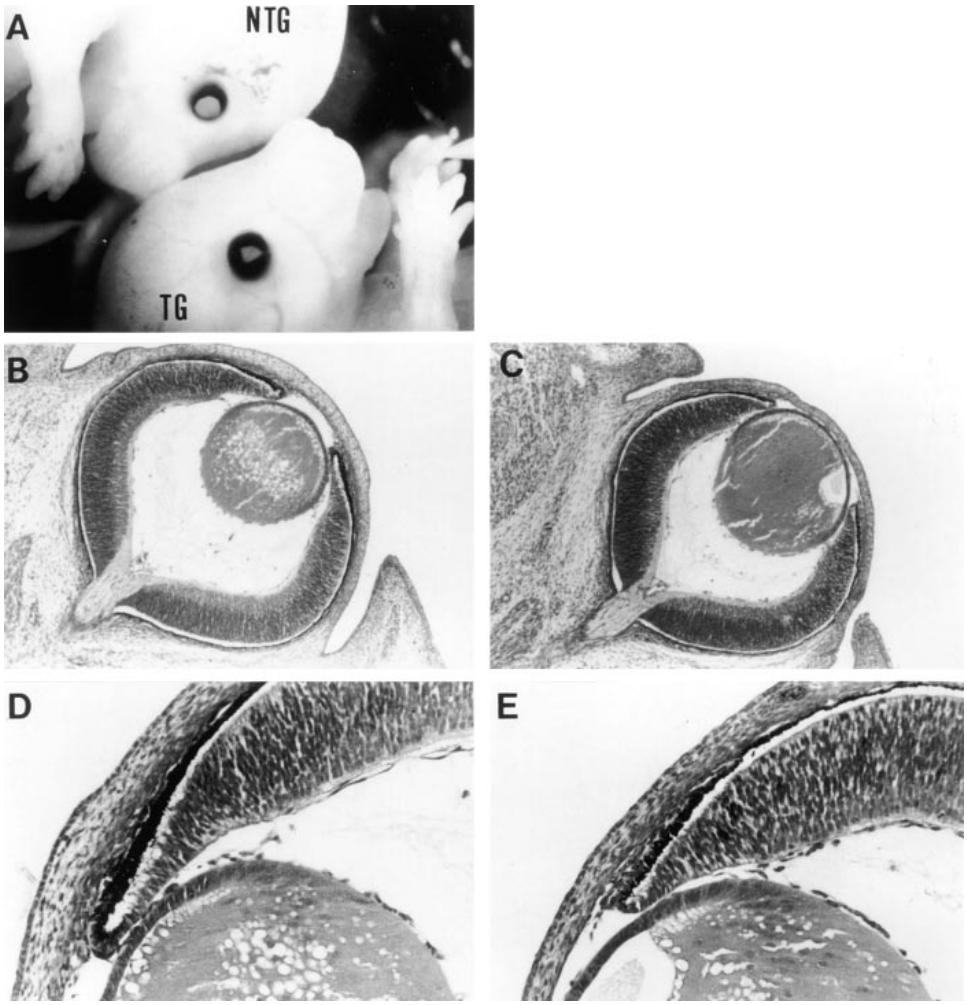

Figure 5. Histological analysis of eyes at embryonic day 15.5. (**A**) External observation of eyes in Mx-c-myc embryo (TG) and control littermate (NTG) (×4). Mx-c-myc embryo exhibits narrow and irregular margin of developing pupil. Histological comparison of eye from Mx-c-myc embryo (**B**) and control littermate (**C**) (×20, hematoxylin-eosin stain). Developing anterior chamber from Mx-c-myc embryo (**D**) and control littermate (**E**) (×100, hematoxylin-eosin stain). Mx-c-myc eye (B and D) exhibits elongated tip of optic cup.

the iris and ciliary body, leading to disorders of the angle. In the Mx-c-myc mice in this study, expression of the exogenous c-*myc* gene was detected in ocular tissues without induction by interferon α/β. Because inducibility of the exogenous gene was confirmed by stimulation of interferon α/β (data not shown), endogenous interferon α/β may induce the expression in ocular tissues. The severity of the phenotypes in the Mx-c-myc mice may be dependent on the amount of c-*myc* expressed in ocular tissues during development. The second possibility is the function by insertional mutagenesis of the endogenous gene(s) that is related with development of the anterior segment. If the endogenous gene(s) is important for eye development, the ocular malformation in the homozygotes should be more severe than that in the heterozygotes. However, we found no significant difference in the phenotype between homozygotes and heterozygotes of the exogenous gene (data not shown). Therefore, we believe that the overexpression of the c-*myc* transgene could be a better explanation for occurrence of the ocular malformation in the Mx-c-myc mice.

Microphakia is one of the most common phenotypes of ocular malformations in mutant mice.[2] The Mx-c-myc mice also exhibited microphakia. c-*Myc* transcripts are abundant in normal lens epithelial cells at the mitotically active undifferentiated stage.[13]

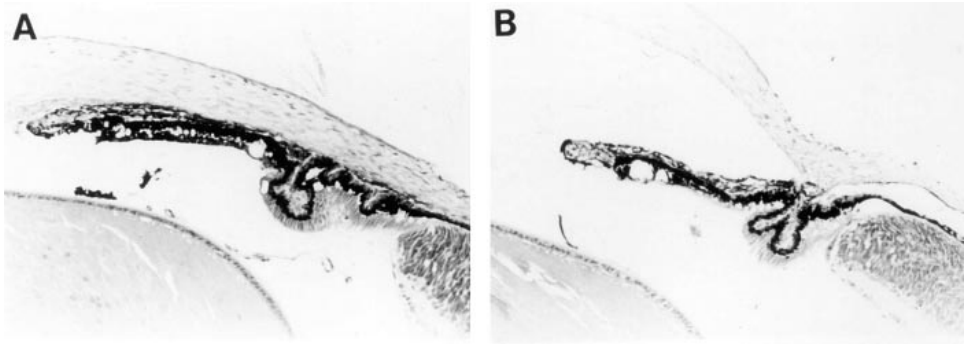

Figure 6. Histological analysis of eyes from the Mx-c-myc mouse and control at 1 week of age. Anterior chamber angle of Mx-c-myc mouse (**A**) and control littermate (**B**) (×50, hematoxylin-eosin stain).

When these cells enter growth arrest and differentiate into mature elongated lens fiber cells at their terminally differentiated stage, c-*myc* is downregulated. Therefore, the downregulation of c-*myc* is important for normal development of the lens cells. Because microphakia was also found in the transgenic mice with the exogenous c-*myc* gene controlled by the lens-specific α/A-crystalline promoter,[14] microphakia in the Mx-c-myc mice may be caused by the overexpression of c-*myc* in the lens cells.

In summary, we have presented a line of the Mx-c-myc transgenic mice that exhibited progressive enlargement of the globe accompanied by other ocular malformations. We suggest the possibility that the Mx-c-myc mice could be a suitable model for investigation of the development of the anterior segment and buphthalmos.

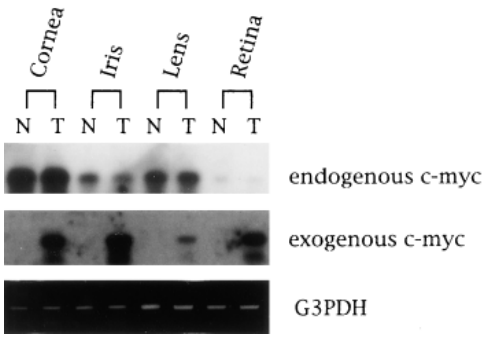

Figure 7. Expression of exogenous and endogenous c-*myc* in ocular structures of Mx-c-myc mouse and nontransgenic littermate. Total RNA was isolated from each ocular structure of Mx-c-myc mouse and control littermate at 4 weeks of age. Both exogenous and endogenous c-*myc* RNA were detected by reverse transcriptase-polymerase chain reaction and Southern blot analysis. Expression of glyceraldehyde-3-phosphate dehydrogenase RNA is shown as amount control of cDNA. N: nontransgenic littermate, T: transgenic mouse, G3PDH: glyceraldehyde-3-phosphate dehydrogenase.

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