Reports

In Situ Detection of β-Galactosidase in Lenses of Transgenic Mice with a γ-Crystallin/*lacZ* Gene

D. R. GORING, J. ROSSANT, S. CLAPOFF, M. L. BREITMAN, L.-C. TSUI

Transgenic mice carrying the γ2-crystallin promoter fused to the coding region of the bacterial *lacZ* gene were generated. The offspring of three founder mice expressed high levels of the enzyme solely in the central nuclear fiber cells of the lens as measured by an in situ assay for the detection of β-galactosidase activity. These results suggest that γ2-crystallin sequences between −759 to +45 contain essential information required for appropriate tissue-specific and temporal regulation of the mouse γ2-crystallin gene. In a broader context, this study also demonstrates the utility of β-galactosidase hybrid gene constructs for monitoring the activity of gene regulatory elements in transgenic mice.

THE γ-CRYSTALLINS COMPRISE A family of closely related polypeptides found exclusively in the terminally differentiated fiber cells of the lens in the mammalian eye (*1, 2*). Individual γ-crystallin genes are differentially regulated during development, resulting in a non-uniform spatial distribution of these proteins within the lens (*3, 4*). In recent studies, we have shown that sequences immediately 5′ of the γ-crystallin genes are at least in part responsible for their tissue-specific expression within the lens (*5, 6*). However, these studies in vitro do not allow us to define the sequences responsible for temporal regulation. To gain insight into the elements regulating developmental expression of these genes and to identify the cells within the lens in which individual γ-crystallin genes are expressed, we have generated transgenic mice containing the 5′ flanking segment of a mouse γ2-crystallin gene (sequences −759 to +45 relative to the transcription initiation site) coupled to the *lacZ* gene of *Escherichia coli*.

Two hundred and fifty-six fertilized eggs from CD-1 mice were microinjected with a 4.5-kb DNA fragment containing mouse γ2-crystallin sequences from −759 to +45 joined to the coding sequences of a *trpS–lacZ* fusion gene (*7*) (Fig. 1A). Southern blot analysis of restriction enzyme–digested DNA from the tails of 69 live-born animals revealed that five contained integrated copies of the hybrid gene. Two of these mice (B1 and B5) carried a single, apparently intact, copy of the injected sequences (Fig. 1A and Fig. 1B, lanes 1–3 and 13–15, respectively). One mouse, B3, carried a single copy with a deletion at the 3′ end (Fig. 1A and Fig. 1B, lanes 7–9); B4 carried two copies in a head-to-tail dimer with a 3′ end deletion in the second copy (Fig. 1A and Fig. 1B, lanes 10–12); B2 contained three copies integrated at two discrete sites, one corresponding to a head-to-tail dimer (Fig. 1A and Fig. 1B, lanes 4–6).

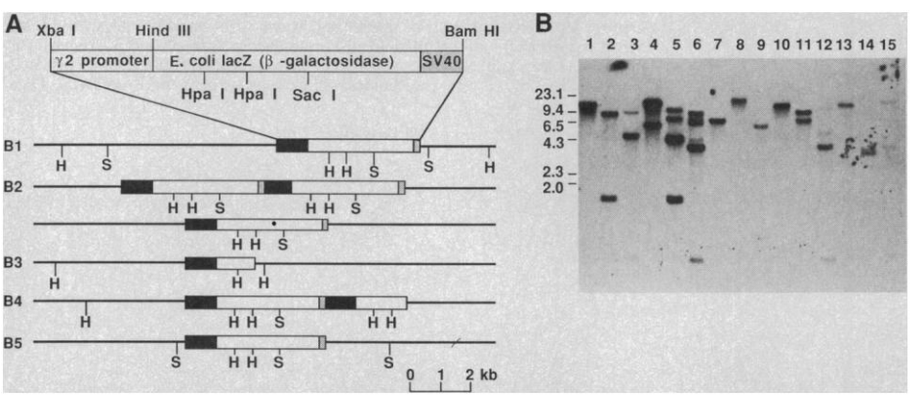

Fig. 1. (**A**) Restriction maps of the injected γ2-crystallin–β-gal fragment and its integrated forms in the five transgenic mice. The 4.5-kb Xba I–Bam HI fragment microinjected into fertilized eggs is shown at the top (*16*). Indicated segments of the hybrid gene are: sequences −759 to +45 of the mouse γ2-crystallin gene (*5*) (black), the *lacZ* fusion gene described by Hall *et al.* (*7*) (white), and the SV40 polyadenylation signal contained in SV40 sequences 2669 to 2534 (stippled). Restriction maps of the integrated DNA were derived from the data in (B). B1 and B5 carry single, apparently intact copies of the hybrid gene; B3 carries a head-to-tail dimer with a 3′ deletion; B4 carries a head-to-tail dimer with a 3′ deletion in the second copy; B2 contains two insertions, one corresponding to a head-to-tail dimer. Abbreviations are H, Hpa I, and S, Sac I. (**B**) Detection of γ2-crystallin–β-gal sequences in transgenic mice by Southern blot analysis (*17*). The 3.7-kb Hind III–Bam HI fragment spanning the *lacZ* fusion gene (A) was used as a probe. Lanes 1–3, 4–6, 7–9, 10–12, and 13–15: DNA samples from transgenic mice B1, B2, B3, B4, and B5, digested with Pst I, Sac I, and Hpa I, respectively. The sizes of Hind III–digested λ DNA are indicated on the left, in kilobases.

To investigate the regulation of expression of the hybrid gene, we assayed bacterial β-galactosidase (β-gal) activity in situ in individual cells by enzymatic cleavage of the indigogenic substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (*8*). G1 progeny of the original transgenic mice carrying γ2-crystallin-*lacZ* sequences were sacrificed and cryostat sections were examined for β-gal activity. High levels of β-gal activity, as evidenced by intense staining, were present in the central nuclear fiber cells in lens sections from three of the transgenic lines: B1, B2, and B4 (Fig. 2). No activity was detectable in other tissues from these animals (including brain, heart, kidney, liver, and spleen), even though low levels of endogenous β-gal activity could be detected with appropriate assay conditions (*9*). For transgenic lines B3 and B5, no β-gal activity was detectable in any of the tissues examined, including lens. Inability to detect the bacterial enzyme in mouse line B3 is consistent with the deletion found in the coding sequence of the single integrated copy of the hybrid gene (see Fig. 1). The reason for the lack of activity in line B5 remains unclear. It is possible that the injected sequence had become incorporated into a region of inactive chromatin or that it had sustained an undetectable sequence alteration.

The similarity of the staining pattern in the lenses of the three expressing lines is striking. In each case, dark blue cells were localized only in the nucleus and not in the cortex or epithelial cells at the anterior of the lens. In the lenses of B1, the darkly staining core was surrounded by a halo of lighter staining cells. Such variations between trans-

D. R. Goring and L.-C. Tsui, Department of Medical Genetics, University of Toronto, Toronto, Ontario, M5S 1A8, Canada, and Department of Genetics, Hospital for Sick Children, Toronto, Ontario, M5G 1X8, Canada.
J. Rossant and M. L. Breitman, Department of Medical Genetics, University of Toronto, Toronto, Ontario, M5S 1A8, Canada, and Research Institute, Mount Sinai Hospital, Toronto, Ontario, M5G 1X5, Canada.
S. Clapoff, Research Institute, Mount Sinai Hospital, Toronto, Ontario, M5G 1X5, Canada.

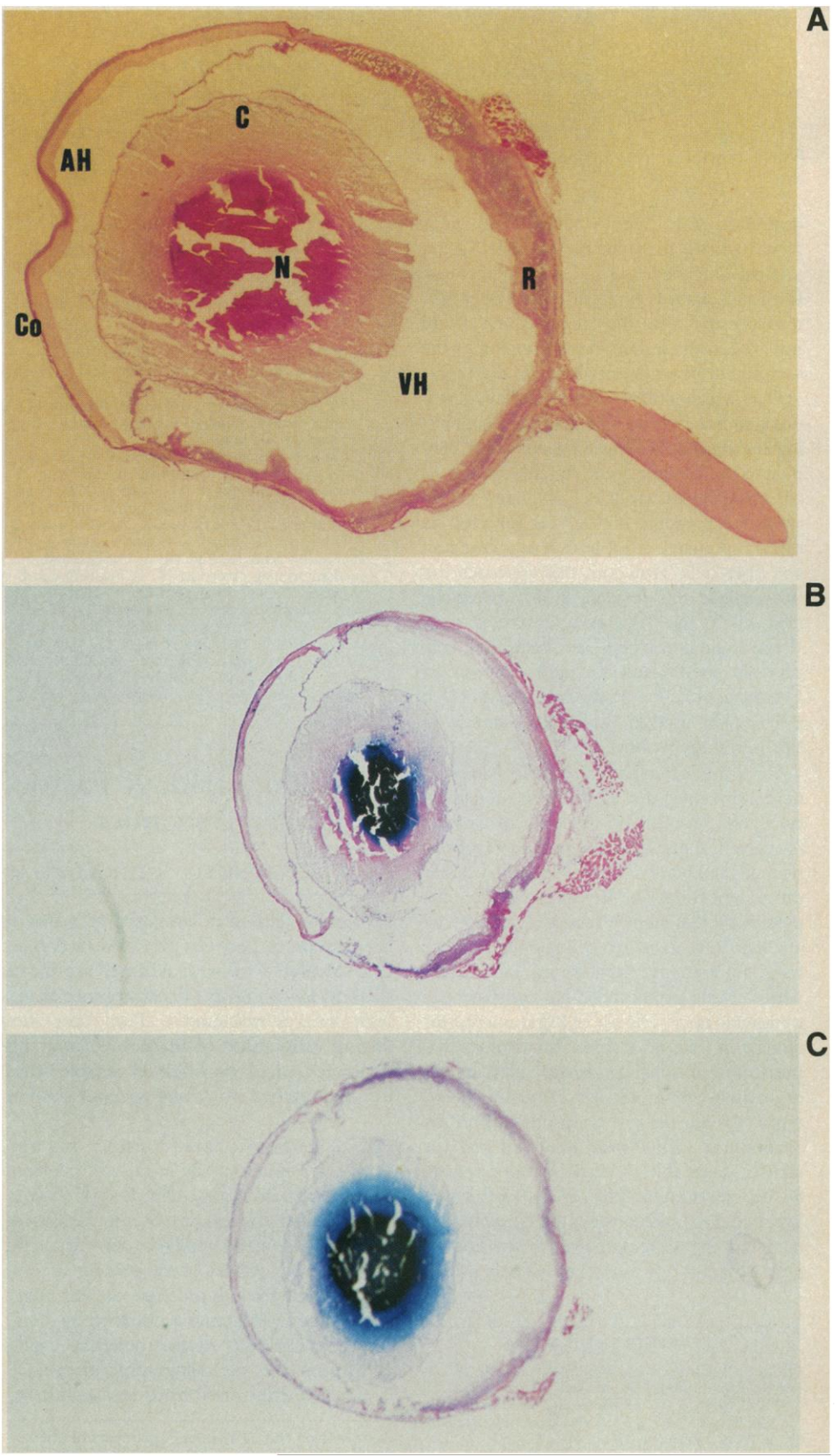

Fig. 2. In situ assay of bacterial β-gal activity in eye sections prepared from transgenic mouse lines (*18*). (**A**) Transverse section of the eye of a 6-week-old mouse. The section was air-dried and stained with hematoxylin and eosin. Abbreviations are Co, cornea; C, cortex; N, nucleus; AH, aqueous humor; VH, vitreous humor; and R, retina. (**B**) Transverse section of the eye of a 6-week-old mouse from line B4. Blue staining was confined to the dense central nucleus of the lens. A similar pattern was observed for line B2. (**C**) Transverse section of the eye of a 6-week-old mouse from line B1. A halo of light blue cells surrounds the darkly staining lens nucleus. It was difficult to avoid stress fractures in the sections because of the hardness of the lens.

genic lines may be due to chromosomal position effects resulting in differences in the level or timing of expression of the hybrid gene. The absence of β-gal activity in the epithelium is consistent with immunocytochemical observations that the latter cells do not contain any of the γ-crystallins (*10*).

The spatial distribution of the hybrid gene product within the lens can be interpreted in terms of temporal regulation of the γ2-crystallin gene. Growth of the lens occurs throughout development by successive deposition of new fiber cells that differentiate continuously from the anterior lens epithelium (*1, 2*). Consequently, fiber cells occupying the central nuclear region of the lens correspond to those formed early in embryonic development while those located in the more cortical, peripheral regions of the lens correspond to fiber cells formed at later developmental stages (see Fig. 3). The fiber cells undergo nuclear breakdown as they move towards the center of the lens and hence the nuclear fiber cells are presumed to be no longer transcriptionally active (*2, 3*). The intense staining of these cells must therefore reflect earlier transcriptional events. This finding is consistent with our previous observation that the mouse γ2 transcript is one of the more abundant γ-crystallin species in the embryonic mouse lens (*11*).

Our study suggests that sequences extending from −759 to +45 contain essential information for appropriate developmental regulation of the mouse γ2-crystallin gene. With the same approach, it should be possible to define further the sequences governing lens-specific expression and those involved in regulating the differential expression of the different γ-crystallin genes during development. The limited information available from DNA transfection assays in primary lens explants has indicated that sequences between −226 and +45 of the mouse γ2-crystallin gene are essential for lens-specific promoter activity (*5, 12*). Furthermore, we have identified an enhancer-like activity in the mouse γ2-crystallin gene between −396 and −126 (*12*). Since se-

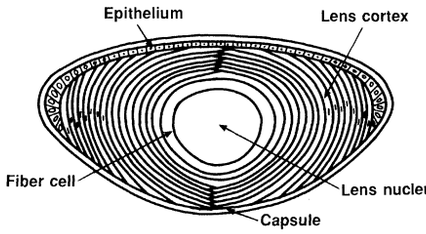

Fig. 3. Schematic cross section of a mammalian lens.

quences from −75 to −26 are highly conserved among the different members of the γ-crystallin gene family (*11, 13*), while those further upstream show little homology (*6*), it is possible that the latter sequences are involved in the differential expression of the γ-crystallin genes during lens development.

Overbeek and co-workers (*14*) have previously shown that a DNA fragment containing the mouse αA-crystallin promoter coupled to the bacterial gene for chloramphenicol acetyltransferase (CAT) directed tissue-specific expression of CAT as detected in extracts prepared from both lens epithelium and fiber cells. However, this approach did not provide the detailed in situ resolution afforded by the β-gal assay described here. While β-gal constructs have been used successfully in P-element transformation of *Drosophila* (*15*), our study demonstrates that β-gal can be used to localize gene activity in situ in transgenic mice.

Slit lamp examination of the lenses of mice expressing the γ2-crystallin–β-gal hybrid gene revealed no evidence of cataract formation, suggesting that high level expression of the bacterial enzyme is not deleterious to lens function. We cannot exclude the possibility that high levels of β-gal activity are injurious in other developmental contexts, a possibility that is being investigated with various promoter–β-gal constructs in transgenic mice. The use of β-gal as a reporter gene in transgenic mice also assumes that post-transcriptional processes affecting its expression would be the same in all cell types. At the present time this is only an assumption. The lower limits of enzyme activity detectable in tissue sections remain to be determined. However, β-gal–based hybrid genes hold considerable potential for studying the developmental regulation of gene expression in situ in transgenic mice.

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16. The γ2-crystallin sequences were excised from pγ2CAT1281 (*5*) and inserted into the Hind III site in the β-gal vector pCH126. The plasmid pCH126 is identical to pCH110 as described (*7*) except that the SV40 promoter (Pvu II–Hind III fragment) was removed and the Hind III site regenerated (F. Lee, personal communication).
17. Three-week-old animals were screened for the insertions of the γ2-crystallin–β-gal hybrid gene by Southern blot analysis [E. M. Southern, *J. Mol. Biol.* **98**, 503 (1975)] of tail DNA digested with Pst I, a non-cutter of the test construct. The probe was a 3.7-kb Hind III–Bam HI fragment spanning the *lacZ* fusion gene (see Fig. 1A) and labeled with the random-priming procedure [A. P. Feinberg and B. Vogelstein, *Anal. Biochem.* **132**, 6 (1983)]. The single integrations detected in animals B1, B3, B4, and B5 and the two present in B2 were subsequently characterized by digestion of tail DNA with Sac I and Hpa I; enzymes that produced one and two cuts in the hybrid gene, respectively (see Fig. 1A). Digested DNA's (5 μg) were separated by electrophoresis through 0.7% agarose gels, transferred to Zetabind filters (AMF Cuno), and prehybridized and hybridized as described [G. M. Wahl, M. Stern, G. R. Stark, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3683 (1979)]. Filters were washed at room temperature once in 2× standard saline citrate (SSC), 0.1% SDS and once in 0.1× SSC, 0.1% SDS; followed by four washes at 65°C in 0.1× SSC, 0.1% SDS. Dried filters were exposed to Kodak XAR-5 film for 1 to 3 days at −70°C between two Dupont Lightning Plus intensifying screens.
18. Whole eyes were frozen in liquid nitrogen and mounted in OCT compound (Fisher) for cryosectioning. Sections (7 μm) were cut, air-dried, and incubated overnight in a buffer containing X-gal [2 m*M* 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Sigma), 3 m*M* potassium ferricyanide, and 3 m*M* potassium ferrocyanide in phosphate-buffered saline, *p*H 7.0]. Sections were then counterstained with hematoxylin and eosin and mounted in Canada balsam. Tissue sections prepared similarly from brain, heart, liver, kidney, and spleen showed no detectable bacterial enzyme activity, although endogenous β-gal activity was detectable under a different assay condition with McIlvaine buffer (120 m*M* NaCl, 3 m*M* potassium ferricyanide, 3 m*M* potassium ferrocyanide, and 2 m*M* X-gal, *p*H 4.2) (D. R. Goring *et al.*, unpublished data).

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Crystallographic *R* Factor Refinement by Molecular Dynamics

AXEL T. BRÜNGER, JOHN KURIYAN, MARTIN KARPLUS

Molecular dynamics was used to refine macromolecular structures by incorporating the difference between the observed crystallographic structure factor amplitude and that calculated from an assumed atomic model into the total energy of the system. The method has a radius of convergence that is larger than that of conventional restrained least-squares refinement. Test cases showed that the need for manual corrections during refinement of macromolecular crystal structures is reduced. In crambin, the dynamics calculation moved residues that were misplaced by more than 3 angstroms into the correct positions without human intervention.

CRYSTALLOGRAPHIC STRUCTURE DETERMINATIONS by x-ray or neutron diffraction generally proceed in two stages. First, the phases of the measured reflections are estimated and a low- to medium-resolution model of the protein is constructed and second, more precise information about the structure is obtained by refining the parameters of the molecular model against the crystallographic data (*1*). The refinement is performed by minimizing the crystallographic *R* factor, which is defined as the difference between the observed [$|F_{obs}(h\ k\ l)|$] and calculated [$|F_{calc}(h\ k\ l)|$] structure factor amplitudes,

$$R = \frac{\sum_{h\,k\,l} ||F_{obs}(h\ k\ l)| - |F_{calc}(h\ k\ l)||}{\sum_{h\,k\,l} |F_{obs}(h\ k\ l)|} \quad (1)$$

where $h\ k\ l$ are the reciprocal lattice points of the crystal.

Conventional refinement involves a series of steps, each of which consists of a few cycles of least-squares refinement with stereochemical and internal packing constraints or restraints (*2–5*) that are followed

A. T. Brünger and M. Karplus, Department of Chemistry, Harvard University, Cambridge, MA 02138.
J. Kuriyan, Department of Chemistry, Harvard University, Cambridge, MA 02138, and Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139.