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Reporter Gene Construct Containing 1.4-kB α1-Proteinase Inhibitor Promoter Confers Expression in the Cornea of Transgenic Mice

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ABSTRACT

A 1.4-kb human α1-proteinase inhibitor (α1-PI) 5′-flanking sequence fused to the *E. coli LacZ* gene was used to generate transgenic mice. The 1.4-kb α1-PI fragment was found to target *LacZ* expression preferentially in the epithelium and stroma of the mouse cornea, and moderately or weakly in white blood cells and a few other tissues, such as the skin and brain. This finding implies that the α1-PI promoter may offer an option for targeting foreign genes in both the epithelial and stromal layers of the cornea in future transgenic experiments. Anat Rec 266:5–9, 2002.

Key words: α1-proteinase inhibitor; cornea; promoter; expression; transgenic mouse

α1-Proteinase inhibitor (α1-PI) is a major protease inhibitor in human serum (Travis and Salvesen, 1983). One of its primary physiological roles is to protect the elastic fibers in lung alveoli from excessive digestion by neutrophil elastase (Olsen et al., 1975). The importance of this protein was proposed based on observations that genetically α1-PI-deficient patients developed early-onset degenerative lung disease (Eriksson, 1964) or liver disease (Sharp et al., 1969). The liver is the predominant site of α1-PI synthesis (Laurell and Jeppsson, 1975). This protein is also found synthesized in blood monocytes and macrophages (Perlmutter et al., 1985), and other extrahepatic sites such as the cornea (Twining et al., 1994), a transparent connective tissue located at the front of the eye.

The human α1-PI gene contains seven exons: Ia, Ib, Ic, and II–V (Brantly et al., 1988). Multiple transcription initiation sites and α1-PI transcripts that comprise different numbers of exons have been identified. The use of different transcription start sites and the alternative splicing in different cells suggest that the gene transcription may respond to tissue- or cell-specific regulatory mechanisms (Perlino et al., 1987; Long et al., 1984; Hafeez et al., 1991).

In keratoconus, an ocular disease that thins and distorts the central portion of the cornea (Rabinowitz, 1998), a markedly reduced expression of the α1-PI gene has been demonstrated (Sawaguchi et al., 1990; Whitelock et al., 1997). During the course of studying keratoconus, we cloned and sequenced a 2.7-kilobase (kb) region of human α1-PI gene upstream of the corneal transcription start site. Transient transfection experiments showed that the 2.7-kb 5′-flanking DNA is functional in human corneal stromal cells, and that the proximal 1.4-kb is sufficient for full promoter activity (Li et al., 1998). No promoter activity was found for either the 2.7- or the 1.4-kb segments in human skin or scleral and conjunctival fibroblasts, suggesting that the 5′-flanking promoter element we identified may be specific for corneal cells.

In this report, we advanced the promoter study to the next level from in vitro cell cultures to in vivo transgenic mice carrying the 1.4 kb-α1-PI promoter-*LacZ* gene. In these mice, corneal epithelial and stromal expression was

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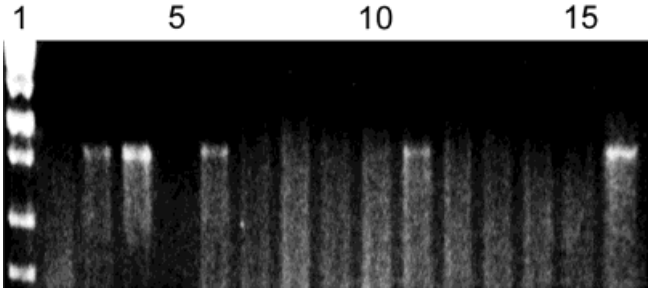

Fig. 1. Detection of α1-PI promoter-β-galactosidase sequences in transgenic mice. PCR analysis of transgenic mouse tail DNA was performed with primers Pr1 and Pr2. Lane 1 shows the 1-kb ladder. Lanes 2–4 contained 0.4 μg normal mouse DNA and 0, 1, and 5 gene copies/cell equivalent, respectively, of the Kpn I/BamH I fragment. Lanes 6–16 were DNA samples from mouse tails. Lanes 6, 11, and 16 showed a positive 1.0-kb band, and the copy number was estimated to be 2–5. Two mice (one male and one female) with positive reactions (founders, lanes 6 and 16) were used for generation of progenies. The 1.0-kb product was not observed in nontransgenic mouse DNA.

conferred by the 1.4-kb α1-PI promoter fragment. The spatial and temporal distribution of the *LacZ* reporter gene was determined.

MATERIALS AND METHODS

Preparation of Construct for Microinjection

A 1,406-basepair (bp) (–1397 to +9) 5′-flanking sequence of the human α1-PI gene was ligated into the Kpn I and Bgl II cloning sites of the pβgal-Basic vector (Clontech, Palo Alto, CA), yielding a p1.4α1-PI-βgal plasmid. A 6,010-bp linear DNA fragment, containing the 1,406-bp α1-PI promoter, the *LacZ* reporter gene, and the SV40 early poly (A) tail was excised from the p1.4α1-PI-βgal plasmid with restriction enzymes Kpn I and BamH I. After digestion, the DNA was extracted from agarose gel using the QIAEX II Gel Extraction kit (Qiagen, Valencia, CA). The purified α1-PI promoter/*LacZ* fusion gene fragment was microinjected into 472 C57BL/6 × SJL F2 hybrid mouse eggs by Xenogen BioSciences (Cranbury, NJ).

Putative transgenic founder pups were analyzed when the pups reached 7 weeks of age. The animals were screened by polymerase chain reaction (PCR) analysis of genomic DNA prepared from tails (Hogan et al., 1986) using a forward primer (pr1: TTTTCCGTGACGTCTCGTTGCTG) complementary to the human α1-PI gene promoter sequences and a reverse primer (Pr2: GCTGATGTGCCCGGCTTCTGACC) specific to the *LacZ* gene. PCR reactions were run for 40 cycles at 94°C for 30 sec, 55°C for 1 min, and 72°C for 3 min, followed by a 10-min extension at 72°C in a Perkin-Elmer 9700 thermocycler (Norwalk, CT). The presence of an approximately 1.0-kb PCR product indicated a positive result. For Southern blot analysis, the genomic DNA from tails was digested with Hind III and separated on a 1% agarose gel. Southern blotting was performed according to the Boehringer-Mannheim protocol using Hybond N membranes (Amersham, Arlington Heights, IL). The DNA probe was either the ^{32}P-labeled 1.0-kb α1-PI-β-gal PCR product mentioned above or the 6-kb Kpn I/BamH I transgene fragment used for microinjection. As a positive control, the 6-kb fragment was run alongside the genomic digests. To determine whether the integrated DNA was transmitted to progeny, founders that expressed α1-PI-β-gal were mated with wild-type C57BL/6J, and tail DNA from the first generation (F1) progeny was analyzed by PCR as above. The transgene-carrying F1 mice were bred to produce second-generation (F2) and subsequent third-generation (F3) progenies.

Immunohistochemical Analysis of Tissues

Adult animals were anesthetized by inhalation of ether and killed by cervical dislocation. Tissues, including the eye, skin, liver, kidney, heart, brain, and blood, were dissected or collected from both transgenic and nontransgenic mice. They were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight. The cornea and lens were excised from the eye after fixation. The tissues were embedded in paraffin.

For immunohistochemistry, 5-μm-thick paraffin sections were deparaffinized and immunostained with polyclonal rabbit anti-β-galactosidase (Cortex Biochem, San Leandro, CA) at a dilution of 1:100. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA) was used at 1:250 as a secondary antibody, and the signal was amplified by using the tyramide signal amplification (TSA)™-direct tetramethylrhodamine (NEN Life Science Products, Boston, MA).

For developmental studies, transgenic mice were set up for timed mating. F3 embryos were dissected from the uterus of the pregnant mice on day 10.5 (E10.5), 12.5 (E12.5), 15.5 (E15.5), and 18.5 (E18.5) of pregnancy, fixed in 4% paraformaldehyde overnight at 4°C, and prepared for whole-mount β-galactosidase immunostaining. Corneal tissues collected from mice at birth (P0), and on postnatal days 7 (P7), 11 (P11), and 30 (P30) were also fixed, processed for paraffin sections, and stained with rabbit polyclonal β-galactosidase antibody as above.

Fig. 2. Immunostaining for β-galactosidase in the (**A–F**) eye, (**G**) skin, (**H**) liver, (**I**) kidney, (**J**) heart, (**K**) brain, and (**L**) blood smear of a 4-month-old F2 transgenic mouse. The 1.4-kb α1-PI promoter drove the *LacZ* gene expression (red staining) in the (A and B) corneal epithelium and (C) stroma, but not in the endothelium (not shown). Prominent staining was observed in the central area (arrowhead in A) of the corneal epithelium, and the reactivity tapered off toward the peripheral region (arrow in A). D: In nontransgenic mice, neither the epithelium nor the stroma of the cornea showed staining. In the (E) lens (arrow heads) and (F) retina, no specific stains were detected. Moderate staining for β-galactosidase was noted in the (G) subcutaneous connective tissues of the skin, (K) cerebral cortex in the brain, and (L) a few white blood cells in the blood smear. Weak staining was also observed in (J) the perimysium of cardiac muscles. Only background staining was found in the (H) liver and (I) kidney, and in control sections incubated with normal rabbit IgG (not shown). The nuclei in L were stained in blue with DAPI. Magnification: (A and E–K) ×10, (B, D, and L) ×40, and (C) ×63.

Fig. 4. Immunostaining for β-galactosidase in the cornea of transgenic mice during embryonic and postnatal stages. Corneal tissues were collected from F3 embryos on days (**A**) 10.5, (**B**) 12.5, (**C**) 15.5, and (**D**) 18.5 of pregnancy, and also neonatal mice at (**E**) birth, and on postnatal days (**F**) 7, (**G**) 11, and (**H**) 30. The α1-PI promoter-driven *LacZ* expression (red) in the corneal epithelium began at E15.5. The β-galactosidase staining was (C) initially sporadic; it then (D–H) increased and remained constant after E18.5. The expression in the stroma began on (F) P7 and was the most prominent on (G) P11. The nuclei (blue) were DAPI-stained. Magnification: ×20. The lens (L) was also seen in some micrographs.

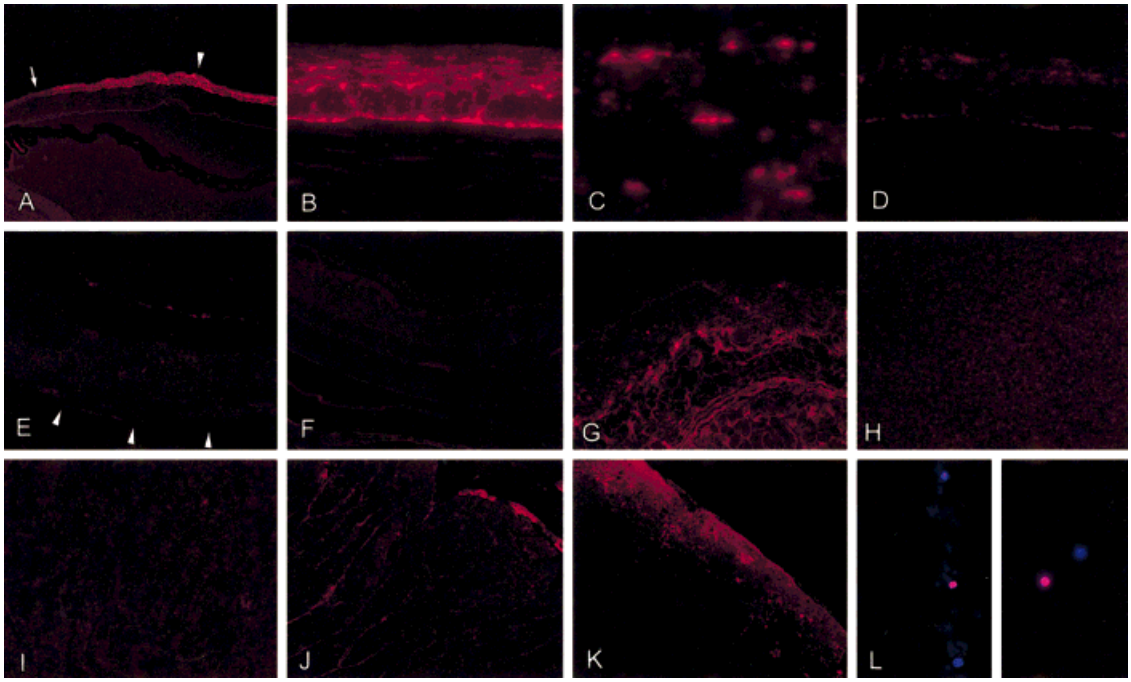

Figure 2.

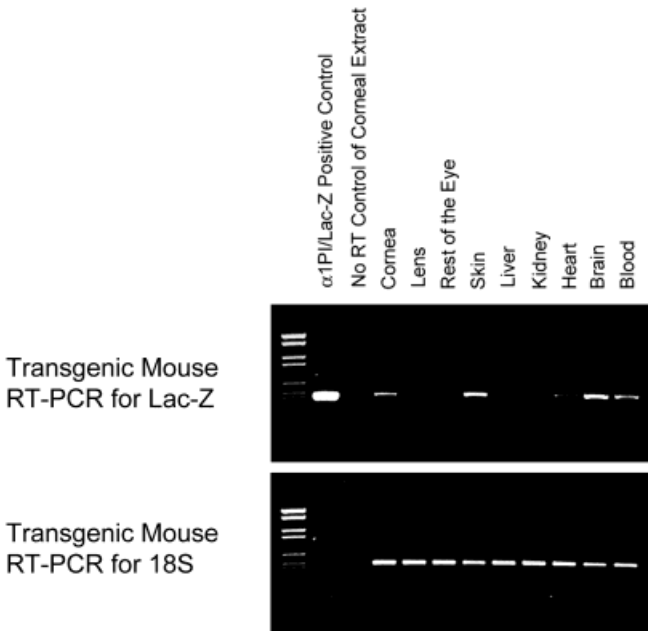

Fig. 3. RT-PCR analysis to detect the expression of *LacZ* gene in various tissues of transgenic mouse. β-Galactosidase-specific primer sets were used in PCR. The DNA molecular marker (lane 1) was run in parallel with the α1-PI promoter/*LacZ* fusion gene fragment (positive control, lane 2), corneal RNA sample omitting the RT reaction (negative control, lane 3), and cDNA samples from the cornea (lane 4), lens (lane 5), the rest of the eye (lane 6), skin (lane 7), liver (lane 8), kidney (lane 9), heart (lane 10), brain (lane 11), and blood cells (lane 12) of an F2 transgenic mouse. The 0.5-kb expected PCR product was found in the cornea, skin, brain, and blood, and faintly in the heart sample, but not in the lens, the rest of the eye, liver, or kidney. The *LacZ* gene was not detected in samples from nontransgenic mice (not shown). The 18S ribosomal band was obtained in all samples, confirming the quality of cDNAs.

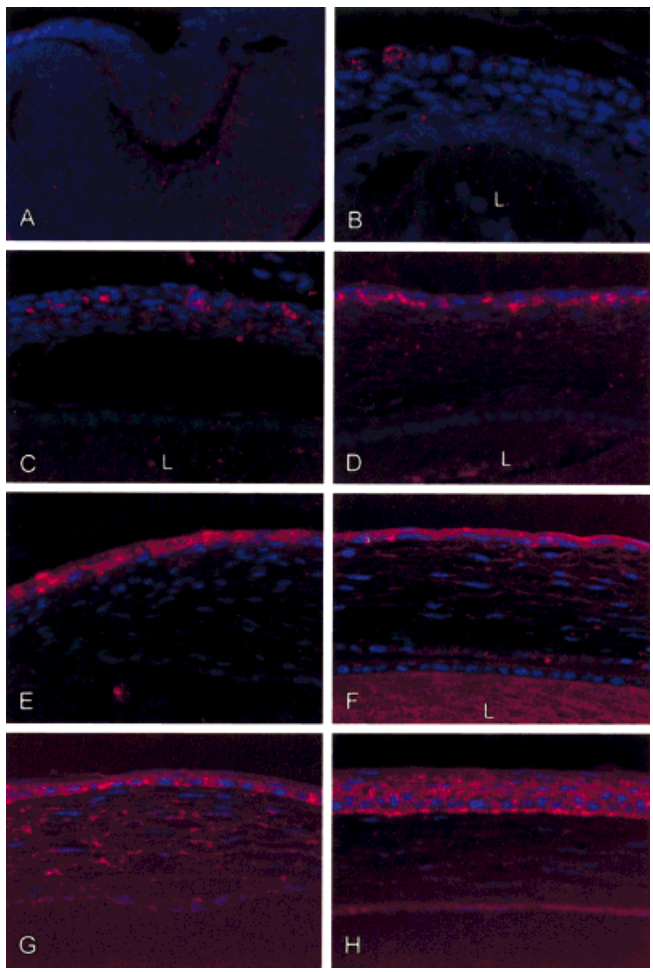

Figure 4.

Reverse Transcription (RT)-PCR

Tissues, including the cornea, lens, the rest of the eye, skin, liver, kidney, heart, brain, and blood, were collected. Total RNA was isolated from these tissues using the StrataPrep Total RNA Miniprep Kit (Stratagene, La Jolla, CA). RT was performed with random hexamers using the Superscript™ first-strand synthesis system for RT-PCR (Life Technologies, Rockville, MD). For PCR analysis, a *LacZ*-specific forward primer (Pr3: GTCGGCTTACGGCGGTGATTT) and a reverse primer (Pr4: TTGCCAACGCTTATTATTACCCAGC) were used (Lem et al., 1991). PCR reactions were run for 40 cycles at 92°C for 1 min, 64°C for 30 sec, and 72°C for 1 min, followed by a 10-min extension at 72°C. Amplified DNA fragments were approximately 500 bp in size. PCR reactions were also performed using primer sets for 18S ribosomal RNA (Ambion, Austin, TX) to confirm the quality of cDNAs. The expected size of the 18S ribosomal PCR product was 488 bp.

RESULTS AND DISCUSSION

A total of five transgenic founders were identified by PCR. Two founders, one male and one female, harboring approximately two to five copies of transgene (Fig. 1), were selected to generate the F1 and the subsequent F2 and F3 mice for analysis. Both transmitted the *LacZ* transgene to approximately 50% of their offspring. Southern blot analysis indicated integration of transgene into a single integration site (data not shown).

The transgenic mice appeared to be normal. The eyes of these mice were also normal in size. To examine tissue-specific expression of *LacZ* driven by the 1.4-kb α1-PI promoter-*LacZ* construct, immunohistochemical experiments for β-galactosidase were performed using the TSA method on both the F1 and F2 progenies. Positive staining was observed in all layers of the corneal epithelium (Fig. 2A and C) and also in the stroma (Fig. 2B). The strong immunoreactivity in the epithelium in the central portion of the cornea was noticeably diminished toward the periphery (Fig. 2A). Minimal staining was seen in the limbal and conjunctival region. In the stroma, the staining was in general weaker than that in the epithelium, extending from the central to the peripheral cornea. The corneal endothelium was negative; occasional staining was probably nonspecific, as it was also observed in negative controls. In nontransgenic mice, neither the corneal epithelium nor the stroma showed any reaction (Fig. 2D). The 1.4-kb α1-PI promoter fragment was thus concluded to target expression to the epithelium and stroma of mouse cornea. This finding is consistent with our culture data, which indicated that the α1-PI promoter fragment is functional in both corneal stromal (Li et al., 1998) and epithelial (Maruyama et al., 2001) cells.

No specific β-galactosidase staining was found in other ocular tissues, such as the lens and retina (Fig. 2E and F), or in nonocular tissues, such as the liver and kidney (Fig. 2H and I). Moderate staining was noted in the subcutaneous connective tissues of the skin (Fig. 2G), cerebral cortex in the brain (Fig. 2K), and a few white blood cells in the blood smear (Fig. 2L). Weak staining was also observed in the perimysium of cardiac muscles (Fig. 2J). Overall, the cornea showed the strongest immunoreactivity.

To further confirm the expression pattern, total RNA isolated from various tissues was reverse transcribed and analyzed by PCR. Using a β-galactosidase-specific primer set, a 0.5-kb PCR product was found in the samples from the cornea, skin, brain, and blood, and faintly in the heart sample, but not in cDNA samples of the lens, the rest of the eye, liver, or kidney (Fig. 3). When an 18S ribosomal RNA-specific primer set was used, all tissue samples yielded a 488-bp PCR band, attesting to the RNA/cDNA integrity. Neither the β-galactosidase staining nor the PCR product was observed in tissues of nontransgenic mice.

Developmental studies indicated that the α1-PI promoter-driven *LacZ* expression in the corneal epithelium began at E15.5. The staining was initially sporadic; it then increased and remained constant after E18.5. The expression in the stroma started on P7 and was the most prominent on P11 (Fig. 4).

The current work indicates that the 1.4-kb α1-PI promoter is functional in the cornea. This implies that it may target genes in the cornea but not in other eye tissues. The α1-PI promoter is also expressed in a few nonocular tissues, and is not strictly cornea-specific. However, it is cornea-preferred and may be considered to be an additional option for gene manipulation in the cornea of transgenic animals. While aldehyde dehydrogenase class 3 gene promoter (Kays and Piatigorsky, 1997) targets, preferrably, the corneal epithelium, and keratocan gene promoter (Liu et al., 2000) targets the corneal stroma, the α1-PI promoter can drive gene expression in both the corneal epithelium and stroma.

The 1.4-kb α1-PI fragment is not a strong promoter. An initial examination of the β-galactosidase reporter gene activity by histochemical X-gal staining resulted in very faint staining in the cornea. The positive outcome was evident immunohistochemically only after amplification by the TSA method. It is possible to modify the promoter construct—for example, by including an enhancer—to heighten the expression. On the other hand, the relatively weak activity may be an advantage in that a foreign gene, when introduced into the cornea, would not induce side effects from over-expression. At any rate, a promoter that targets expression in the corneal epithelium and stroma may be very useful for future gene manipulation studies in the evaluation of epithelial-stromal interactions and corneal wound healing, and for studies of corneal diseases such as keratoconus.

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