Developmental Biology 222, 41–54 (2000)
doi:10.1006/dbio.2000.9694, available online at http://www.idealibrary.com on IDEAL®

Pax-6 Expression and Activity Are Induced in the Reepithelializing Cornea and Control Activity of the Transcriptional Promoter for Matrix Metalloproteinase Gelatinase B

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Recent evidence supports the idea that matrix metalloproteinases (MMPs) act as morphogenetic regulators in embryonic and adult events of tissue remodeling. MMP activity is controlled primarily at the level of gene expression. In a recent study we characterized the transcriptional promoter of the MMP gene, gelatinase B (gelB), in transgenic mice, demonstrating the requirement for DNA sequences between −522 and +19 for appropriate activity. In this study we investigated factors required for gelB promoter activity in the developing eye and reepithelializing adult cornea. Pax-6 is a homeobox and paired domain transcription factor that acts at the top of the hierarchy of genes controlling eye development. Pax-6 is also expressed in the adult eye. We show here that the tissue expression pattern of Pax-6 overlaps extensively with gelB promoter activity in the developing and adult eye. In addition Pax-6 is observed to be upregulated in repairing corneal epithelium, as is gelB promoter activity. In cell culture transfection experiments, we identified two promoter regions which mediate positive response to Pax-6. By electrophoretic mobility shift assay, we further pinpoint two Pax-6 binding sites within these response regions and demonstrate direct interaction of the Pax-6 paired domain with one of these sites. These data suggest a mechanism by which Pax-6 may direct gelB expression in an eye-specific manner. © 2000 Academic Press

Key Words: **cornea; epithelium; eye; gelatinase B; matrix metalloproteinase; MMP-9; Pax-6; wound healing.**

INTRODUCTION

The Pax proteins are a family of developmental regulators characterized by the paired domain (PD;[1] Bopp *et al.*, 1986; Treisman *et al.*, 1991), an evolutionarily conserved DNA-binding motif, first identified in the prototype *Drosophila gene paired* (*prd*; Frigerio *et al.*, 1986). One of these family members, Pax-6, resides at the highest level of a genetic hierarchy controlling eye development in species as diverse as flies and humans (Glaser *et al.*, 1994,1995; Halder *et al.*, 1995a,b; Fini *et al.*, 1997). Mutations in Pax-6 are the basis of mouse and rat *small eye* and *Drosophila eyeless*. Homozygosity for these loss-of-function mutations is lethal, and the phenotype includes brain defects, lack of nasal cavities, and complete lack of eyes (Sander *et al.*, 1997). Heterozygosity (haploinsufficiency) is associated with a spectrum of eye defects in animals and in humans, most involving structures in the anterior segment including the iris and cornea (Ton *et al.*, 1991; Glaser *et al.*, 1992; Hansen *et al.*, 1994). Strikingly, increased Pax-6 gene dosage (delivered in transgenic mice) causes developmental abnormalities of the eye similar to those associated with haploinsufficiency (Schedl *et al.*, 1996). These observations suggest that cells within the eye are exceptionally sensitive to changes in levels of Pax-6 activity. Pax-6 protein has also been shown to be present in the adult eye (Koroma *et al.*, 1997). Thus constitutive Pax-6 may be involved in the

[1] Abbreviations used: β-gal, β-galactosidase; CAT, chloramphenicol acetyl transferase; ECM, extracellular matrix; EMSA, electrophoretic mobility shift assay; gelB, gelatinase B; MMPs, matrix metalloproteinases; PD, paired domain; TPA, phorbol acetate; TRE, TPA response element.

0012-1606/00 $35.00

maintenance and remodeling of adult tissues; however, few data have been presented so far concerning its function in this regard.

Matrix metalloproteinases (MMPs) are a family of zinc endopeptidases characterized by reactivity toward components of the extracellular matrix. MMPs were first identified in tissues undergoing normal developmental or adult remodeling and in tissues undergoing pathological destruction. Primarily because of MMP substrate specificity, it was traditionally held that their morphogenetic role was as effector: to mediate normal ECM dismantling when properly regulated or to destroy ECM structures when disregulated (Gross, 1981). Evidence has been accumulating, however, to support the idea that MMPs act as true morphogenetic regulators. Thus, it has been increasingly appreciated that interaction with ECM can control cell behavior and differentiation and that this interaction can be exquisitely modulated by MMPs (Boudreau and Bissel, 1998). Moreover, MMP substrate specificity is now known to be much broader than originally thought and includes such morphogenetic regulators as cytokines and cell adhesion molecules (Wilson and Matrisian, 1996; Vu and Werb, 1998; Werb and Chin, 1998). In view of these advances, it is not surprising that recent studies examining perturbation of MMP activity have revealed dramatic effects on tissue patterning, in both embryo and adult (Chin and Werb, 1997; Rudolph-Owen *et al.*, 1998; Thomasset *et al.*, 1998; Vu *et al.*, 1998).

MMP activity is modulated at multiple levels; however, gene expression is the key regulatory event (Fini *et al.*, 1998). Thus, MMPs are expressed according to a precise temporospatial pattern in the embryo and adult that is thought to reflect functional requirements. MMP expression can also be induced in most tissues by inflammatory, stress, or repair stimuli. A large body of work over the past 10 years has defined factors controlling MMP gene expression in response to stress-related stimuli (Fini *et al.*, 1998). Expression of several of the MMP genes, including collagenase (MMP-1), stromelysin (MMP-3), and gelatinase B (gelB; MMP-9) can be stimulated by tumor-promoting phorbol esters such as TPA or by growth factors and inflammatory cytokines. In transient transfection assays, basal or stimulated activity of the transcriptional promoter typically requires a TPA-response element (TRE), which binds transcription factor AP-1. Additional elements which modulate transcription have been identified. For example; in the gelB gene, there are two TREs present in the first 600 bp upstream of the transcriptional start site, and at least one of these must be intact for promoter activity (Sato and Seiki, 1993; Fini *et al.*, 1994). Deletion of other DNA sequences, including but not restricted to response sites for transcription factors NF-κB, Sp1, and AP-2, affect the activity of the gelB gene, but are not absolutely essential for promoter activity in transient transfection assay (Sato and Seiki, 1993; Sato *et al.*, 1993; Fini *et al.*, 1994; Gum *et al.*, 1996).

In contrast to the large amount of work on stress regulators, very few studies have considered mechanisms of MMP regulation during development. We recently began to investigate this territory by undertaking the first analysis of a MMP gene transcriptional promoter *in vivo:* the promoter for the rabbit gene for gelB. We found that DNA sequences between −522 and +19 of the rabbit gelB promoter, as exemplified in our transgenic mouse line 3445, are sufficient for developmental, adult, and repair-specific reporter gene activity according to a pattern which closely parallels expression of the endogenous gelB gene (Mohan *et al.*, 1998). In contrast to requirements in transient transfection assay, truncation or internal deletion of promoter sequences between −522 and +19, even when both TREs were left intact, eliminated promoter function *in vivo.* These studies emphasize the importance of elements other than the TRE in expression of the gelB gene for both developmental and stress-related promoter activity *in vivo.*

Pax-6 has a highly localized expression pattern during development in distinct regions of the forebrain and hindbrain, the spinal cord, the pituitary, the pancreas, the nasal epithelium, and the developing and adult eye (Koroma *et al.*, 1997; Sander *et al.*, 1997). *In situ* hybridization and immunolocalization studies have revealed that like Pax-6, gelB is also expressed developmentally in the brain, spinal cord, and eye (Reponen *et al.*, 1994; Canete-Soler *et al.*, 1995a,b) and is induced in many adult tissues during healing or regenerative processes (Fini *et al.*, 1998). During studies to characterize gelB promoter activity in our transgenic mouse line 3445, we were struck by the similarities between promoter activity and Pax-6 gene expression patterns (Mohan *et al.*, 1998). In particular, the whole-mount staining technique for assay of gelB promoter activity prominently highlighted the eye at embryonic day 13. Strong promoter activity also occurred at this time in the nasal cavities, a gelB expression site which had not been previously reported. Together with the localized expression in specific areas of the brain and spinal cord, a picture emerged of striking overlap with the developmental expression pattern for Pax-6 and hinted that gelB might be a downstream target for Pax-6.

In this study, we investigate the hypothesis that Pax-6 regulates activity of the gelB promoter and further characterize the expression and activity of Pax-6 in the reepithelializing cornea.

MATERIALS AND METHODS

Assay of Transgene Expression in Mouse Line 3445

Construction of mouse line 3445, which harbors the Pr22 transgene, was previously described (Mohan *et al.*, 1998). The transgene consists of bases −522 to +19 of the gelB promoter linked to a β-gal reporter. Promoter activity in these mice closely parallels the activity of the endogenous gelB gene (Mohan *et al.*, 1998). Embryos from matings between line 3445 mice heterozygous for the transgene and from wild-type CD-1 mice were collected at staged intervals, fixed in buffered 4% paraformaldehyde for 25 min, washed three times in PBS, and stained as whole mounts in buffered 2% X-gal solution at 30°C for 5 to 12 h

TABLE 1
Oligonucleotide Probes Used for EMSA

GelB promoter probes
RM82/83, bases −570 to −524
CACTGCCCTGAAGATTCAGCTGTCGGAGACAGGGGTTACCCCGGTG
RM80/81, bases −515 to −467
CAAATCCTGCCTCAGAGAGCCCACTCCTTCCGCCCAGCTGGAGCCGGGA
BF10/11, bases −408 to −388
CCTCAGGAGGGGGTGTCACAG
RM72/73, bases −385 to −359
TCAAGGGTGGGCCTGGGGTGGCACTCA
BF8/9, bases −233 to −206
GGCCTGTGGTTTCCTGTGGGTCTGGGGT
Control probes
PDcon (optimized Pax-6 paired domain binding site)
TGGAATTCAGGAAAAATTTTCACGCTTGAGTTCACAGCTCGTCGAGTA
AP-2con (canonical AP2 binding site)
GATCGAACTGACCGCCCGCGGCCCGT
AP1con (canonical AP1 binding site)
CGCTTGATGAGTCAGCCGGAA
SP1con (canonical SP1 binding site)
ATTCGATCGGGGCGGGGCCAGC
NFκBcon (canonical NFκB binding site)
AGTTGAGGGGACTTTCCCAGGC

Note. Underlined in RM80/81 is an Sp1 binding site conserved between human and rabbit. The second underline is a second site which fits the Sp1 binding consensus. Underlined in RM72/73 is a region which is protected by footprinting with AP-2 (Fini *et al.*, 1994). This element also binds Sp1 (Mohan and Fini, unpublished data).

(Wasserman *et al.*, 1993). Genetics predicts that half of these embryos will not carry the transgene and therefore will serve as a control for nonspecific staining. Adult eyes were surgically removed from the mice and fixed and stained as above. After staining, the tissues were washed in PBS and refixed in 4% paraformaldehyde, then embedded in paraffin and sectioned (6 μm).

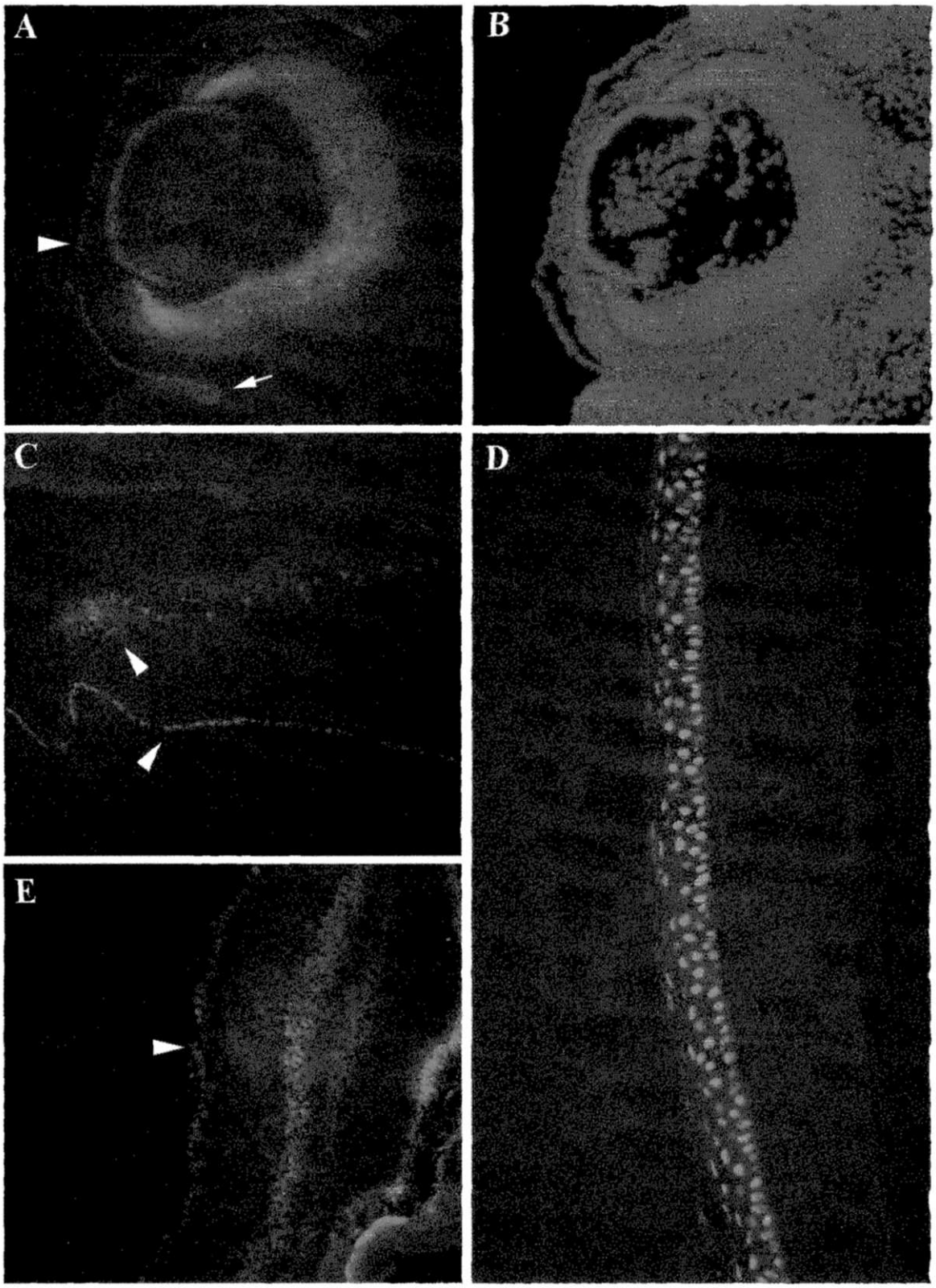
Immunohistochemistry

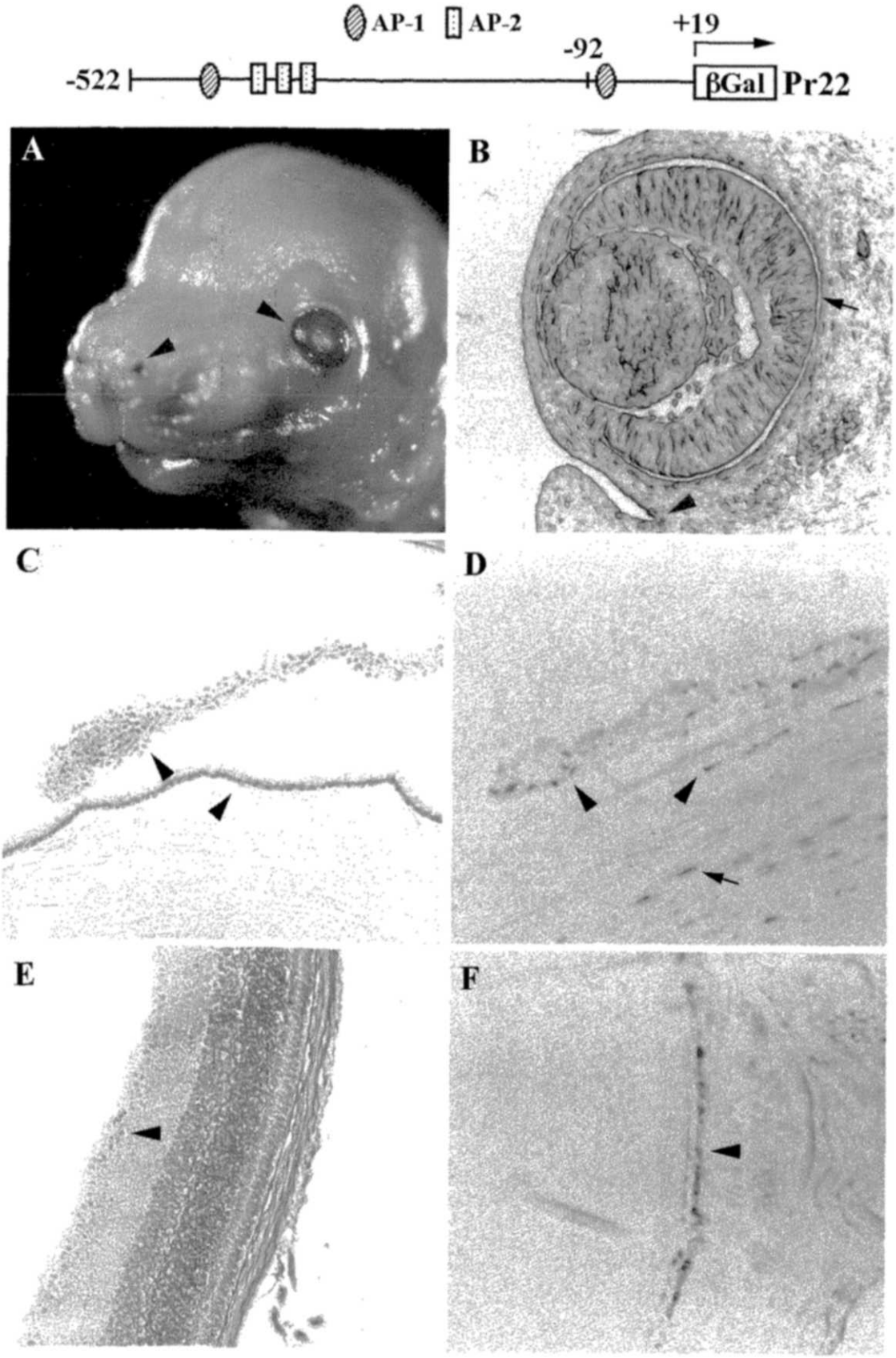
Wild-type embryonic day 13 (E13) mice and adult mouse eyes were frozen in liquid nitrogen and sectioned at 5 and 7 μm on a cryostat, respectively. Sections were fixed in 4% paraformaldehyde for 10 min (4°C) followed by permeabilization in 0.5% NP-40/PBS for 5 min and blocking in 3% goat serum for 1 h at room temp. Incubation with polyclonal Pax-6 antibody (purchased from Covance Co., Richmond CA) was performed in 1.5% goat serum for 1.5 h at a dilution of 1:200 in PBS. Then FITC-conjugated secondary antibody was applied for 1 h at 1:50. After incubation, sections were washed three times in PBS, with Hoechst nuclear stain included in the final wash (1:5000 dilution). In each experiment a section was stained without primary antibody to serve as a negative control. Fluorescent staining was visualized on a Nikon E400 microscope and captured using a color digital camera (Digital Instruments, MI). Some representative sections of each experiment were stained instead with hematoxylin and eosin by standard methods.

Corneal Surgeries

Adult mice (6–8 weeks of age) were anesthetized by an intraperitoneal injection of 2% Avertin (0.017 ml/g body weight). Residual eye reflexes were blocked by topical application of 0.5% proparacaine to the corneal surface (Alcon, TX). A circular demar-

FIG. 1. Localization of Pax-6 in embryonic and adult eyes. Immunohistochemical detection of Pax-6 protein in E13 (A and B) and adult (C–E) wild-type mouse eyes. (A) Sagittal E13 section stained for Pax-6 in the presumptive retina, conjunctiva (arrow), lens epithelium, and corneal epithelium (arrowhead), to be compared with Fig. 2B. (B) Identical section as in (A) counterstained with Hoechst nuclear dye to visualize the locations of cell nuclei. (C) Pax-6 staining of adult iris and lens epithelium (arrowheads), to be compared with Figs. 2C and 2D. (D) Constitutive Pax-6 staining of adult corneal epithelial cells in cross section. (E) Staining of ganglion cells (arrowhead) and inner nuclear layer retinal cells for Pax-6, to be compared with Figs. 2E and 2F. All images were at 20× original magnification except (A) and (B), which were at 10×.





cation was created in the central portion of one cornea of each mouse by excimer laser keratectomy to a depth of 40 μm, removing the epithelium, basement membrane, and anterior stroma to a diameter of 1.5 mm. This is similar to the photorefractive keratectomy procedure used in humans to correct refractive error. Antibiotic ointment was applied to the eyes after surgery. Mice were sacrificed 18 h after surgery at a time when the migrating epithelial sheet had almost resurfaced the corneal defect. Reepithelializing and control eyes were removed and processed for β-gal staining or immunohistochemistry, as described above. A modified procedure was performed on adult New Zealand White rabbits (Charles River Laboratories, Wilmington, MA) with the aim of obtaining tissue to prepare lysates for EMSA or Western blotting. Rabbits were anesthetized with xylazine and ketamine. Residual reflexes were blocked by topical application of 0.5% proparacaine. In this case the wound was created by gently pressing an 8-mm trephine on the surface of the cornea followed by gentle scraping with a scalpel within the circumscribed area. Animals were sacrificed 20 h after surgery, and epithelial tissue was immediately collected from the area of the wound, or from uninjured corneas, by gentle scraping with a scalpel as described (Mohan *et al.*, 1998).

Electrophoretic Mobility Shift Assay

Nuclear extracts from cells or corneal epithelial tissues were prepared as described (Mohan *et al.*, 1998), essentially followed the procedure of Dignam *et al.* (1983). Protein concentration was measured by Bio-Rad dye binding assay (Bio-Rad, Hercules, CA). Aliquots of equal protein were frozen at −70°C and thawed just prior to use. The sequences of double-stranded oligonucleotides used for EMSA are delineated in Table 1. Probes containing the canonical DNA binding sites for transcription factors AP-1, Sp1, and AP-2 were purchased from Promega Co. (Madison, WI). PDcon encodes the optimized Pax-6 paired domain binding site as described (Epstein *et al.*, 1994a). The double-stranded oligo probes derived from the gelB promoter were synthesized by the Tufts University oligonucleotide facility.

For each EMSA 0.03 pmol radiolabeled DNA was incubated for 20 min at room temperature with equal amounts of nuclear extracts (5–10 μg or 1–2 μl) from tissues or cell cultures in a high-salt DNA binding buffer containing poly(dI–dC) (Promega), according to established protocol. The protein–DNA complexes were resolved by electrophoresis on a 4% nondenaturing polyacrylamide gel, dried, and exposed to X-ray film for autoradiography. In competition experiments, 50× molar excess of unlabeled probe was incubated with nuclear lysates for 10 min before addition of labeled oligos. For supershift EMSA, protein–DNA complexes were incubated with antibodies for an additional 40 min at room temperature. Polyclonal Sp1 and AP-2 antibodies for supershifts were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Two Pax-6 antibodies were used in this study. The Pax-6A (Covance Co., Richmond, CA) and Pax-6B antibodies were both raised to the C-terminal domain of mouse Pax-6 as respectively described (Davis and Reed, 1996; Xu *et al.*, 1999). Both antibodies recognize similar epitopes; however, Pax6A supershifted Pax-6-containing complexes, while Pax6B removed them. Pax-6 paired domain/GST fusions (PD/GST and GST control) have been previously described (Epstein *et al.*, 1994a). Recombinant protein was purified on glutathione–Sepharose 4B columns as recommended by the manufacturer (Pharmacia Biotech, Piscataway, NJ).

Western Blotting and Densitometry

Nuclear lysates from normal or resurfacing corneal epithelium were prepared as for EMSA. Equal amounts of protein (5 μg) were separated by 11% SDS–PAGE and transferred to Immobilon P membrane (Millipore, Bedford, MA). Blots were probed with Pax-6 antibody at a 1:500 dilution followed by an HRP-conjugated secondary and ECL detection reagents as recommended by the manufacturer (Amersham, Arlington Heights, IL). After film exposure, blots were stained with 0.1% amido black to confirm the equal loading of total proteins between lanes. Densitometry was performed using an Eagle Eye image analyzer (Stratagene), and values were calculated for duplicate wound and control lanes by comparison to a standard curve.

Cell Culture and Transient Transfection Analysis

The rabbit corneal epithelial-derived cell line SIRC was cultured as recommended by the supplier (American Type Culture Collection, Rockville, MD). Cells were plated for transfection as described (Fini *et al.*, 1994). GelB promoter–reporter DNA constructs used in this study were derived from the rabbit gelB gene (Fini *et al.*, 1994). The development of Pr22, Pr34, Pr35, and Pr42 was previously described (Mohan *et al.*, 1998). Development of Pr15 was also previously described (Fini *et al.*, 1994). A new construct, Pr23, was created by insertion of a 2.4-kb fragment of genomic DNA into the *Eco*RI site of Pr22; this extends the gelB 5′ flanking DNA an additional 2.4 kb upstream. The reporter gene used in each construct is the gene for either β-galactosidase (β-gal) or chloramphenicol acetyltransferase (CAT). A full-length Pax-6 expression plasmid was previously described (Epstein *et al.*, 1994a). Plasmid DNAs were purified using the Qiagen plasmid DNA purification kit (Qiagen, Santa Clarita, CA). In a standard transfection, 1 μg of a gelB construct was cotransfected with either 0.4 μg of the Pax-6 expression plasmid or control DNA. A second reporter plasmid (0.8 μg) driven by a strong and ubiquitously active promoter was included to control for any differences in transfection efficiencies. The control vector used was either the CMV–β-Galactosidase Control Vector (Stratagene, La Jolla, CA) or the pCAT control

FIG. 2. Localization of gelB promoter activity in the eyes of transgenic mouse line3445. Gelatinase B promoter activity in mouse line 3445 detected by histochemical localization of β-gal expression in E13 (A and B) and adult (C–F) mice. (Top) The Pr22 transgene. (A) Strong β-gal expression is observed in E13 eyes and nasal cavities (arrowheads). (B) Sagittal section through an E13 eye showing β-gal staining of retinal precursor cells in the inner layer of the optic cup (arrow) and epithelium of the cleft between the corneal limbus and eyelid (future conjunctiva) (arrowhead). (C) Hematoxylin- and eosin-stained section showing adult iris and lens, followed by an equivalent section showing the iris and lens epithelium stained for β-gal (D) (arrowheads). Note the arrow in (D) indicating artifactual dark marks in the lens fibers. (E) Hematoxylin- and eosin-stained section showing adult retina, with the arrowhead indicating the ganglion cell layer, followed by an equivalent section staining in the ganglion cells for β-gal (F). All images were at 20× original magnification except (B), which was at 10×.

plasmid (Promega), the choice being the alternative to whichever reporter was being utilized in the gelB construct under analysis. Total DNA transfected was equalized in all cases with empty expression vector. DNA was introduced into cells using Lipofectamine reagent following the procedure recommended by the manufacturer (Life Technologies, Gaithersburg, MD). All transfections were done at least three separate times in triplicate. Cells were collected 24 h after treatment and cell lysates were assayed for β-galactosidase activity (Holcomb and Silhavy, 1972). An equivalent volume of each lysate was then assayed for CAT activity using [^{14}C]chloramphenicol as a substrate and quantified by scintillation counter (Gorman *et al.*, 1982; Seed and Sheen, 1988). Results were then normalized to β-galactosidase expression.

RESULTS

Pax-6 Expression Patterns Overlap GelB Promoter Activity in Embryonic and Adult Mouse Eyes

Pax-6 expression patterns were observed in E13 and adult mouse eyes by immunofluorescence localization (Fig. 1). The Pax-6 expression profile essentially matched that described in detail for chick and mouse eyes by Koroma *et al.* (1997). Briefly, nuclear staining was present in E13 corneal epithelium, future conjunctiva, and retina, as well as in adult corneal epithelium, conjunctiva, lens epithelium, and ganglion and inner nuclear retinal layers (Fig. 1). Localization of gelB transcriptional promoter activity in embryonic and adult eyes was investigated by assaying expression of the β-gal reporter gene driven by the gelB promoter in line 3445 transgenic mice (Fig. 2). We previously reported strong histochemical staining for β-gal in the brain of these mice at E11 (Mohan *et al.*, 1998). At E13, staining in the brain was reduced; however, staining in the eye and nasal cavities was intense (Fig. 2A). At this and all other times assayed, littermates that did not carry the transgene were negative for β-gal staining (Mohan *et al.*, 1998).

To compare the activity pattern of the gelB promoter and the expression of Pax-6 in the different tissues of the eye, we examined sagittal sections through E13 and adult eyes of line 3445 and control mice. Histochemical staining for β-gal at E13 was localized in the retinal precursor cells which make up the inner layer of the optic cup and to a specific area of the future conjunctival epithelium within the cleft between eyelid and cornea (Fig. 2B). Additionally, in some sections, weak staining was observed in lens epithelial cells (not shown). This pattern overlaps with the localization of Pax-6 in presumptive conjunctiva, retina, and lens epithelium; however, the gelB promoter was not active in the corneal epithelium, a prominent Pax-6 staining site (Figs. 1A and 1B).

In adult eyes, staining for β-gal was localized to the lens epithelium, the stromal layer of the iris, the mesenchymally derived tissues of the ciliary body (Fig. 2C), and the ganglion cell layer of the retina (Fig. 2E). The expression pattern for Pax-6 overlaps with the gelB activity pattern in the lens epithelium, iris, and ciliary body (Fig. 1C) and ganglion cell layer of the adult retina (Fig. 1E), but Pax-6 was also expressed in the cells of the inner nuclear layer (Fig. 1E). Pax-6 continued to be expressed in the corneal epithelium of the adult eye (Fig. 1D); however, as in embryos, the gelB promoter continued to be inactive at this location (not shown).

These data demonstrate considerable overlap between the tissue localization patterns of gelB promoter activity and Pax-6 expression in the eye, yet also reveal significant differences.

Colocalization of GelB Promoter Activity and Pax-6 Expression in Reepithelializing Corneas

While gelB is not normally expressed in the cornea of the developing or adult eye, expression is induced in adult corneal epithelium migrating to repair a wound (Fini *et al.*, 1996). This induction provides a useful model for studying gelB transcriptional regulation, as the corneal epithelium is a tissue with clearly delineated margins that is easy to isolate. We investigated the localization of gelB promoter activity in repairing corneal epithelium of line 3445 transgenic mice (Mohan *et al.*, 1998). As predicted by the expression pattern of the endogenous gelB gene, uninjured corneas were negative for β-gal activity; however, activity was detectable in the corneal epithelium migrating to heal a wound (Figs. 3A and 3B). Activity was localized to the basal cell layer, similar to the reported pattern of endogenous gelB expression (Matsubara *et al.*, 1991; Fini *et al.*, 1996). As described above, Pax-6 is constitutively expressed throughout the cells of the corneal epithelium (Fig. 1D). Sections from reepithelializing corneas stained for Pax-6 showed no major changes in localization; however, an increase in staining intensity was observed in cells at the migrating wound edge (Fig. 3C). These findings demonstrate overlap between Pax-6 expression in corneal epithelium and gelB promoter activity induced as part of the repair response in the cornea.

Increase in Pax-6 Protein and DNA Binding Activity in Reepithelializing Corneas

To investigate potential changes in the expression levels of Pax-6 in epithelium during repair, a Western blot analysis was performed (Fig. 4A). Equal amounts of control or wound rabbit epithelial lysates were loaded into duplicate lanes. In both cases the Pax-6 antibody bound proteins of 46–48 kDa corresponding to the two major Pax-6 isoforms (Fig. 4A, lanes C and W). A control lysate from corneal fibroblasts was negative for immunoreactive Pax-6 (Fig. 4A, lane F). Plotting of experimental values against a standard curve revealed a threefold increase of Pax-6 protein in wound lysates compared to lysates from uninjured cells (Fig. 4B).

To investigate relative DNA binding activity for the Pax-6 paired domain in normal and wounded corneal epithelium, EMSA was performed on nuclear extracts

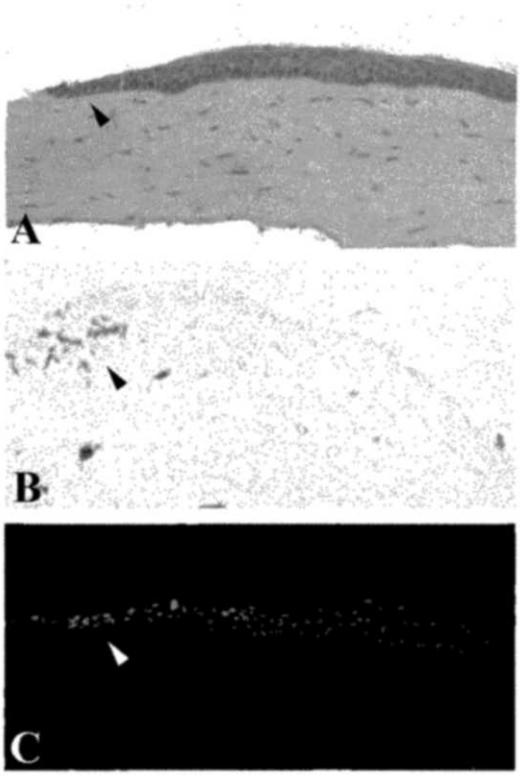


FIG. 3. Colocalization of gelB promoter activity with Pax-6 protein in wounded corneas. Cross sections through laser-wounded adult mouse corneas showing the edge of epithelial regrowth (arrowheads in (A–C)). (A) Hematoxylin- and eosin-stained section and equivalent sections in (B) showing β-gal promoter activity in the migrating wound epithelium of a line 3445 mouse and (C) intensified Pax-6 staining at the wound edge. Note that the contrast in (C) has been reduced to diminish the constitutive background epithelial Pax-6 staining. All images were at 20× original magnification.

derived from the same lysates used for Western blotting described above. The double-stranded oligonucleotide, PDcon, presenting an optimized Pax-6 DNA binding site, served as probe for this experiment (Table 1). Nuclear extracts prepared from uninjured epithelium revealed a single major protein:DNA complex which was competed with an excess of unlabeled PDcon, but not an unrelated oligonucleotide (Fig. 4C). The mobility of the complex was supershifted by preincubation with one Pax-6 antibody (Pax-6A) and abolished by a second Pax-6 antibody (Pax-6B), demonstrating that it contains Pax-6 (Fig. 4C). Antibodies recognizing transcription factors AP-2 or Sp1 did not affect the DNA binding activity of the protein complex to the PDcon probe (Fig. 4C). An identical complex formed on PDcon when nuclear extracts prepared from migrating wound epithelium were used for EMSA (Fig. 4D). However, the amount of binding to the PDcon probe was increased with extracts from wound epithelium compared to extracts from uninjured epithelium. EMSA using probes for other transcription factors known to regulate gelatinase B activity are shown for comparison. The increased binding of Pax-6 was greater than the increase in NF-κB or AP-1 activities in wound lysates, but quite modest in comparison with the change in Sp1 DNA binding activity (Fig. 4D).

These data reveal that both the dosage of Pax-6 and the level of Pax-6 DNA binding activity are increased in repairing corneal epithelium.

Pax-6 Activates the GelB Promoter in Cultured Corneal Epithelial Cells

To test the hypothesis that increased dosage of Pax-6 protein stimulates gelB promoter activity in corneal epithelial cells, we performed cell co-transfection experiments with a set of gelB reporter constructs and an expression plasmid encoding the 46 kDa form of Pax-6 (Epstein *et al.*, 1994a). To provide sufficient material for analysis, experiments were done using a spontaneously immortalized cell line derived from rabbit corneal epithelium (SIRC), which constitutively expresses gelB (Fini *et al.*, 1994). These cells were also found to express Pax-6 endogenously, as determined by Western blot analysis (Fig. 5, top). Cotransfections were initially performed with a construct driven by approximately 3 kb of gelB promoter DNA (Pr23, Fig. 5), and the Pax-6 expression plasmid at a range of doses. We found that the maximal Pax-6 induction of the reporter genes occurred with 0.4 μg of Pax-6 expression vector per transfection. Increased dosages reduced the induction back to basal levels; however, they did not repress reporter expression up to 2.0 μg (not shown). This narrow window of activity is consistent with the importance of Pax-6 dosage to proper developmental activity (Schedl *et al.*, 1996). Subsequent experiments were performed with the inductive 0.4-μg plasmid dose. The results are summarized in Fig. 5.

While cotransfection with Pax-6 increased expression of Pr23 by about threefold, Pax-6 did not alter activity of Pr22, a construct driven by a considerably shortened fragment of promoter DNA located between −522 and +19 of the transcription start site of the gelB gene. This suggests that there is a positive response element for Pax-6 in the gelB promoter located 5′ of base −522. However, cotransfection with Pax-6 also induced expression of Pr42, a modified version of Pr22 generated by internal deletion between bases −312 and −92. This suggested that the promoter DNA in Pr22 also contains a positive Pax-6-response element, but the positive response is cancelled out by a negative response element located between bases −312 and −92. In support of this hypothesis, expression of Pr15, which contains promoter DNA up to the proximal end of the deletion in Pr42 (−333 to +19), was *repressed* by cotransfection with Pax-6, and expression of Pr34, which

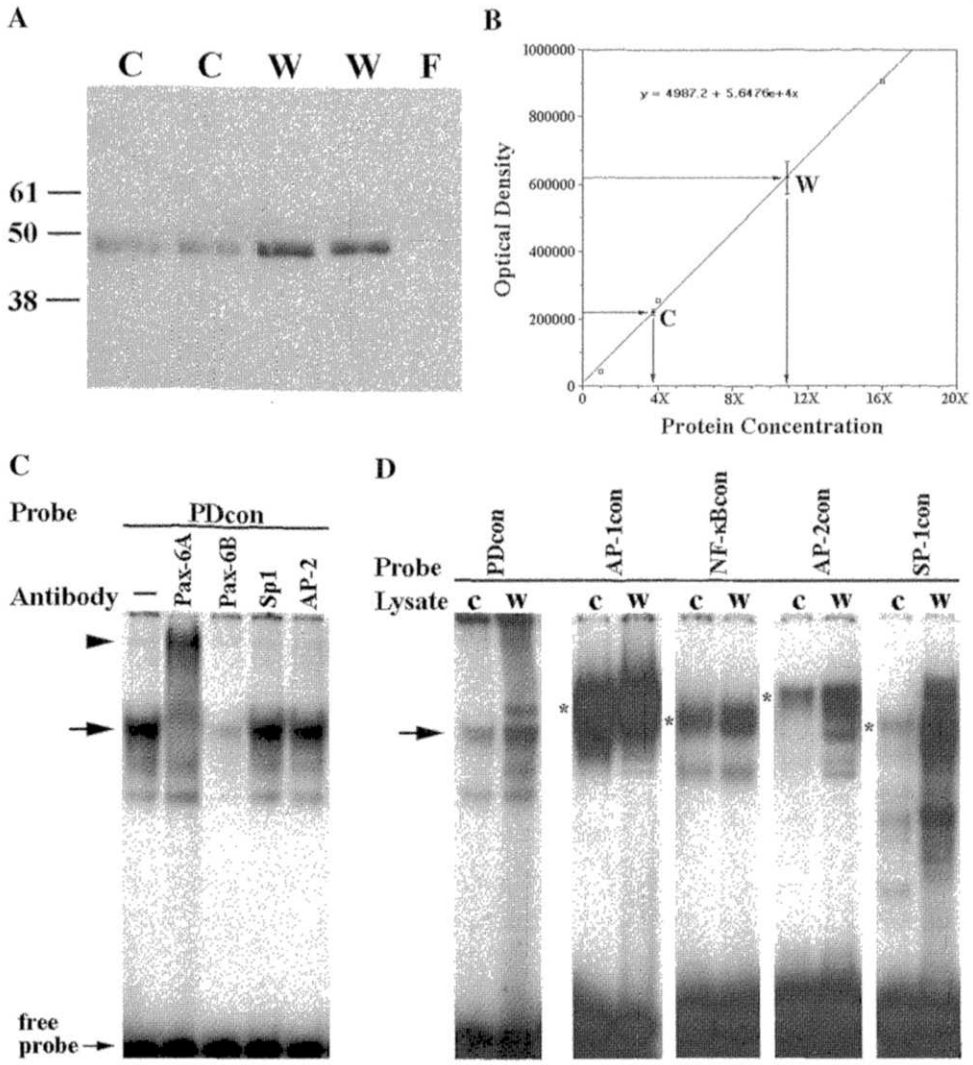


FIG. 4. Induction of Pax-6 protein and DNA binding activity in normal and wounded corneas. (A) Western blot probes with Pax-6 antibody to nuclear lysates from control (lanes C) and wounded (lanes W) rabbit corneal epithelium. (Lane F) A control lysate from corneal stromal cells which do not express Pax-6. (B) Densitometric analysis of the blot in (A) plotted against a standard curve showing a threefold increase in Pax-6 wound lysates (w) over controls (c). (C and D) EMSAs showing an increase in Pax-6 DNA binding activity in wounded corneal rabbit epithelium. (C) When a Pax-6 paired domain binding consensus probe (PDcon) was incubated with nuclear lysates from control rabbit corneal epithelium, a clear Pax-6 binding band was produced (arrow). One Pax-6 antibody supershifted the band (arrowhead; lane Pax-6A), and one removed the band (lane Pax-6B). Antibodies for Sp1 or AP-2 had no effect. (D) Control (lanes C) and wound (lanes W) nuclear lysates incubated with Pax-6 paired domain binding consensus probe (PDcon) show an increase in DNA binding in the wound lysates, compared with other transcription factors AP-1, NF-κB, AP-2, and Sp1 binding to their consensus probes.

contains promoter DNA from base −92 to +19, was not significantly affected by cotransfection with Pax-6. Together, these data define a region of positive response to Pax-6 between bases −522 and −312. Pr35, a construct generated from Pr42 by internal deletion between −438 and +19 and addition of 5′ sequences (−566 to −522), also responded positively to cotransfection with Pax-6, consistent with our conclusions about the location of response elements.

Pax-6 Protein Binds to DNA Elements Located in the Positive Response Region of the GelB Promoter

To identify DNA elements involved in Pax-6 interaction which might be involved in positive response, we scanned the sequence of the gelB promoter between bases −600 and −92 with the aim of identifying candidate regions with similarity to the canonical Pax-6 binding site, as well as regions of overall high cross-species conservation (Fini *et*

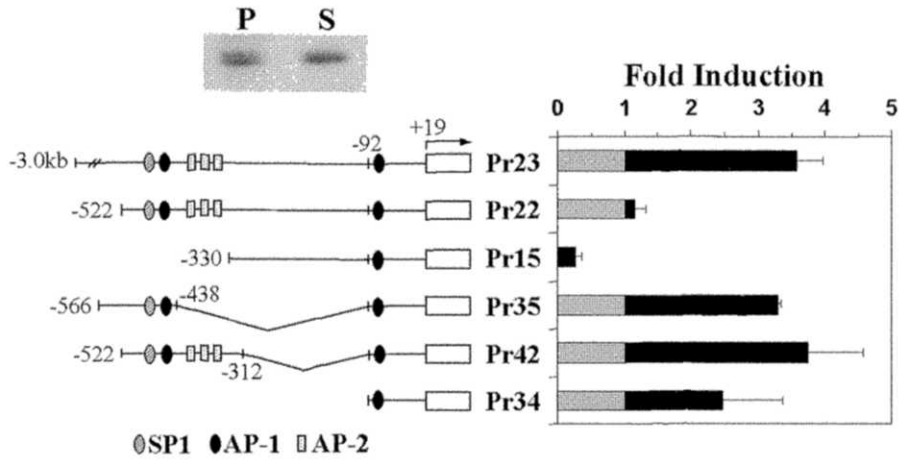


FIG. 5. Pax-6 activates the gelB promoter in corneal epithelial cells. Various gelB promoter constructs were transiently transfected with and without a cotransfected Pax-6 expression vector, into a corneal epithelial cell line (SIRC). The top shows a Western blot with Pax-6 antibody comparing equal lysates from primary corneal epithelial cells (P) and SIRC cells (S). Below are gelB constructs depicted with key AP-1, Sp1, and AP-2 binding sites beside each corresponding construct name and Pax-6-induced response on the right. Fold gelB induction was calculated by dividing multiple Pax-6-transfected responses with controls (set to 1).

al., 1994). Five oligonucleotide probes were prepared from these regions (Table 1) and tested for their capacity to bind Pax-6 by EMSA, using cell lysates from SIRC cells transfected with the Pax-6 expression plasmid. Cells were transfected at the same positive dose used for the promoter activity analysis described above, since these cells were the ones in which the gelB promoter was induced. A representative experiment using probe RM72/73 is shown in Fig. 6A. Several protein–DNA complexes formed on this probe, all of which were competed with 50-fold excess of cold probe. However, a 50-fold excess of an unrelated oligonucleotide (AP-1con) did not compete away any of the complexes, indicating specificity for DNA sequences in RM72/73. In contrast, an excess of unlabeled PDcon efficiently and specifically competed the slowest mobility complex (arrow). Pretreatment with Pax-6 antibody also completely eliminated this complex (Pax-6B). These data indicate that Pax-6 protein is part of the slowest protein–DNA complex that forms with RM72/73. Similar results were obtained when lysates from untransfected cells were used (not shown). Similar results were also obtained with RM80/81; in contrast, the other three probes did not bind Pax-6 (data not shown). These data identify two binding sites for Pax-6 within the positive response region of the gelB promoter.

Pax-6 could bind directly to its interaction sites in the gelB promoter; however, it could also bind indirectly via another protein or proteins. To distinguish between these two possibilities, we performed EMSA using a purified Pax-6 paired domain, expressed as a GST fusion protein. Results are shown in Fig. 6B. Incubation of the PDcon probe with the fusion protein resulted in formation of a DNA–protein complex doublet. In contrast, GST alone did not bind the PDcon probe. This shows that fusion of the paired domain to the GST protein confers appropriate binding specificity for PDcon. GST alone did not bind to RM72/73; however, the fusion protein did bind to RM72/73. This experiment shows direct binding of the Pax-6 paired domain to RM72/73, without the aid of other proteins.

DISCUSSION

Evidence has been accumulating to support the idea that MMPs act as true morphogenetic regulators (Werb and Chin, 1998). For MMPs to function in this way, their activity must be precisely controlled; regulation occurs primarily at the level of transcription (Fini *et al.*, 1998). A large body of work has defined transcription factors controlling MMP gene expression in response to stress-related stimuli in cultured cells (Fini *et al.*, 1998); however, factors controlling MMP gene expression in response to morphogenetic signals are essentially unknown. In this study, we provide the first such example, to our knowledge, implicating the product of a gene which resides at the very top of the genetic hierarchy for eye development: the gene encoding the homeo- and paired domain-containing transcription factor Pax-6. We correlated gelB promoter activity *in vivo* in embryonic and adult eyes with the pattern of Pax-6 expression. We further documented upregulation of Pax-6 expression and activity in repairing corneal wounds, in correlation with induction of gelB promoter activity. Then, using cell transfection studies, we directly demonstrated that Pax-6 stimulates gelB promoter activity and defined three regions within the promoter for Pax-6 response. EMSA with cell lysates allowed us to identify two specific DNA elements that bind immunoreactive Pax-6, located within a positive

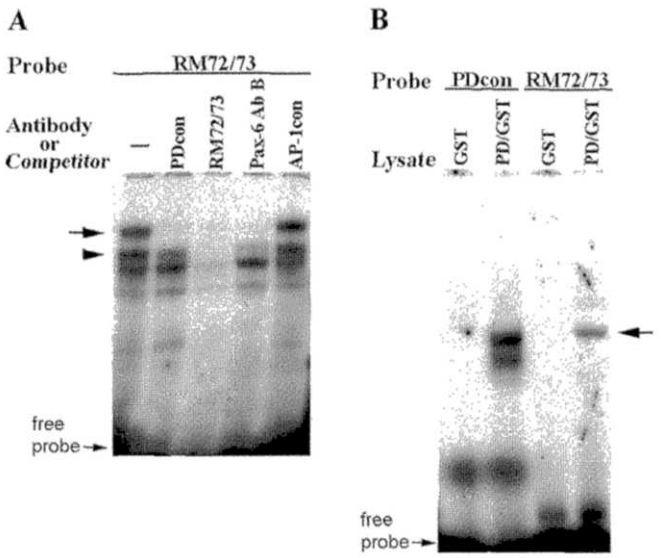


FIG. 6. Pax-6 binds specifically to sequences located in the positive response region of the gelB promoter. (A) EMSA showing the RM72/73 gelB promoter probe binding proteins from nuclear lysates made from Pax-6-transfected SIRC cells. Arrow points to a band that is specifically competed by a Pax-6 paired domain binding consensus sequence (PDcon) and a Pax-6 antibody (Pax-6B). (B) EMSA showing specific binding of a Pax-6 paired domain GST fusion protein (PD/GST) to both a paired domain binding consensus probe (PDcon) and the RM72/73 probe from the gelB promoter (arrow).

Pax-6 response region of the gelB promoter. Finally, EMSA with a purified Pax-6 PD allowed us to show that Pax-6 has the potential to bind directly with the gelB promoter DNA within this region. These data suggest a mechanism by which gelB expression may be directed in an eye-specific manner.

Correlation of GelB Promoter Activity in Vivo with Pax-6 Expression and Activity

Transgenic mouse line 3445, which harbors a β-gal reporter gene construct driven by −522 to +19 of the gelB promoter (Mohan *et al.*, 1998), was used to map gelB promoter activity in eyes. We have previously demonstrated that gelB promoter activity reflects the endogenous gelB gene expression pattern in this line of mice (Mohan *et al.*, 1998). In this study we found considerable overlap between the pattern of gelB promoter activity and the pattern of Pax-6 expression. On the other hand, there were several prominent sites of Pax-6 expression where the promoter was not active, in particular, the corneal epithelium.

Genetic studies have revealed that Pax-6 gene dosage profoundly influences phenotype of embryos and adult (Read, 1995; Schedl *et al.*, 1996). This appears to reflect an exquisite sensitivity of the cells in a tissue to the level of Pax-6 activity. Expression and activity of many transcription factors increase in the epithelium migrating to resurface a corneal wound (Mohan *et al.*, 1998), and the consequences of this might be similar in some cases to an increase in genetic dosage. Since the corneal epithelium of the eye is a major expression site for Pax-6 in both embryos and adults, we investigated changes in Pax-6 expression and activity during corneal reepithelialization. We found that the levels of both Pax-6 protein and DNA binding activity for the Pax-6 PD were increased in extracts from migrating repair epithelium compared to uninjured epithelium. Interestingly, this change correlated with induction of gelB promoter activity in the repairing corneal epithelium of line 3445 transgenic mice. Considering the results of our transfection analysis, these findings suggest that increased dosage of Pax-6 activity could contribute to induction of gelB expression during reepithelialization.

In the cell transfection analysis as performed in this study, positive and negative response regions in the −522 to +19 portion of the gelB promoter precisely balanced each other's activity, resulting in a net zero effect. It is this promoter that drives reporter gene expression in the DNA construct harbored by our line 3445 mice (Mohan *et al.*, 1998). This does not argue against a role for Pax-6 in controlling gelB promoter activity *in vivo*; we previously showed that cell culture cannot perfectly reproduce the conditions associated with gelB promoter regulation in wounds (Mohan *et al.*, 1998). Other transcription factors, such as AP-2 and Sp1 are also induced in the healing corneal epithelium and have been shown to affect gelB promoter activity (Sato and Seiki, 1993; Fini *et al.*, 1994). It is interesting to note that the two potential Pax-6 binding sites identified here are situated next to either an AP-2 or an Sp1 site. Ultimately, the final promoter response to Pax-6 would depend on the DNA context of each of the Pax-6-response elements; i.e., the capacity to bind interacting protein factors and the availability of these factors under different *in vivo* conditions.

Implications of GelB as a Pax-6 Target Gene

In its role as a key regulator of eye development, Pax-6 must ultimately influence the activity of a large number of downstream genes which contribute to morphogenesis of the eye. Indeed, it is well known that Pax-6 directly controls expression of other regulatory genes at the top of the developmental hierarchy, including Dachshund, sine oculis, and eyes absent (Fini *et al.*, 1997). A second class of target genes for Pax-6 lies downstream in the hierarchical cascade and these genes function in cell differentiation. Those identified to date are the crystallins of the mammalian lens (Richardson *et al.*, 1995; Cvekl *et al.*, 1995); neural cell adhesion molecule L1 (Chalepakis *et al.*, 1994) and rhodopsin (Sheng *et al.*, 1997) of the mammalian and *Drosophila* retinas, respectively; and keratin 13 of the cornea (Liu *et al.*, 1999). The function of MMPs as morpho-

genetic regulators places gelB in the first class of Pax-6 target genes. In addition, gelB has some features which make it unique as a Pax-6 target gene. Perhaps the most important of these is that, although it is expressed in eye tissues according to a specific developmental and adult pattern, gelB is not an eye-specific protein. In addition, expression of gelB can be induced in virtually any tissue in response to stress-related stimuli (Fini *et al.*, 1998). This suggests the interesting possibility that Pax-6 functions to modulate gelB gene expression so that it will be expressed in an eye-appropriate manner.

GelB is a 92-kDa MMP with proteolytic reactivity against extracellular matrix components of basement membranes. Based on its embryonic expression pattern, it has been postulated to be involved in implantation of the embryo into the uterus, as well as bone, brain, and eye development. In support of these ideas, it was recently shown that delayed conversion of hypertrophic cartilage to bone occurs in developing long bones of mice made genetically deficient for gelB. This defect, which is eventually compensated by an unknown secondary mechanism, appears to occur as a result of impaired vascular invasion of the cartilagenous zone (Vu *et al.*, 1998). Interestingly, the developmental localization of gelB expression in the brain and the eye is also consistent with a possible role in vascularization (Canete-Soler *et al.*, 1995b). Unfortunately, gene targeting has not been useful in elucidating the function of gelB in these organs, which appear to develop normally in gelB-deficient mice.

In adult animals, gelB is synthesized by inflammatory cells and gelB-deficient mice have impaired neutrophil function (Liu *et al.*, 1998). GelB expression is also induced in many adult tissues under conditions of stress in a pattern which suggests a role in epithelial repair and angiogenesis (Fini *et al.*, 1998). Overexpression or inappropriate expression of gelB has been connected with failure to heal (Matsubara *et al.*, 1991; Fini *et al.*, 1996,1998) as well as tumor invasion and metastasis (Vu and Werb, 1998).

Given the known functions, what role might gelB play, if any, in the phenotypes associated with Pax-6 mutations? Homozygosity for a mutant Pax-6 gene results in lethality due to severe brain and nasal defects. However, these Pax-6 mutations are semidominant, resulting in phenotypes of haploinsufficiency in heterozygotes. Thus, mice heterozygously deficient for Pax-6 have small eyes (Hill *et al.*, 1991). In humans, a heterozygous Pax-6 phenotype, aniridia, is characterized by various ocular malformations, including the lens, iris, cornea, and retina, resulting in cataracts and glaucoma (Ton *et al.*, 1991; Glaser *et al.*, 1992). Peter's anomaly is another human Pax-6-deficient phenotype resulting in central corneal opacification and angiogenesis, as well as adhesion between the lens and the cornea (Hansen *et al.*, 1994). Mice overexpressing Pax-6 also have a microphthalmia phenotype with reduction in corneal size, iris abnormalities, a disorganized ciliary body, and lens defects (Schedl *et al.*, 1996). Cell lines overexpressing Pax-6 become oncogenically transformed (Maulbecker and Gruss, 1993). These phenotypes overlap some of the phenotypes associated with gelB deficiency or its excessive or inappropriate expression. Studies to investigate the connection between Pax-6-deficient phenotypes and altered gelB expression are under way in our lab.

While recent work has provided insight into the role of Pax-6 in embryonic development, essentially nothing is known about its role in adult organs. Our demonstration that Pax-6 activity is induced with the repair response in cornea is novel, to our knowledge. This finding suggests that Pax-6 might exert control over stress-related remodeling in the eye. Pax-6 has two major splice variants—46- and 48-kDa forms, each with unique DNA binding properties (Epstein *et al.*, 1994b). In the present study we have focused on the major 46-kDa variant and its effect on the gelB promoter; however, the intriguing possibility exists that a change in the relative expression levels of the two forms may have a physiological significance. We were unable to clearly distinguish the forms in our Western blots presented here, but our experience with them suggests that the cannonical form of Pax-6 is induced in the wounds, while the alternate splice is repressed. Studies are under way to investigate this possibility. These and other experiments might be undertaken by using viral vectors to alter Pax-6 activity and dosage in the adult eye. The cornea offers a tissue with unique assessibility for such an approach.

ACKNOWLEDGMENTS

We thank Dr. S. K. Chintala for his help with the mouse laser wounding experiment. This work was supported by National Institutes of Health Grants DK39428, AR42981, and EY12651 (M.E.F.) and EY10123 (R.L.M.). M.E.F. is a Jules and Doris Stein Research to Prevent Blindness Professor.

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Received for publication September 14, 1999
Revised February 7, 2000
Accepted February 28, 2000