Journal of Cellular Biochemistry 77:135–148 (2000)

Transgenic Mice With a Mutated Collagen Promoter Display Normal Response During Bleomycin-Induced Fibrosis and Possess Neurological Abnormalities

John H. Stoddart, Jr.,[1] Daniel Ladd,[1] Roderick T. Bronson,[3] Michael Harmon,[1,2] James Jaworski,[1,2] Carole Pritzker,[1,2] Niels Lausen,[1] and Barbara D. Smith[1,2]*

[1]Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118
[2]Boston Veterans Administration Medical Center, Boston, Massachusetts 02118
[3]Department of Pathology, Tufts University School of Medicine and Veterinary Medicine, Boston, Massachusetts 02111

Abstract We have previously identified a potential TGF-β activation element (TAE) in the rat collagen α1(I) promoter at −1624 upstream of the transcriptional start site [Ritzenthaler et al., 1991, 1993]. To determine the importance of the TAE in vivo, we produced transgenic mice carrying 3.6 kb of the rat collagen α1(I) promoter linked to the reporter gene chloramphenicol acetyl transferase with and without site-directed mutations that eliminate DNA-protein binding at the TAE site. Tissue-specific expression of the reporter gene in transgenic mice with the mutated collagen promoter was similar to that of transgenic mice with the normal promoter in two genetic backgrounds as judged by in situ hybridization, reporter assays, and immunochemistry. Endotracheal instillation of bleomycin induces lung fibrosis, mediated in part by TGF-β. Earlier studies indicated that expression of wild-type collagen-reporter gene was upregulated in transgenic mice lungs in response to endotracheal instillation of bleomycin. A similar level of reporter gene upregulation was observed in transgenic mice carrying the mutation in the TAE. Two lines of transgenic mice carrying the mutated promoter construct displayed unexpected neurological abnormalities. In the FVB genetic background, there was a higher than normal incidence of mortality, spontaneous seizures, and an inability to nurture offspring. Histological evidence demonstrated clear abnormalities, including disorderly arrangement of neurons in the hippocampus and significant laminar cortical necrosis in the cerebrum in animals after seizures. In the C57Bl/6 background, there was a high incidence of severe communicating hydrocephalus, early runting, and increased mortality similar to that in transgenic animals with astroglial overexpression of TGF-β. These animals provide an interesting model system to investigate molecular mechanisms responsible for seizures and hydrocephalus. J. Cell. Biochem. 77:135–148, 2000. © 2000 Wiley-Liss, Inc.

Key words: collagen; transgenic mice; lung fibrosis; neuropathology; TGF-β

The expression of extracellular matrix proteins is tightly regulated in complex tissue-specific and developmental patterns. Inappropriate expression of matrix proteins in different tissues is associated with developmental abnormalities and disease states, including pulmonary fibrosis, sclerosis, and Alzheimer's disease [Fillit and Leveugle, 1995; Gauldie et al., 1993]. Type I collagen, composed of two α1(I) and one α2(I) polypeptides, is one of the most abundant extracellular matrix proteins in the body found in high amounts in bone, skin, and tendons with lower amounts in lung. Although expressed in low amounts in brain tissue, type I collagen is a vital component of the extracellular matrix of the central and peripheral nervous systems, and thus plays a role in the development of the nervous system [Rutka et al., 1988].

The collagen type I genes (COL1A1 and COL1A2) are on separate chromosomes with clearly different promoter sequences, although the two genes are usually coordinately regulated. The primary site of regulation of this molecule is believed to be transcriptional. Several groups have analyzed the transcriptional

Grant sponsor: National Institutes of Health; Grant number: P50-HL56386; Grant sponsor: VA merit review program.

*Correspondence to: B. Smith, Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118.

Received 2 July 1999; Accepted 13 October 1999

Print compilation © 2000 Wiley-Liss, Inc.
This article published in Wiley InterScience, February 2000.

regulation of COL1A1, using transgenic animals with various portions of the COL1A1 gene linked to reporter genes [Bedalov, 1995; Bogdanovic et al., 1994; Dodig et al., 1996; Pavlin et al., 1992; Rossert et al., 1995, 1996; Slack et al., 1991; Sokolov et al., 1995]. Transcriptional regulation of COL1A1 is influenced by distal upstream regions of the promoter [Dodig et al., 1996; Ritzenthaler et al., 1991, 1993], the first intron [Bornstein, 1996], the body of the gene [Breault et al., 1997], and in the 3′ flanking region [Rippe et al., 1997; Stefanovic et al., 1997]. The murine COL1A1 promoter (3.6 kb) confers tissue-specific expression of type I collagen [Liska et al., 1994; Pavlin et al., 1992; Rossert et al., 1995, 1996] with expression patterns of reporter genes similar but not identical to known distributions of type I collagen. Expression of reporter genes was high in bone, teeth, tendon or skin and very low in certain adult organs (liver and brain) similar to the endogenous gene. Expression was not detected in selected tissues known to express type I collagen such as the aorta [Bedalov et al., 1994].

Using deletion analysis and co-transfection studies with the rat collagen α1(I) promoter driving the CAT gene, we previously located a cis-acting DNA element 1.6 kb upstream of the transcription start site that confers a three- to fourfold stimulation in the presence of transforming growth factor-β (TGF-β) [Ritzenthaler, 1991]. This element, referred to as α(I)-TAE, is a binding site for a unique protein complex, as judged by DNA electrophoretic mobility shift, cross-linking, and Southwestern assays [Ritzenthaler et al., 1993]. The TAE sequence has consensus binding sites for the transcription factor AP-2, although this protein does not appear to be expressed in lung fibroblasts [Ritzenthaler et al., 1993]. In addition, bleomycin stimulates collagen expression through this element in cell culture [King et al., 1994]. Several different TGF-β response elements have been described in collagen type I genes. For example, a SP-1 site may be involved in the human COL1A1 promoter [Jimenez et al., 1994]. In the COL1A2 promoter, there is a more active site described as an NF1-like site [Rossi et al., 1988], an Sp-1 site [Chung et al., 1996], a Jun binding site [Chung et al., 1996], or SMAD-dependent element [Chen et al., 1999]. This region, which is not well conserved in the COL1A1 gene, may bind several proteins that act cooperatively to activate transcription. These findings suggest that more than one transcription factor may be activating collagen type I gene expression during TGF-β stimulation. There are certainly major differences between the two type I promoters and there may be species differences between human and murine promoters.

We employed transgenic animals to examine the function of the collagen α1(I) promoter fragment in vivo [Agarwal et al., 1996]. When transgenic mice carrying 3.6 kb of rat collagen promoter driving CAT reporter gene (ColCAT3.6) were challenged with subcutaneous injections of TGF-β or endotracheal instillation of bleomycin to induce lung fibrosis, CAT expression in skin, and lung was increased [Agarwal et al., 1996]. Our results indicated in vivo activation of α1(I) promoter by bleomycin treatment in a subset of lung fibroblasts. In order to determine whether the TAE in COL1A1 promoter functions in vivo during fibrosis, we generated transgenic mice containing 3.6 kb of the rat COL1A1 promoter mutated at the TAE site linked to CAT (mTAEColCAT3.6). The mutated transgene was generally expressed in a tissue-specific manner similar to that of the normal transgene (ColCAT3.6). The mutation did not abolish the upregulation of reporter gene expression in lungs of transgenic mice instilled with bleomycin in a model of pulmonary fibrosis. Furthermore, the introduction of the mutated transgene produced unexpected phenotypes in animals including seizures, increased mortality, and hydrocephalus.

MATERIALS AND METHODS

DNA Construct Description and Transgenic Animals

The ColCAT3.6 plasmid containing 3.6 kb of the rat COL1A1 promoter [Lichtler et al., 1989] was kindly provided by A. Lichtler and D. Rowe (University of Connecticut, Farmington, CT). The mTAEColCAT3.6 construct was prepared by site-directed mutagenesis [Kunkel et al., 1987], using a commercial kit according to the manufacturer's instructions (Bio-Rad, Hercules, CA). The primer used to generate the mutation was 5′-AGCTTGTGCGCGGCCGCT-3′. The mTAEColCAT3.6 differs from the normal ColCAT 3.6 in 6 base pairs (bp) starting at position −1624 (Fig. 1). The sequence of the mutated construct was verified by DNA sequencing. In addition, the mutation introduced a *Not*I restriction site in the plasmid that was used for routine screening of the plasmid. Plas-

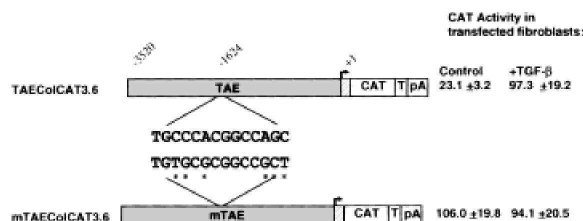

Fig. 1. Depiction of the normal (ColCAT3.6) and mutated (mTAEcolCAT3.6) collagen promoter constructs used to produce transgenic mouse lines. The normal and mutated promoter transgene constructs contain 3.6 kb of the COL1A1 promoter (shaded box), including 115 base pairs of the gene downstream of the transcriptional start site (diagonally shaded box), followed by the chloramphenicol acetyl transferase coding sequence (CAT), the SV40 T antigen splice site (T), and polyadenylation signal (pA). The numbers above the promoter indicate position relative to the transcriptional start site (+1). The nucleotide sequences of the TAE and corresponding mutated TAE are indicated, where (*) highlights bases altered from the wild-type. Values on the right with standard error indicate CAT activity of constructs transfected into fibroblasts.

mid DNAs were prepared by standard procedures including double cesium chloride fractionation. The transgene used for microinjection was prepared by *Hae*II digestion and isolation of the 5.3-kb fragment containing the 3.6-kb promoter and CAT coding sequence from an agarose gel.

Transgenic mice were generated at the Boston University transgenic facility by microinjection of fertilized pronuclei eggs of the FVB or C57Bl/6 strains (Taconic). Microinjected eggs were then implanted into outbred Swiss Webster FBr (Taconic) foster mothers. Transgenic mice were identified by CAT assay of tail homogenates from 18- to 22-day old animals. Cells used for primary culture were obtained from the brains of newborn (1- to 3-day) transgenic animals according to previously described methods [Arenander and de Vellis, 1992], and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS).

Histology and In Situ Hybridization

The tails of newborn (2- to 3-day-old) animals were fixed in fresh, 4% paraformaldehyde/phosphate-buffered saline (PBS), embedded in paraffin, and cut into sections of 4-µm thickness. Animals used for brain histology were placed under anesthesia with pentobarbitol (60 mg/kg body weight) and transcardially perfused with 5.0 ml heparinized saline, followed by 10 ml Bouin's solution (Polysciences, Warrington, PA), then stored in Bouin's solution for at least 10 days for complete fixation. Mouse brains were embedded in paraffin and cut into 10-µm serial sections. Samples were processed for in situ hybridization of α1(I) collagen or CAT [Agarwal et al., 1996]. Sections were also stained with hematoxylin & eosin (H&E) or ver Hoff's van Geisen stains as recommended by the manufacturer (Sigma Chemical Co., St. Louis, MO). Immunofluorescence for CAT antigen was performed by incubating tissue sections with a rabbit anti-CAT antibody (5 Prime, 3 Prime Corp., Boulder, CO) at 1:300 or 1:50 dilution (where indicated) in PBS overnight at 4°C, followed by a goat anti-rabbit Alexa 488 secondary antibody (Molecular Probes, Eugene, OR) at 1:100 dilution in PBS for 2 h at room temperature in the dark. Fluorescence in situ hybridization of primary brain cultures was performed with a digoxygenin-labeled CAT probe (the 1.4-kb *Eco*RI fragment of ColCAT3.6) and fluorescein-labeled anti-digoxygenin secondary antibody, followed by counterstaining with propidium iodide [Wyandt et al., 1998]. Microscopy was performed on an Olympus IX70 or an Olympus BH-2 microscope using a Kodak EOS DCS 5C digital camera utilizing Adobe Photoshop software.

CAT Assays

Protein extracts were obtained from mouse tissues homogenized with a Brinkman polytron at high speed in Tris buffer (0.25 M Tris pH 8.0, with freshly diluted phenylmethyl sulfonyl fluoride [PMSF], 1 mM), and sonicated 15–20 s with a Bronson 450 sonifier. Extracts were then centrifuged for 10 min at 12,000*g* to remove particulate particles. Protein concentrations were determined by Bradford method (Bio-Rad). The amount of protein analyzed for CAT activity was typically 5–10 µg per high-expressing tissue (tail, skin, and bone), and 50–100 µg per low-producing tissue (lung and brain).

The primary method to assay for CAT activity involved measuring acetylated chloramphenicol isolated by an organic extraction. In brief, protein to be analyzed was pre-heated at 65°C for 10 min, then incubated with (0.15 µCi) [^{3}H]-labeled acetyl CoA (New England Nuclear, Boston, MA), 1 mM unlabeled acetyl CoA, and 1.5 mM chloramphenicol in 0.25 M Tris buffer, pH 7.6, in a total volume of 0.10 ml for 60 min at 37°C. The reaction mixture was extracted with ethyl acetate and the radioactivity in the organic phase, containing [^{3}H]-acetylated chlor-

amphenicol, was measured by liquid scintillation. Purified chloramphenicol acetyl transferase standards (Pharmacia, Uppsala, Sweden) were included for each experiment. There were typically 365,000–465,000 cpm per CAT unit. Samples were assayed in duplicate.

An alternative method used to analyze CAT activity involved measurement of acetate into [^{14}C]-labeled chloramphenicol by thin-layer chromatography (TLC) as described [Agarwal et al., 1996]. Quantitation was performed by counting radioactivity on chromatographic plates by an InstantImager™ electronic autoradiography recorder (Packard, Meriden, CT). In control experiments, it was determined that normal nontransgenic tissues had no detectable CAT activity, and no detectable chloramphenicol de-acetylase activity (data not shown).

Southern Blotting

Genomic DNA isolated from tail was analyzed for transgene copy number by Southern blotting with one of two probes as described [Pavlin et al., 1992]. In brief, mouse genomic DNA digested with either *Pvu*II or *Eco*RI was separated on 1.0% agarose gel and transferred to nylon membranes (Hybond N^+; Amersham) by capillary blotting in 10× SSC solution. Hybridization with radiolabeled probes was performed in the presence of 2× SSC, 1% dextran sulfate, 10% sodium dodecyl sulfate (SDS), and 1 mg/ml salmon sperm DNA at 65°C for 18 h and washed under stringent conditions: 0.2× SSC, 1% SDS, at 65°C. The *Pvu*II-*Xba*I promoter fragment of COL1A1 (containing only collagen sequence −944 to +115) or the 1.4-kb *Eco*I fragment of ColCAT3.6 (containing only the CAT coding sequence) were radiolabeled with the [^{32}P]-α CTP (Multi-prime TM kit, Amersham). *Pvu*II-digested genomic DNA was hybridized with the *Pvu*II-*Xba*I probe, yielding the endogenous promoter at 2.6 kb and transgenic promoter at 1.0 kb. The ratio of transgene to endogenous collagen promoter was calculated as an index of transgene copy number. *Eco*RI-digested genomic DNA was analyzed for transgene copy number using the CAT-derived probe and measured using serial dilutions of CAT DNA. Radioactivity signals were analyzed by the InstantImager™ (Packard). The two approaches yielded similar results. Several individuals from the F1 and subsequent generations were evaluated for DNA copy number.

Bleomycin Experiments

Adult transgenic mice were treated with bleomycin by endotracheal instillation as described [Agarwal et al., 1996], then sacrificed at 7–21 days, as indicated in the text. CAT enzyme activity present in whole lung homogenates was determined as described above. There were at least four animals in each group. In selected experiments, animals were anesthetized and perfused with 4% paraformaldehyde and their lungs were processed for in situ hybridization as described.

RESULTS

We have previously identified a cis-acting site at position −1624 of the COL1A1 promoter that showed selective responsiveness to TGF-β in cell culture [Ritzenthaler et al., 1991, 1993]. To determine whether this site is active in vivo, we have prepared transgenic mice carrying 3.6 kb of the promoter with mutations in the TAE region linked to the CAT reporter gene. Representation of the wild-type (ColCAT3.6) and mutated (mTAEColCAT3.6) transgene constructs are depicted in Figure 1. The mTAEColCAT3.6 differs from the wild-type ColCAT3.6 by six base substitutions as indicated. These mutations were chosen based on our results that this particular mutated 14-bp sequence does not (1) abrogate the TGF-β response in co-transfection assays, (2) compete for protein binding, or (3) form a binding site for proteins by gel-shift assays or Southwestern analysis [Ritzenthaler et al., 1993]. The mTAEColCAT3.6 construct was tested by transient transfection of human lung fibroblasts. There was a higher baseline CAT activity in mTAEColCAT 3.6-transfected fibroblasts than in ColCAT3.6-transfected fibroblasts (Fig. 1). In addition, there was no TGF-β stimulation of the mTAEColCAT3.6 construct. Since the construct was active in transfection assays, we sought to determine whether the mutation would change expression of CAT in vivo.

In order to test the TAE site during injury in this model of fibrosis, transgenic mouse lines were established in two genetic backgrounds, FVB and C57Bl/6. The FVB background, used extensively for transgenic animal production, was selected because the FVB egg nuclei are easily injected with DNA and the strain breeds more vigorously than most other strains. The C57Bl/6 strain was also selected since earlier

bleomycin studies were performed in this background. Four separate transgenic lines from independent founders were generated in FVB background: two carrying the mTAEColCAT3.6 transgene (designated lines 1 and 2), and two carrying the normal ColCAT3.6 (designated lines 3 and 4). In the C57Bl/6 background, one line with mTAEColCAT3.6 (line 5) and two lines carrying ColCAT3.6 (lines 6 and 7) were produced. Transgenic founders were bred with wild-type animals, which produced heterozygote offspring in typical Mendelian ratios. To produce a second strain in C57Bl/6 carrying mTAEColCAT3.6, we backcrossed the line 1 transgene (mTAEColCAT3.6/FVB) into wild-type C57Bl/6, and designated this line 1B. After four generations, greater than 90% of the genetic background from line 1B was derived from C57Bl/6.

The relative transgene copy numbers and expression of CAT in each of the lines are shown in Table I. The CAT activity values were determined from the tails of weanling animals in each line. There was an overall linear relationship between transgene copy number and relative expression of CAT protein for most of the lines produced with line 6 as the exception. High levels of transgene expression were observed in both backgrounds of mice (FVB and C57Bl/6) with normal or mutated promoters. In general, the transgene copy numbers and CAT activity within each line did not change through four to five generations suggesting transgene integration into the mouse genome at a single locus and segregation as a unit. The restriction digest patterns from southern blots of each line were consistent with multiple copies of transgene arranged in a head to tail fashion. Flanking regions were different sizes, suggesting separate integration sites. Karyotype analysis by fluorescence in situ hybridization (FISH) performed on cultured astrocytes derived from transgenic lines 1, 4, and 5 suggested single, unique transgene integration sites with each line in different chromosome locations.

High levels of CAT enzyme activity were observed in bone and skin, and very low levels were found in lung, liver, and brain for all lines carrying the normal or mutated promoter. Expression of the CAT reporter gene in the tails of newborn (2- to 3-day-old) transgenic animals was examined further by histological methods. CAT protein was observed in the dermis (particularly the papillary dermis) and outlining regions of developing bone, as judged by immunofluorescence of CAT protein. The staining pattern observed in line 1 (mTAEColCAT3.6) tail (Fig. 2a) was identical to that observed in line 4 (ColCAT3.6) tail (Fig. 2b). The distribution of CAT and collagen RNA in representative tail sections by in situ hybridization under dark field microscopy is shown in Figure 3. CAT mRNA was primarily located in the papillary dermis (Fig. 3a,b), whereas collagen mRNA was located in the reticular dermis, deep dermis, and tendon (Fig. 3c,d). Notably, the CAT mRNA levels were low in tail tendon. The staining patterns for CAT mRNA or protein were similar for animals carrying the mutated or normal promoter transgene and absent in nontransgenic mice.

Animals were instilled by intratracheal instillation with bleomycin and sacrificed after 11 days to determine the activity of the reporter gene. At 11 days after bleomycin treatment, there was significant development of fibrosis in the lungs of mice from both FVB and C57Bl/6 backgrounds with either transgene construct as judged by histology. The CAT enzyme activity of lung homogenates from treated animals is

TABLE I. Transgene Copy Number and Expression in Transgenic Mouse Lines

Line	Mouse background	Transgene	Copy no.[a]	CAT expression[b] CAT U/mg tail protein[c] (±SE)
1	FVB	mColCAT3.6	50–100	79.3 ± 8.06 (n = 18)
2	FVB	mColCAT3.6	3–10	0.43 ± 0.04 (n = 31)
3	FVB	ColCAT3.6	5–20	42.6 ± 5.76 (n = 24)
4	FVB	ColCAT3.6	20–50	51.8 ± 5.02 (n = 24)
5	C57Bl/6	mColCAT3.6	20–50	41.5 ± 3.25 (n = 30)
6	C57Bl/6	ColCAT3.6	50–100	4.43 ± 0.55 (n = 35)
7	C57Bl/6	ColCAT3.6	40–80	39.0 ± 2.84 (n = 66)

[a]Transgene copy number determined by Southern blotting as described under Materials and Methods.
[b]Expression in weanling animals. CAT assays typically yielded 365,000–465,000 CPM per CAT unit.
[c]n = number of animals.

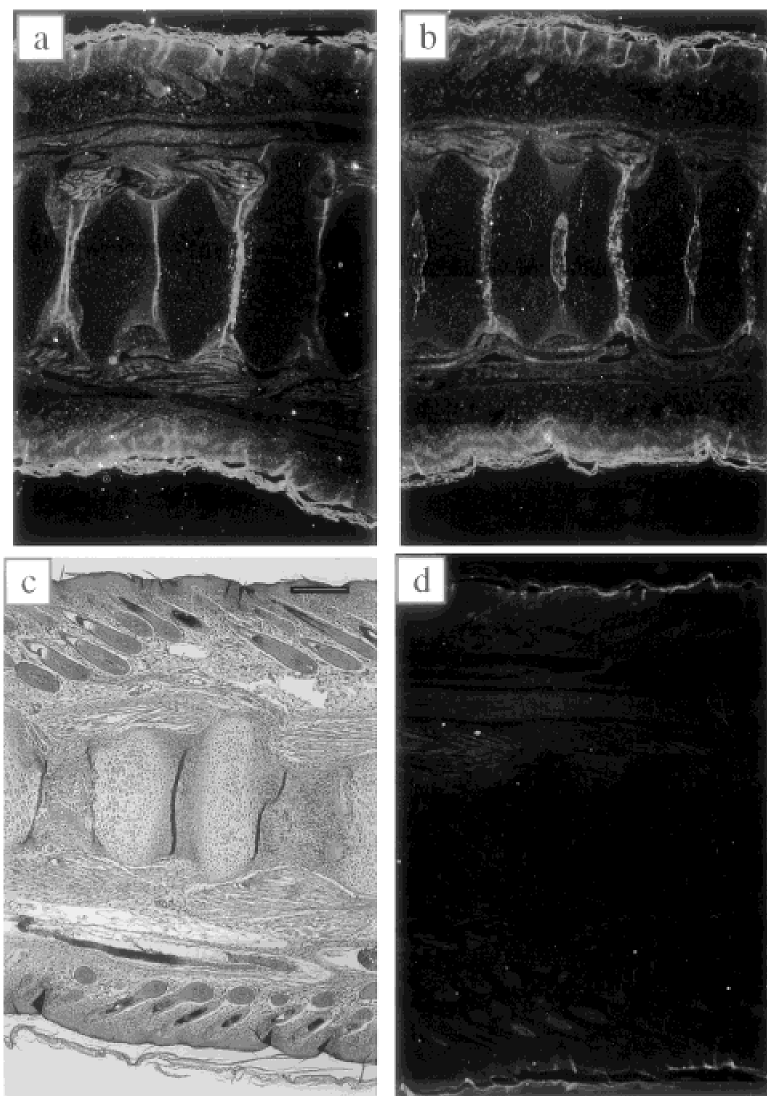

Fig. 2. CAT protein levels in tails of transgenic mice detected by immunofluorescence. Immunofluorescence staining of CAT protein in newborn tails from a line 1 animal (mTAE-ColCAT3.6/FVB) (**a**), line 4 animal (ColCAT36. FVB) (**b**), and nontransgenic control (**d**). CAT antibody was used at 1:200 dilution. Also shown is a ver Hoff's van Geisen stained tail from a nontransgenic mouse (**c**). Images in each panel were made from composites of three individual digital images. Scale bar = 250 μm.

shown in Figure 4. The data are expressed as a ratio of the relative CAT activity from the lungs of bleomycin-treated animals compared with saline-treated animals of the same strain in order to normalize for differences in CAT activity between transgenic lines. Bleomycin treatment caused an increase in CAT activity in lungs of all transgenic animals, although the fold increase varied from between 3- to 16-fold in the different lines. There was no significant loss of CAT induction in transgenic mice carrying the mutated promoter.

Animals carrying the normal promoter transgene in the FVB and C57Bl/6 backgrounds appeared normal and bred successfully, in agreement with the work of others who have produced transgenic mouse lines with this construct [Bedalov et al., 1994; Pavlin et al., 1992]. However, a significant number of animals carrying the mutated promoter transgene in lines 1 and 5 displayed unusual behavior and increased mortality. Several line 1 female mice failed to nurture their litters, demonstrating inattention to pups scattered about the cage. The non-nurturing trait was observed through the course of many (at least seven) generations for a subset of line 1 mothers. The lack of nurturing was generally followed by death of most or all pups in the non-nurtured litter. The frequency of line 1 animals born that were not nurtured (and therefore died) was significantly higher (22%) than control transgenics (2–4%). Matings between two heterozygote line 1 mice were less successful at yielding viable offspring than were matings between a transgenic heterozygote and a wild-type animal. Homozygotes of line 1 or

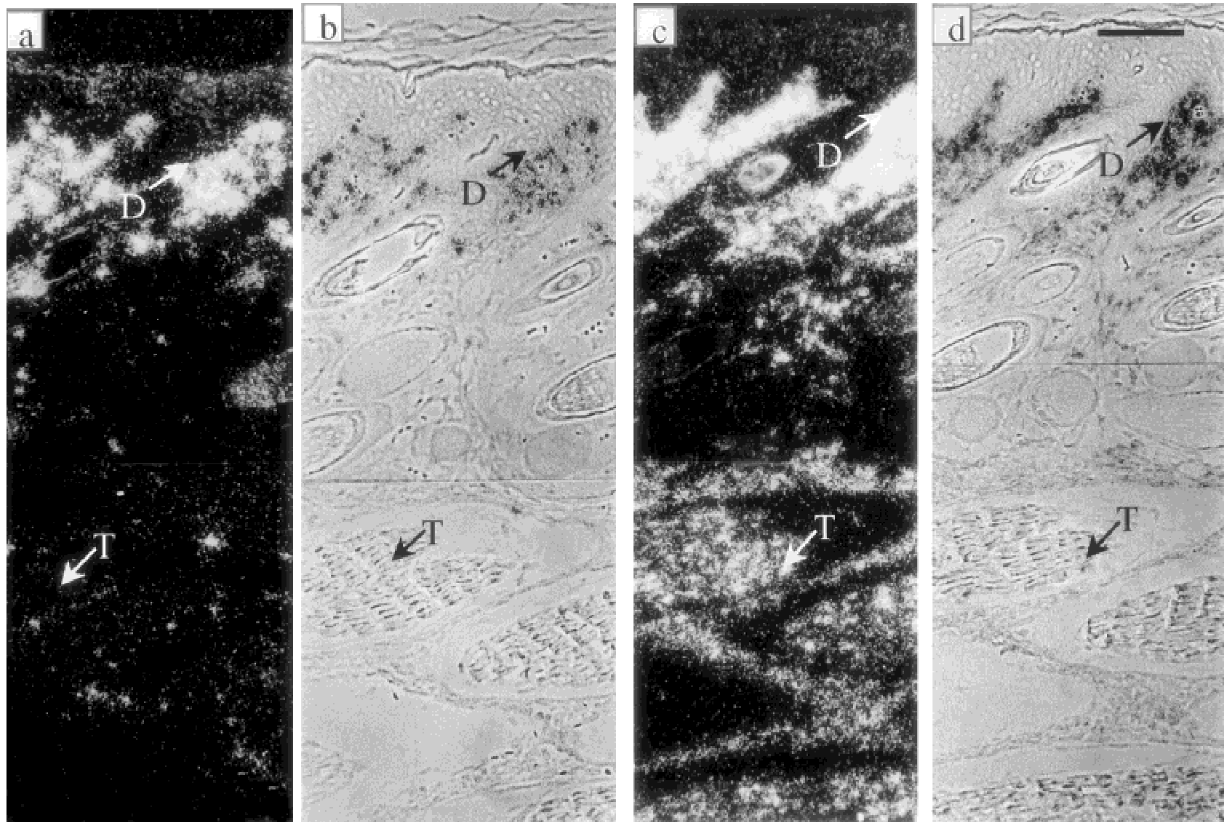

Fig. 3. CAT and collagen mRNA levels in the tails of transgenic mice. In situ hybridization performed on tail of newborn transgenic line 4 (ColCAT3.6) mouse for CAT mRNA (**a,b**) or collagen mRNA (**c,d**) shown under dark field (a,c) or phase contrast (b,d). Signal of CAT mRNA was intense in the dermis (indicated D) and of collagen mRNA was intense in the dermis and tendon (indicated T). The staining pattern in the tail for all transgenics (normal or mutated promoter) was similar. Scale bar = 250 μm.

line 5 animals (mTAEColCAT3.6 in C57Bl/6) have not been produced. By contrast, all the control transgenic lines carrying ColCAT3.6 have included several homozygote animals that have bred normally.

The second striking behavioral anomaly observed in a portion of line 1 animals was the appearance of seizures that often resulted in death within several (3 to 10) days. During seizures there was extensive visible musculature contraction of the forelimbs and hindlimbs, excessive salivation, and abnormal posture and countenance. Unusual behavioral patterns associated with seizures included extreme hyperactivity in response to movement of the cage or touch, alternating with a lethargic, catatonic disposition. Several instances of self-scratching to the point of bleeding were observed. Over the course of several generations, the frequency of animals which displayed seizures or related signs was significantly higher for line 1 (24%) compared with control transgenics (1–3%). Animals that displayed these abnormal features were adults, generally between 60–120 days of age. In several instances, both males and females were observed to undergo seizures immediately following mating situations.

Histological analysis was performed on brains of line 1 mice after seizures. As compared with a phenotypically normal transgenic mouse (Fig. 5a), the cerebrum from a line 1 mouse that underwent seizures displayed significant laminar cortical necrosis (Fig. 5b). In addition, the orderly dense arrangement of neurons in the CA3 region of the hippocampus normally present and seen in the control animal (Fig. 5c) was lost in the line 1 animal (Fig. 5d). The hippocampus of the line 1 animal showed significant neuronal cell death and loss of tissue architecture. This region of the brain is particularly sensitive to damage induced by seizures and/or hypoxia [Klempt et al., 1992; Nichols et al., 1991].

Although most mTAEColCAT3.6/C57Bl/6 (line 5) mice appeared normal, a subset of ani-

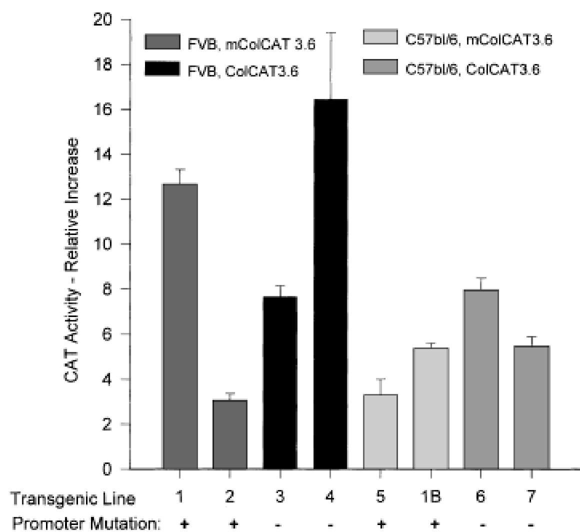

Fig. 4. CAT activity in the lungs of transgenic mice treated with bleomycin. Adult transgenic mice were treated by intratracheal instillation with bleomycin or saline. After 11 days, animals were sacrificed and the CAT activity from whole lung homogenates was determined as described under Materials and Methods. Data are expressed as relative fold increase in CAT activity after bleomycin treatment for each transgenic line as compared with saline-instilled animals from the same line. Transgenic construct mColCAT3.6 is carried by lines 1, 2, 5, and 1B, and control construct ColCAT3.6 is carried by lines 3, 4, 6, and 7.

mals displayed a developmental defect(s), including hydrocephalus. Selected line 5 offspring had small body size with dome-shaped enlarged heads. An elevated level of cerebrospinal fluid (CSF) released during autopsy of selected line 5 animals was consistent with hydrocephalus. Histological analysis was performed on several line 5 transgenic animals, some of which appeared to have hydrocephalus. The brain of a 30-day-old line 5 mouse exhibited greatly enlarged lateral ventricles, as compared with controls, as shown in Figure 6a,b. The arrow in Figure 6a points to a normal lateral ventricle from a nontransgenic littermate. In addition, the cerebellum of the affected animal was reduced in size with corresponding reduced number of folia (Fig. 6c,d). In another extreme case, a severely hydrocephalic 30-day-old line 5 animal possessed brain tissue in the region of the nasal cavity (data not shown), suggesting that elevated cerebral spinal fluid pressure dramatically affected the normal brain development. Several additional animals derived from line 5 transgenic parents were sacrificed and the heads serially sectioned. Hydrocephalus was evident at birth, and no obstructions were observed in the serial sections which is consistent with nonobstructive/communicating hydrocephalus.

The hydrocephalus defect was observed primarily in mice derived from matings between two line 5 heterozygote parents, a situation expected to produce 25% homozygote offspring. There was a higher incidence than normal of early mortality from such litters (approximately 15% of total). In addition, viable homozygote line 5 animals (similar to line 1) have not been produced despite many breeding attempts. These observations raised the possibility that the phenotypic conditions may have been related to the dosage of transgene.

The C57Bl/6 mice carrying mTAEColCAT3.6 did not have the apparent nurturing defect or seizures present in the selected FVB/mTAEColCAT3.6 mice. To determine whether the nurturing/seizure defect could be observed in the C57Bl/6 background, we monitored the line 1B animals (original line 1 transgene backcrossed in the C57Bl/6 background). Through greater than six generations, there have been no observations of seizures or related symptoms in line 1B animals, female or male. Breeding of line 1B females has proceeded normally in seven out of nine females tested. In addition, there have been no apparent cases of hydrocephalus in the line 1B animals. The non-nurturing phenotype was more consistently observed in the FVB background carrying mTAEColCAT3.6 (line 1), suggesting that the mouse genetic background may play a role in the susceptibility to the condition.

To determine whether the abnormal neurological conditions observed were associated with elevated expression of CAT in the brain, whole brain extracts from adult transgenic animals were analyzed for expression CAT activity. The CAT activities from the brains of all transgenic strains were within the same range. Thus there was no apparent global increase in reporter gene expression in the brains of these animals. In situ and immunohistological analysis also did not indicate any localized increase in CAT activity or increase in collagen deposition in the brain.

DISCUSSION

We have produced transgenic mice carrying 3.6 kb of the rat COL1A1 promoter with (or without) mutations to characterize regulatory elements of the COL1A1 gene. The element we have analyzed in our transgenic mice was origi-

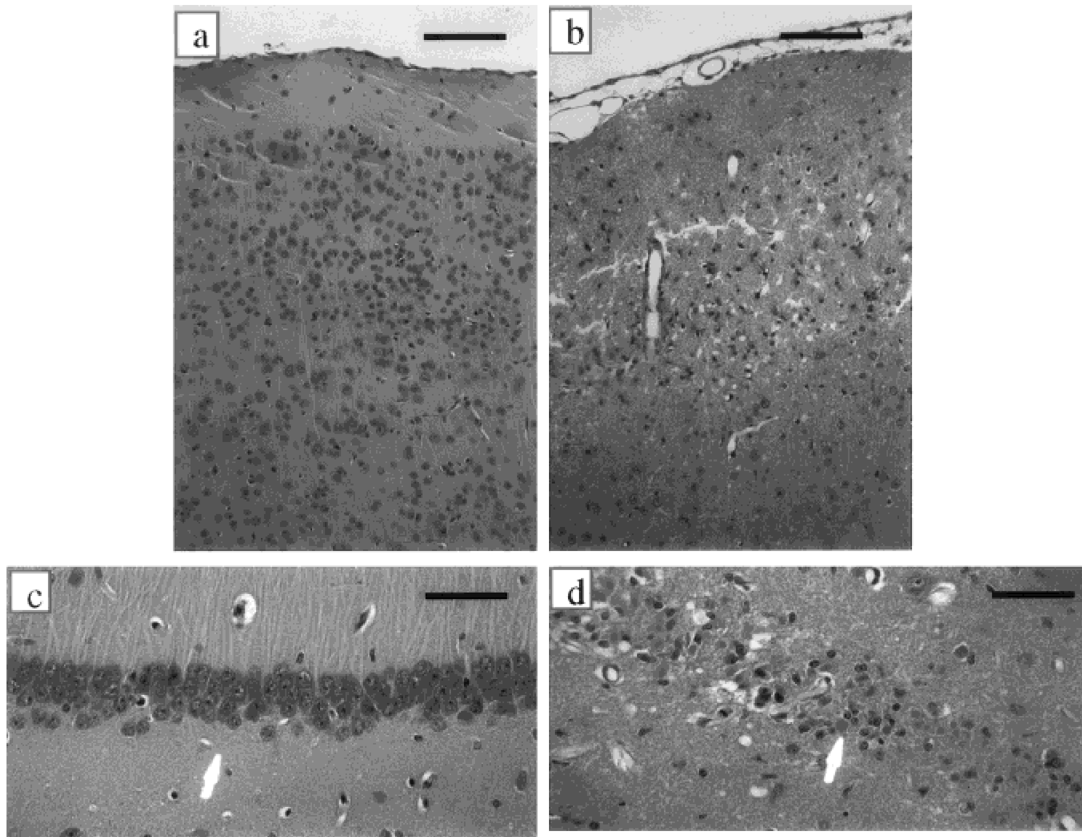

Fig. 5. Neuropathology characteristic of seizures evident in brains of transgenic mTAEColCAT3.6/FVB (line 1) animal. Hematoxylin/eosin stained transverse sections of the cerebrum from line 4 transgenic control (ColCAT3.6/FVB) (**a**) and line 1 (mTAEColCAT3.6/FVB) transgenic mice (**b**). Loss of neurons and tissue necrosis are evident in the brain from the line 1 animal. Matching transverse sections from the CA3 region of the hippocampus of line 4 (**c**) and line 1 mice (**d**). The orderly array of viable neuronal nuclei normally observed in control animals (**c**, arrow) is missing in the brain from the line 1 mouse. Scale bars = 125 μm (a, b) or 62.5 μm (**c,d**).

nally identified as a region responding to TGF-β in lung fibroblasts [Ritzenthaler et al., 1991, 1993]. This element, called TAE, is located between two tissue-specific regulatory regions containing an osteoblast specific element [Dodig et al., 1996; Rossert et al., 1996] and an enhancer-like element [Rossert et al., 1996]. In addition, it was described as a region that responds, in part, to bleomycin treatment of fibroblasts in culture [King et al., 1994]. To evaluate the potential function of this element, we produced a promoter-reporter construct with site directed mutations designed to eliminate DNA protein binding [Ritzenthaler et al., 1991, 1993]. This construct in transient transfection assays did not respond to TGF-β and the mutations conferred a higher level of basal activity (Fig. 1), suggesting that this is an inhibitory region, rather than and an activation region. It is possible that a repressor complex is present at this site, which becomes inactivated by the mutation. Therefore, this mutated construct was used to produce transgenic mice to determine its activity in vivo as well as in cell culture.

We found that the transgene driven by the mutated promoter was expressed in a tissue-restricted pattern similar to transgenic lines with wild-type promoter [Bedalov et al., 1995; Bogdanovic et al., 1994; Dodig et al., 1996; Houglum et al., 1995; Pavlin et al., 1992; Slack et al., 1991]. Absolute levels of expression varied between transgenic lines, in which CAT activity was typically related to transgene copy number (Table 1). The mutations did not cause a dramatic increase in activity in vivo. Our observations concur with previous reports that ColCAT3.6 has cis-acting elements necessary for specificity within most connective tissues with the exception of vascular smooth muscle [Bedalov et al., 1994; Pavlin et al., 1992]. It is noteworthy that expression of the transgene (normal or mutated) in the tendon of all lines

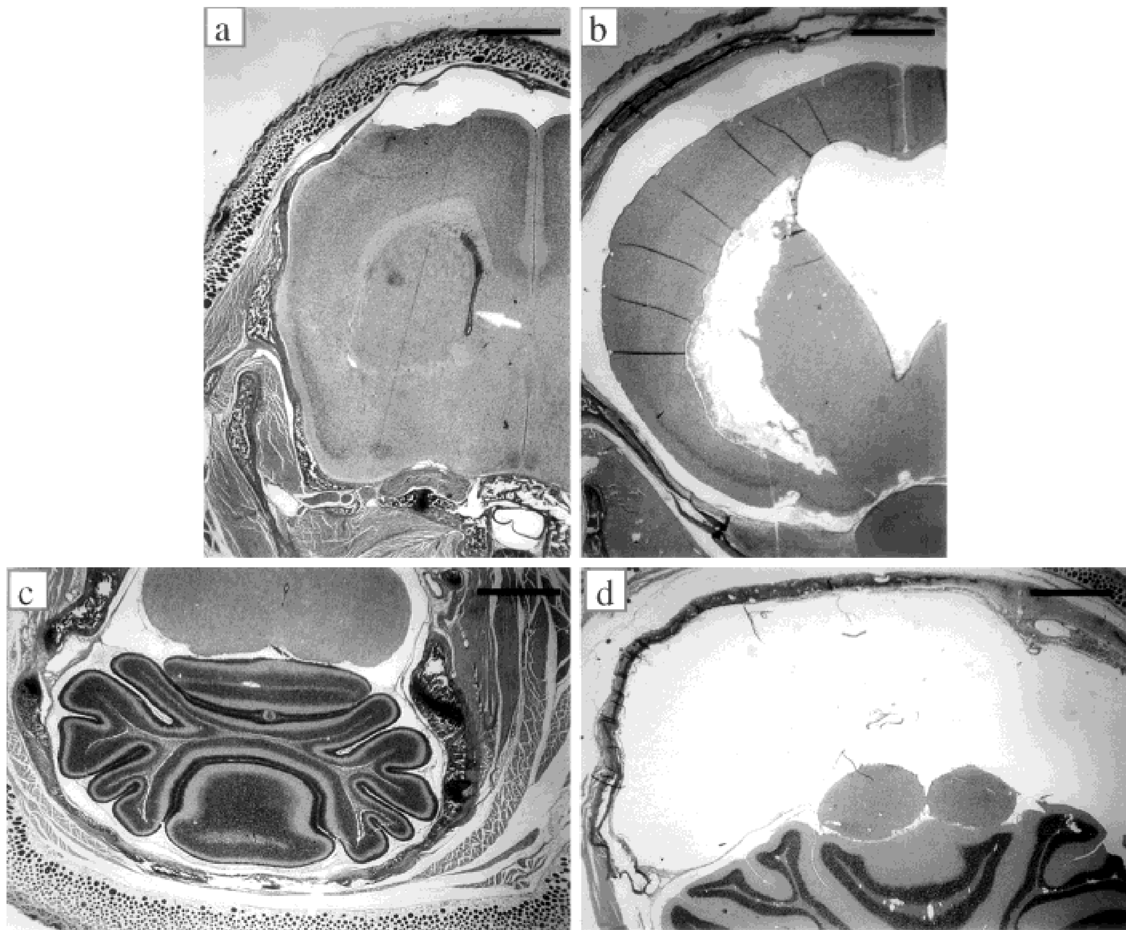

Fig. 6. Evidence of hydrocephalus in brain of line 5 (mTAEColCAT3.6/C57Bl/6) animal. Hematoxylin & eosin (H&E)-stained transverse sections of brains of a 30-day old nontransgenic (**a**) and line 5 (mTAEColCAT3.6/C57Bl/6) transgenic littermate (**b**). The lateral ventricle of the control animal (a, arrow) is greatly enlarged in the line 5 transgenic (b). H&E-stained transverse sections of the cerebellum of nontransgenic littermate (**c**) and line 5 transgenic mouse (**d**) showed that the cerebellum of the transgenic mouse was significantly smaller, with a reduced number of folia. Scale bars = 1.00 mm.

we produced was lower than expected (Fig. 3). This observation is in contrasts with previous investigations by others who observed that 3.6 Kb of the COL1A1 promoter directed expression to tendon [Houglum et al., 1995; Liska et al., 1994; Pavlin et al., 1992]. The reason for this disparity remains unclear.

The pulmonary response after bleomycin treatment is associated with elevated expression of a variety of extracellular matrix components, including alpha 1(I) and alpha 2(I) collagens, elastin, tenascin, and proteoglycans. [Lucey et al., 1996; Shahzeidi et al., 1994; Westergren-Thorsson et al., 1993; Zhang et al., 1995; Zhao et al., 1998]. We have previously demonstrated that collagen promoter-reporter activity increased in certain lung cells in response to bleomycin treatment of transgenic mice and in skin after TGF-β injections [Agarwal et al., 1996]. TGF-β is a primary intercellular mediator involved in bleomycin-induced fibrosis. TGF-β levels are elevated after bleomycin treatment [Gauldie et al., 1993], and antibodies against TGF-β partially prevent the onset of fibrosis [Giri et al., 1993]. Since TAE element responds to TGF-β and bleomycin in cell culture, we instilled bleomycin into transgenic mice with mutated and wild-type collagen promoters to ascertain whether the TAE element plays a role in the response to fibrotic signals in vivo. Transgenic animals carrying the mutated promoter construct (mTAEColCAT3.6) developed lung fibrosis with an upregulation of reporter gene similar to control mice (Fig. 4). The mutations did not block the fibrotic response of the transgene which is consistent with the idea that bleomycin mediates its effect in part through the action of cytokines other than

TGF-β. For example, it is known that bleomycin treatment is associated with increased levels of chemokines MIP-1α and MCP-1 [Smith et al., 1996], as well as circulating levels of the proinflammatory cytokine TNF [Gauldie et al., 1993].

Alternatively, the COL1A1 promoter may contain other elements outside of the TAE region that contributes to the control of expression during bleomycin-induced injury. Most likely, these additional elements are not in the first intron. The role of the first intron in tissue-specific expression has remained controversial. Selected reports have shown that the COL1A1 first intron was required for tissue specificity [Liska et al., 1990, 1992, 1994], although others have not observed this requirement [Olsen et al., 1991; Sokolov et al., 1993, 1995]. Recently it was demonstrated that genetically engineered mice lacking the COL1A1 first intron expressed a reduced amount of collagen in the lung of adult animals and were otherwise normal [Hormuzdi et al., 1998]. However, intratracheal instillation of bleomycin in mice with the intron targeted deletion caused increased collagen synthesis in fibrotic lungs, suggesting that the fibrotic response elements are not in the intron but elsewhere in the promoter or gene [Hormuzdi et al., 1999].

Neurological abnormalities, including a high rate of mortality, seizures, a nurturing problem, and hydrocephalus were observed in a subset of two independent transgenic lines carrying mTAEColCAT3.6. The integration sites of all the lines were different as judged by FISH analysis that demonstrated separate chromosome integration sites and by Southern analysis with different-sized flanking sequences. The neurological findings were unexpected, as several lines of transgenic mice carrying the nonmutated and mutated ColCAT3.6 constructs never had these abnormalities. [Bedalov et al., 1995; Bogdanovic et al., 1994; Dodig et al., 1996; Pavlin et al., 1992] Furthermore, other similar constructs with mouse [Rossert et al., 1995, 1996], or human COL1A1 promoter reporter genes [Houglum et al., 1995; Slack et al., 1991] have not resulted in abnormal phenotypes. It seemed unlikely that the same construct was coincidentally located in two different genes that caused neurological symptoms.

We considered the hypothesis that the phenotype was related to the transgene per se, as opposed to a DNA integration effect. Two observations suggested that the phenotype might be related to transgene "dosage". First, the abnormalities were observed in two lines carrying an elevated copy number (lines 1 and 5), but not a third line with a low copy number (line 2). Second, viable homozygote animals from the high copy number affected lines could not be produced, despite many attempts, whereas several viable homozygotes carrying elevated copy numbers of the control transgene and homozygotes from line 2 carrying low copy numbers of the mutated transgene have been produced.

Next, we investigated the possibility that multiple copies of the mutated promoter transgene may have reduced or altered the levels of cellular transcription factors. There is precedence for induction of an altered neurological phenotype through genetic manipulation of transcription factor levels. For example, mice deficient in fosB have a nurturing defect similar to that in line 1 [Brown et al., 1996]. The TAE sequence has a binding site for AP-2 protein [Ritzenthaler et al., 1993], which is a retinoic acid-responsive protein produced by epithelial and neuroepithelial cells involved in development. Knockouts of AP-2 protein cause defects in neural tube closure, eye, craniofacial, and skeletal development [Schorle et al., 1996; Zhang et al., 1996]. We have recently cloned and characterized a fibroblast TAE binding protein, YB-1, that also binds to this DNA sequence (unpublished data). Therefore, the mutation we introduced in the COL1A1 promoter removed a YB-1 and an AP-2 binding site. In addition, a consensus sequence for CREB/ATF transcription factors was also inadvertently created. Mice deficient in activating transcription factor-2 (ATF-2) also have severe defects on the development of the skeletal and central nervous systems [Reimold et al., 1996], including enlarged brain ventricles and decreased epiphyseal plate ossification. We found that the mutated promoter sequence did not bind AP-2 and appeared to bind a CREB or a CREB-like protein in the brains of transgenic animals. However, the relative amounts of CREB, YB-1, and AP-2 in the brain nuclear extracts were not significantly different between transgenic and nontransgenic groups, as judged by Western analysis and gel-shift data (unpublished data).

The abnormalities present in some of our transgenic mice were remarkably similar to those seen in transgenic mice overexpressing TGF-β, specifically in astrocytes. These mice

displayed seizures, hydrocephalus, uncoordinated movement, and early runting [Galbreath et al., 1995; Wyss-Coray et al., 1995]. To investigate whether our transgenic mice had altered TGF-β signaling, we used primary cultured astrocytes from newborn transgenic and control mice. Cells were treated with TGF-β in culture and RNA was analyzed by Northern blot. The induction of plasminogen activator inhibitor (PAI-1), a TGF-β-regulated gene, and collagen was similar for control and transgenic cell cultures (unpublished data). These observations suggest that a potential defect of the TGF-β signaling pathways would be modest at best.

We considered the possibility that there were alterations in gene expression in the brains of these animals. The TAE element is located within a potentially critical region of the promoter between two tissue-specific elements: an osteoblast-specific element at position −1683 to −1670 [Dodig et al., 1996] and an enhancer-like element −1617 to −1582 [Rossert et al., 1996]. A homeo-domain like protein present in osteoblasts [Dodig et al., 1996] interacts with the sequence from −1670 to −1683. A mutant transgene construct made with a deleted TAE sequence was inappropriately expressed in the brains of 15.5-day transgenic embryos [Rossert et al., 1996]. The abnormally high expression in the brains of transgenic animals raises the possibility that the deleted region may normally serve as a binding site for a repressor protein and, in its absence, allows expression in brain. Thus, it is possible that mutation of this region of the promoter is involved in tissue specific or developmental regulation of collagen expression in the brain. Collagen mRNA isolated directly from mouse brains or associated calvaria bones from transgenic (either normal or mutant construct) was similar to controls. In addition, histological analysis of newborn transgenic mouse brain sections did not demonstrate differences of connective tissue levels, as judged by Masson-trichrome staining or in situ hybridization. In addition, we could not detect a significant elevation in expression of CAT in whole brain extracts. It is possible that subtle localized or developmental-specific changes could have gone undetected. Tissue-specific elements may be necessary during development for certain cells to express collagen, and further studies will be required to determine whether the promoter TAE element is involved in subtle tissue-specific regulation of COL1A1 during development.

It remains possible that the altered phenotype observed in a subset of the mice were related to their specific genetic backgrounds. The seizure/non-nurturing defect observed in FVB (line 1) was not apparent in the C57Bl6 background (lines 5 and 1B), and the hydrocephalus condition in the C57Bl6 background (line 5) was not apparent in the FVB lines. Notably, it has recently been demonstrated that FVB mice are more susceptible to seizures than are other backgrounds of mice [Goelz et al., 1998]. FVB mice undergo seizures at lower doses of kainic acid than do C57Bl/6; they also display a higher degree of excitotoxic cell death than C57Bl/6 after having comparable seizures [Schauwecker and Steward, 1997]. Thus, the most logical explanation may be that the genetic background contributed to the phenotypes observed in these transgenic mice.

In conclusion, several transgenic animal lines have been established with multiple copies of a mutated collagen promoter driving the CAT reporter gene. The mutation, situated between critical regulatory elements of the COL1A1 promoter, was originally identified on the basis of conferring responsiveness to TGF-β. All lines of transgenic mice carrying either ColCAT3.6 or mTAEColCAT3.6 demonstrated up-regulation of reporter gene expression in the lung following bleomycin treatment. This suggests that there are additional elements of the promoter, which confer responsiveness to TGF-β in vivo, and/or that other cytokines in addition to TGF-β play a role in the bleomycin response. Transgenic lines harboring high levels of the mutated construct displayed abnormalities including seizures, hydrocephalus, and a high rate of mortality, a phenotype remarkably similar to that observed in mice that overexpress TGF-β in astrocytes. However, our experiments did not demonstrate a difference in TGF-β signaling among transgenic or control mice. Further, there appeared to be no difference of endogenous levels of transcription factors capable of binding the mutated promoter sequence in the brains of transgenic and normal mice. A logical explanation of the phenotype may relate to the genetic backgrounds of the animals used in this study.

ACKNOWLEDGMENTS

We thank Fuxiang Hou for technical assistance.

REFERENCES

Agarwal AR, Goldstein RH, Lucey E, Ngo HQ, Smith BD. 1996. Cell-specific expression of the alpha 1 (I) collagen promoter-CAT transgene in skin and lung: a response to TGF-beta subcutaneous injection and bleomycin endotracheal instillation. J Cell Biochem 63:135–148.

Arenander A, de Vellis J. 1992. Early response gene induction in astrocytes as a mechanism for encoding and integrating neuronal signals. Prog Brain Res 94:177–188.

Bedalov A. 1995. Differences in regulation of alpha 1(I) collagen promoter in vascular smooth muscle cells in transfection studies and transgenic mice. Biochem Mol Biol Int 37:159–166.

Bedalov A, Breault DT, Sokolov BP, Lichtler AC, Bedalov I, Clark SH, Mack K, Khillan JS, Woody CO, Kream BE, Rowe DW. 1994. Regulation of the alpha 1(I) collagen promoter in vascular smooth muscle cells. Comparison with other alpha 1(I) collagen-producing cells in transgenic animals and cultured cells. J Biol Chem 269:4903–4909.

Bedalov A, Salvatori R, Dodig M, Kronenberg MS, Kapural B, Bogdanovic Z, Kream BE, Woody CO, Clark SH, Mack K, Rowe DW, Lichtler AC. 1995. Regulation of COL1A1 expression in type I collagen producing tissues: identification of a 49 base pair region which is required for transgene expression in bone of transgenic mice. J Bone Miner Res 10:1443–14451.

Bogdanovic Z, Bedalov A, Krebsbach PH, Pavlin D, Woody CO, Clark SH, Thomas HF, Rowe DW, Kream BE, Lichtler AC. 1994. Upstream regulatory elements necessary for expression of the rat COL1A1 promoter in transgenic mice. J Bone Miner Res 9:285–292.

Bornstein P. 1996. Regulation of expression of the alpha 1 (I) collagen gene: a critical appraisal of the role of the first intron [see comments]. Matrix Biol 15:3–10.

Breault DT, Lichtler AC, Rowe DW. 1997. COL1A1 transgene expression in stably transfected osteoblastic cells. Relative contributions of first intron, 3′-flanking sequences, and sequences derived from the body of the human COL1A1 minigene. J Biol Chem 272:31241–31250.

Brown JR, Ye H, Bronson RT, Dikkes P, Greenberg ME. 1996. A defect in nurturing in mice lacking the immediate early gene fosB. Cell 86:297–309.

Chen SJ, Yuan W, Mori Y, Levenson A, Trojanowska M, Varga J. 1999. Stimulation of type I collagen transcription in human skin fibroblasts by TGF-beta: involvement of Smad 3. J Invest Dermatol 112:49–57.

Chung KY, Agarwal A, Uitto J, Mauviel A. 1996. An AP-1 binding sequence is essential for regulation of the human alpha2(I) collagen (COL1A2) promoter activity by transforming growth factor-beta. J Biol Chem 271:3272–3278.

Dodig M, Kronenberg MS, Bedalov A, Kream BE, Gronowicz G, Clark SH, Mack K, Liu YH, Maxon R, Pan ZZ, Upholt WB, Rowe DW, Lichtler AC. 1996. Identification of a TAAT-containing motif required for high level expression of the COL1A1 promoter in differentiated osteoblasts of transgenic mice. J Biol Chem 271:16422–16429.

Fillit H, Leveugle B. 1995. Disorders of the extracellular matrix and the pathogenesis of senile dementia of the Alzheimer's type [editorial]. Lab Invest 72:249–253.

Galbreath E, Kim SJ, Park K, Brenner M, Messing A. 1995. Overexpression of TGF-beta 1 in the central nervous system of transgenic mice results in hydrocephalus. J Neuropathol Exp Neurol 54:339–349.

Gauldie J, Jordana M, Cox G. 1993. Cytokines and pulmonary fibrosis. Thorax 48:931–935.

Giri SN, Hyde DM, Hollinger MA. 1993. Effect of antibody to transforming growth factor beta on bleomycin induced accumulation of lung collagen in mice [see comments]. Thorax 48:959–966.

Goelz MF, Mahler J, Harry J, Myers P, Clark J, Thigpen JE, Forsythe DB. 1998. Neuropathologic findings associated with seizures in FVB mice. Lab Anim Sci 48:34–37.

Hormuzdi SG, Penttinen R, Jaenisch R, Bornstein P. 1998. A gene-targeting approach identifies a function for the first intron in expression of the alpha1(I) collagen gene. Mol Cell Biol 18:3368–3375.

Hormuzdi SG, Strandjord TP, Madtes DK, Bornstein P. 1999. Mice with a targeted intronic deletion in the Col1a1 gene respond to bleomycin-induced pulmonary fibrosis with increased expression of the mutant allele. Matrix Biol 18:287–294.

Houglum K, Buck M, Alcorn J, Contreras S, Bornstein P, Chojkier M. 1995. Two different cis-acting regulatory regions direct cell-specific transcription of the collagen alpha 1(I) gene in hepatic stellate cells and in skin and tendon fibroblasts. J Clin Invest 96:2269–2276.

Jimenez SA, Varga J, Olsen A, Li L, Diaz A, Herhal J, Koch J. 1994. Functional analysis of human alpha 1(I) procollagen gene promoter. Differential activity in collagen-producing and -nonproducing cells and response to transforming growth factor beta 1. J Biol Chem 269: 12684–12691.

King SL, Lichtler AC, Rowe DW, Xie R, Long GL, Absher MP, Cutroneo KR. 1994. Bleomycin stimulates pro-alpha 1 (I) collagen promoter through transforming growth factor beta response element by intracellular and extracellular signaling. J Biol Chem 269:13156–13161.

Klempt ND, Sirimanne E, Gunn AJ, Klempt M, Singh K, Williams C, Gluckman PD. 1992. Hypoxia-ischemia induces transforming growth factor beta 1 mRNA in the infant rat brain. Brain Res Mol Brain Res 13:93–101.

Kunkel TA, Roberts JD, Zakour RA. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol 154:367–382.

Lichtler A, Stover ML, Angilly J, Kream B, Rowe DW. 1989. Isolation and characterization of the rat alpha 1(I) collagen promoter. Regulation by 1,25-dihydroxyvitamin D [published erratum appears in J Biol Chem 1994: 269: 16518]. J Biol Chem 264:3072–3077.

Liska DJ, Slack JL, Bornstein P. 1990. A highly conserved intronic sequence is involved in transcriptional regulation of the alpha 1(I) collagen gene. Cell Regul 1:487–498.

Liska DJ, Robinson VR, Bornstein P. 1992. Elements in the first intron of the alpha 1(I) collagen gene interact with Sp1 to regulate gene expression. Gene Expr 2:379–389.

Liska DJ, Reed MJ, Sage EH, Bornstein P. 1994. Cell-specific expression of alpha 1(I) collagen-hGH minigenes in transgenic mice. J Cell Biol 125:695–704.

Lucey EC, Ngo HQ, Agarwal A, Smith BD, Snider GL, Goldstein RH. 1996. Differential expression of elastin and alpha 1(I) collagen mRNA in mice with bleomycin-induced pulmonary fibrosis. Lab Invest 74:12–20.

Nichols NR, Laping NJ, Day JR, Finch CE. 1991. Increases in transforming growth factor-beta mRNA in hippocampus during response to entorhinal cortex lesions in intact and adrenalectomized rats. J Neurosci Res 28:134–139.

Olsen AS, Geddis AE, Prockop DJ. 1991. High levels of expression of a minigene version of the human pro alpha 1 (I) collagen gene in stably transfected mouse fibroblasts. Effects of deleting putative regulatory sequences in the first intron. J Biol Chem 266:1117–1121.

Pavlin D, Lichtler AC, Bedalov A, Kream BE, Harrison JR, Thomas HF, Gronowicz GA, Clark SH, Woody CO, Rowe DW. 1992. Differential utilization of regulatory domains within the alpha 1(I) collagen promoter in osseous and fibroblastic cells. J Cell Biol 116:227–236.

Reimold AM, Grusby MJ, Kosaras B, Fries JW, Mori R, Maniwa S, Clauss IM, Collins T, Sidman RL, Glimcher MJ, Glimcher LH. 1996. Chondrodysplasia and neurological abnormalities in ATF-2-deficient mice. Nature 379: 262–265.

Rippe RA, Umezawa A, Kimball JP, Breindl M, Brenner DA. 1997. Binding of upstream stimulatory factor to an E-box in the 3′-flanking region stimulates alpha1(I) collagen gene transcription. J Biol Chem 272:1753–1760.

Ritzenthaler JD, Goldstein RH, Fine A, Lichtler A, Rowe DW, Smith BD. 1991. Transforming-growth-factor-beta activation elements in the distal promoter regions of the rat alpha 1 type I collagen gene. Biochem J 280:157–162.

Ritzenthaler JD, Goldstein RH, Fine A, Smith BD. 1993. Regulation of the alpha 1(I) collagen promoter via a transforming growth factor-beta activation element. J Biol Chem 268:13625–13631.

Rossert J, Eberspaecher H, de Crombrugghe B. 1995. Separate cis-acting DNA elements of the mouse pro-alpha 1(I) collagen promoter direct expression of reporter genes to different type I collagen-producing cells in transgenic mice. J Cell Biol 129:1421–1432.

Rossert JA, Chen SS, Eberspaecher H, Smith CN, de Crombrugghe B. 1996. Identification of a minimal sequence of the mouse pro-alpha 1(I) collagen promoter that confers high-level osteoblast expression in transgenic mice and that binds a protein selectively present in osteoblasts. Proc Natl Acad Sci USA 93:1027–1031.

Rossi P, Karsenty G, Roberts AB, Roche NS, Sporn MB, de Crombrugghe B. 1988. A nuclear factor 1 binding site mediates the transcriptional activation of a type I collagen promoter by transforming growth factor-beta. Cell 52:405–414.

Rutka JT, Apodaca G, Stern R, Rosenblum M. 1988. The extracellular matrix of the central and peripheral nervous systems: structure and function. J Neurosurg 69: 155–170.

Schauwecker PE, Steward O. 1997. Genetic determinants of susceptibility to excitotoxic cell death: implications for gene targeting approaches. Proc Natl Acad Sci USA 94: 4103–4108.

Schorle H, Meier P, Buchert M, Jaenisch R, Mitchell PJ. 1996. Transcription factor AP-2 essential for cranial closure and craniofacial development. Nature 381:235–238.

Shahzeidi S, Jeffery PK, Laurent GJ, McAnulty RJ. 1994. Increased type I procollagen mRNA transcripts in the lungs of mice during the development of bleomycin-induced fibrosis. Eur Respir J 7:1938–1943.

Slack JL, Liska DJ, Bornstein P. 1991. An upstream regulatory region mediates high-level, tissue-specific expression of the human alpha 1(I) collagen gene in transgenic mice. Mol Cell Biol 11:2066–2074.

Smith RE, Strieter RM, Phan SH, Kunkel SL. 1996. C-C chemokines: novel mediators of the profibrotic inflammatory response to bleomycin challenge. Am J Respir Cell Mol Biol 15:693–702.

Sokolov BP, Mays PK, Khillan JS, Prockop DJ. 1993. Tissue- and development-specific expression in transgenic mice of a type I procollagen (COL1A1) minigene construct with 2.3 kb of the promoter region and 2 kb of the 3′-flanking region. Specificity is independent of the putative regulatory sequences in the first intron. Biochemistry 32:9242–9249.

Sokolov BP, Ala-Kokko L, Dhulipala R, Arita M, Khillan JS, Prockop DJ. 1995. Tissue-specific expression of the gene for type I procollagen (COL1A1) in transgenic mice. Only 476 base pairs of the promoter are required if collagen genes are used as reporters. J Biol Chem 270:9622–9629.

Stefanovic B, Hellerbrand C, Holcik M, Briendl M, Aliebhaber S, Brenner DA. 1997. Posttranscriptional regulation of collagen alpha1(I) mRNA in hepatic stellate cells. Mol Cell Biol 17:5201–5209.

Westergren-Thorsson G, Hernnas J, Sarnstrand B, Oldberg A, Heinegard D, Malmstrom A. 1993. Altered expression of small proteoglycans, collagen, and transforming growth factor-beta 1 in developing bleomycin-induced pulmonary fibrosis in rats. J Clin Invest 92:632–637.

Wyandt HE, Chinnappan D, Ioannidou S, Salama M, O'Hara C. 1998. Fluorescence in situ hybridization to assess aneuploidy for chromosomes 7 and 8 in hematologic disorders. Cancer Genet Cytogenet 102:114–124.

Wyss-Coray T, Feng L, Masliah E, Ruppe MD, Lee HS, Toggas SM, Rockenstein EM, Mucke L. 1995. Increased central nervous system production of extracellular matrix components and development of hydrocephalus in transgenic mice overexpressing transforming growth factor-beta 1. Am J Pathol 147:53–67.

Zhang K, Garner W, Cohen L, Rodriguez J, Phan S. 1995. Increased types I and III collagen and transforming growth factor-beta 1 mRNA and protein in hypertrophic burn scar. J Invest Dermatol 104:750–754.

Zhang J, Hagopian-Donaldson S, Serbedzija G, Elsemore J, Plehn-Dujowich D, McMahon AP, Flavell RA, Williams T. 1996. Neural tube, skeletal and body wall defects in mice lacking transcription factor AP-2. Nature 381:238–241.

Zhao Y, Young SL, McIntosh JC. 1998. Induction of tenascin in rat lungs undergoing bleomycin-induced pulmonary fibrosis. Am J Physiol 274:L1049–1057.