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Cell-Specific Expression of the α1(I) Collagen Promoter-CAT Transgene in Skin and Lung: A Response to TGF-β Subcutaneous Injection and Bleomycin Endotracheal Instillation

Atulya R. Agarwal, Ronald H. Goldstein, Edgar Lucey, Heip Q. Ngo, and Barbara D. Smith

Department of Biochemistry and the Pulmonary Center, Boston University Medical Center and the Boston Veterans Administration Medical Center, Boston, Massachusetts 02118

Abstract Transgenic mice containing a rat collagen α1(I) promoter (3.6 kilobases) fused to the reporter gene chloramphenicol acetyl transferase (CAT) express the reporter gene parallel to endogenous gene in most connective tissues other than vascular tissue [Pavlin et al. (1992): J Cell Biol 116:227–236; Bedalov et al. (1994): J Biol Chem 269:4903–4909]. We have challenged transgenic mice with subcutaneous injections of transforming growth factor-β (TGF-β) or intratracheal instillation of bleomycin. In situ hybridization studies of skin revealed increased CAT expression in the papillary dermis of TGF-β treated animals. In contrast, α1(I) collagen mRNA was expressed throughout the dermis including granulation tissue and reticular dermis. Therefore, the transgenic promoter responds to TGF-β in a subset of dermal fibroblasts. Endotracheal instillation of bleomycin induces lung fibrosis which is thought to be mediated in part by TGF-β. CAT gene expression in lungs was increased 6–8-fold at 2 weeks post bleomycin treatment. In situ hybridization studies revealed focal areas of cells expressing both CAT and collagen genes in the interstitium. However, most regions, especially around airways, contained a subset of cells expressing the endogenous gene with little or no CAT expression as judged by in situ hybridization. These cells could be myofibroblasts that require additional cis-acting elements to activate α1(I) collagen gene expression similar to smooth muscle cells.

Key words: skin, lung, CAT gene expression, α1(I) collagen promoter, TGF-β

Type I collagen, the most abundant member of the collagen family, is a major fibrillar component of extracellular matrix responsible for the structural integrity of many different tissues. Correct expression of type I collagen requires cell-specific coordinate expression of two proteins, α1(I) and α2(I) [reviewed in Adams, 1989; Bornstein and Sage, 1989; de Crombrugghe, 1991]. The genes for these proteins are located on different chromosomes as single copies which are transcribed in many different cell types, for example fibroblasts, osteoblasts, skeletal muscle and smooth muscle cells. The synthesis of type I collagen is modulated during numerous biological processes including wound healing, morphogenesis, and fibrosis. Several extracellular molecules, such as TGF-β, interleukin-1β (IL-1β), prostaglandins, vitamin C, and vitamin D modulate the synthesis of type I collagen during normal and pathological conditions. Although both transcriptional and post-transcriptional mechanisms are involved in regulation of type I collagen synthesis, most of the control appears to be transcriptional [Penttinen et al., 1988; Harrison et al., 1990; de Crombrugghe et al., 1991].

TGF-β, a strong stimulator of collagen type I synthesis, is involved in wound healing and fibrosis [Roberts et al., 1986; Sporn et al., 1986; Ignotz and Massague, 1986; Border and Ruoslahti, 1992]. Roberts et al. [1986] demonstrated that when this growth factor is injected subcutaneously in newborn mice, there is stimulation of collagen synthesis with formation of granulation tissue. TGF-β has been located in macrophages and epithelial cells in lungs from patients with idiopathic pulmonary fibrosis [Brockelmann et al., 1991; Khalil et al., 1991]. Several investigators have postulated that TGF-β is associated with fibrotic processes by

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Address reprint requests to Barbara D. Smith, Department of Biochemistry, K-124, Boston University School of Medicine, 80 East Concord St., Boston, MA 02118.

stimulating persistent expression of collagen. During bleomycin-induced lung fibrosis in animals, TGF-β expression increases before collagen gene expression [Phan and Kunkel, 1992; Hoyt and Lazo, 1988; Raghow et al., 1989]. In addition, the use of TGF-β blocking antibodies demonstrates that TGF-β is involved in the pulmonary fibrotic process [Giri et al., 1993].

We previously demonstrated that TGF-β stimulates human lung fibroblasts to produce increased amounts of protein [Fine and Goldstein, 1987] and to increase steady state levels of mRNA for collagen α1(I) [Fine et al., 1990; Goldstein et al., 1990]. Nuclear run-on experiments indicate that the increased mRNA levels are, in part, due to transcriptional regulation [Fine et al., 1989]. Deletion analysis and cotransfection studies with the collagen α1(I) promoter driving the CAT gene locates a cis-acting DNA element 1.6 Kb upstream of the transcription start site. This element, referred to as α(I)-TAE, is a binding site for a unique protein complex as judged by DNA electrophoretic mobility shift, crosslinking, and Southwestern assays [Ritzenthaler et al., 1991, 1993]. In addition, bleomycin stimulates collagen through this element in cell culture [King et al., 1994]. However, several different TGF-β response elements have been described in collagen [Rossi et al., 1988; Inagaki et al., 1994; Jimenez et al., 1994] and other promoters [Keeton et al., 1991; Kim et al., 1990; Riccio et al., 1992) suggesting that more than one transcription factor is activated during TGF-β stimulation.

We employed transgenic animals to examine the function of the collagen α1(I) promoter fragment in vivo. Transgenic animals containing the rat α1(I) promoter (3,520 bases) CAT construct used in our transfection studies [Ritzenthaler et al., 1991, 1993] express CAT in several connective tissues (bone, tendon, and skin), but not in blood vessels [Pavlin et al., 1992; Bedalov et al., 1994; Bogdanovic et al., 1994]. In this paper, we describe the expression of the transgene compared to the endogenous collagen gene in skin and lung by in situ hybridization. In addition, we demonstrate the cell specific response of this transgene to subcutaneous injections of TGF-β and intratracheal instillation of bleomycin. Our results indicate activation of α1(I) promoter by TGF-β and bleomycin treatment activates a subset of cells, suggesting differential expression of the α1(I) promoter as compared with the endogenous gene.

MATERIALS AND METHODS

TGF-β Injection

Newborn mice containing the 3.6 ColCAT transgene (line 2) were kindly provided by Dr. David W. Rowe and Dr. Stephen H. Clark. Newborn animals (three per group) were injected with TGF-β (Research Diagnostic Systems, Minneapolis, MN) (800 ng in 20 μl saline) subcutaneously once daily for 2 days into the nape of the neck [Roberts et al., 1986]. Control animals were injected with saline. On the third day, the animals were sacrificed by cervical dislocation and the area surrounding the site of injection was removed and placed in 4% paraformaldehyde, pH 7.4, at 4°C for 16 h. The tissue was then processed for in situ hybridization.

Bleomycin Instillation

Transgenic mice aged 6–8 weeks were anesthetized with methoxyflurane (Pitman-Moore, Mundelein, IL). Bleomycin (Blenoxane, Bristol-Myers Squibb Co., Princeton, NJ) was dissolved at a concentration of 1 U/1 ml 0.14 M sodium chloride and 0.1 U/20 g animal was administered by endotracheal instillation. Control animals received sterile saline. Animals were sacrificed 3–21 days post bleomycin instillation. The mice were first anesthetized with an i.p. injection of 0.1 ml sodium pentobarbital (Anpro Pharmaceutical, Arcadia, CA). The thoracic cavity was opened, the dorsal aorta was cut, and the lungs were vascular perfused with sterile saline followed by freshly prepared 4% paraformaldehyde in phosphate buffered saline (pH 7.4). The lungs were inflation fixed by endotracheal instillation of 4% paraformaldehyde (3 ml). The trachea was ligated just caudal to the larynx. The lungs were excised and placed in 4% paraformaldehyde at 4°C for 16 h.

Tissue Preparation for CAT Assays

Lung tissue was removed and placed in 0.25 M Tris-HCl. The lungs were then homogenized using a Brinkman polytron and sonicated for 15–20 s with a Bronson 450 sonifier. Extracts were frozen at −80°C for 30 min and thawed at 37°C. Aliquots of homogenate was assayed for protein by Bradford method with Coomassie Blue G-250 [Bradford, 1967]. Chloramphenicol acetyl transferase (CAT) assays were performed with aliquots containing equal amounts of protein.

CAT activity was measured according to Gorman [Gorman et al., 1982]. Radio-labeled chloramphenicol (0.125 μCi of D-threo-[dichloroacetyl-1-^{14}C] chloramphenicol) (New England Nuclear, Boston, MA) and 4 mM acetyl coenzyme A (Pharmacia, Piscataway, NJ) were added to samples containing 50–200 μg of protein or standard enzyme (Pharmacia) in 150 μl of 0.25 M Tris-HCl, pH 7.8. The samples were incubated at 37°C for 4–6 hours, which was within the linear range. After ethyl acetate extractions, the samples were resuspended in 15 μl ethyl acetate and spotted on thin layer chromatography plates to separate the chloramphenicol and acetylated products. The plates were incubated for 1–2 h in chloroform/methanol (95:5, v/v) and exposed to X-ray film for autoradiography. Acetylated and non-acetylated radioactive areas were removed from the thin layer plates and placed into scintillation counting fluid, and the radioactivity was quantitated by a Beckman scintillation counter.

Tissue Preparation for Microscopy

The tissue was processed for in situ hybridization using a method developed by Sassoon [Sassoon et al., 1988]. Either skin from the region surrounding TGF-β injections or slabs of lung tissue were washed for 30 min at 4°C in phosphate buffered saline, pH 7.4, followed by 30 min at 4°C in 0.85% saline. The tissue was dehydrated by ethanol followed by xylene at room temperature and embedded in paraffin (Paraplast Plus embedding media, Oxford Laboratories, St. Louis, MO). The embedding molds were stored at 4°C until needed. Approximately 10–20 sections were prepared from each block by serially sectioning (4–6 μ sections). The sections were transferred to Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), air dried at 42°C for 60 min, and stored.

Prior to hybridization several steps were followed to reduce non-specific binding [Sassoon et al., 1988]. Slides were deparaffinized in xylene, rehydrated through a series of ethanol washes with decreasing concentrations followed by immersion in saline and phosphate buffered saline before refixing in 4% paraformaldehyde. To allow optimal entry of probe, sections were washed twice in PBS, and exposed to 1 ml of proteinase K (20 mg/ml) for 15 min, washed in PBS, and fixed again in 4% paraformaldehyde. To decrease surface charges, slides were treated with acetic anhydride in 0.1 M triethanolamine, washed in PBS, dehydrated through a series of ethanol washes and allowed to air dry for at least 2 h.

Riboprobe Preparation

Vectors were linearized for the production of both sense and antisense probes. The collagen rat α1(I) probe contains approximately 520 bp of 3′ non-collagenous coding and 80 bp of 3′ untranslated sequence isolated from pα1R1 [Genovese et al., 1984] cloned into pGem3. The collagen plasmid was linearized with *HinD*III followed by T7 RNA polymerase transcription to produce the anti-sense riboprobe or linearized with *EcoR*I followed by SP6 RNA polymerase to produce the sense riboprobe. Chloramphenicol acetyl transferase (CAT) specific probe [Donoghue et al., 1991] was also prepared. In vitro transcription was performed with 0.5 μg plasmid in the presence of 50 μCi [^{35}S] uridine triphosphate (New England Nuclear, Boston, MA) using the Riboprobe Gemini System (Promega, Madison, WI). Following a 2 h incubation at 37°C, the DNA templates were digested with 1 μl of DNAse RQ1 (RNAse-free DNAse, Promega) for 15 min at 37°C, extracted with phenol/chloroform, precipitated in ethanol, and resuspended in 300 μl hybridization buffer [50% formamide (Ambion, Inc., Austin, TX), 0.3 M NaCl, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA (pH 8.0), 10 mM NaH_2PO_4-H_2O (pH 8.0), 10% Dextran Sulfate, 1 × Denhardt's, 0.5 mg/ml tRNA, and 100 mM DTT].

In Situ Hybridization

Radiolabeled riboprobes were diluted to a concentration of 50,000 CPM/μl in hybridization buffer. Riboprobes were heated to 80°C for 2 min before addition to sections. Slides were incubated at 52°C in a humid atmosphere containing 50% formamide and 4 × SSC for 16 h.

Following hybridization, the slides were washed to reduce non-specific binding. Initially, the slides were washed in 5 × SSC and 10 mM DTT at 50°C followed by 50% de-ionized formamide, 2 × SSC, and 10 mM DTT at 65°C. Sections were incubated with RNAse A (20 μg/ml) for 1 h to digest any unbound probe, washed in 2 × SSC and 0.1 × SSC and dehydrated in decreasing concentrations of ethanol containing 0.3 M ammonium acetate. After two 100% ethanol washes, slides were allowed to air dry for at least 2 h.

Kodak NTB-2 liquid emulsion (Kodak, Rochester, NY) was melted at 45°C in a dark room. Slides were dipped into the emulsion and allowed to dry in a vertical position for 30 min. After exposure for 3–7 days at 4°C, the emulsion coated slides were developed using Kodak D-19 for 3.5 min at 16°C, hardened for 10 s with Kodak hardener, and fixed for 10 min with Kodak rapid fixer. Sections were counter stained with either Toluidine blue (VWR, Boston, MA), or hematoxylin (Gills 3, Shandon Lipshaw, Pittsburgh, PA) and eosin (Eosin B, Shandon Lipshaw) or cover-slipped with no stain to be examined with a phase contrast objective.

For comparison of α1(I) collagen and CAT expression, neighboring serial sections were each hybridized with one of the probes. Identification of lung cells and tissues was aided by comparison with neighboring serial sections stained with hematoxylin. Skin sections were counter stained with Toluidine blue. Entire sections were scanned for grains by phase contrast, bright field, and dark field microscopy with a Leitz microscope. At least 10 randomly chosen noncontiguous and non-overlapping fields were photographed on 4–6 sections for each probe to aid in comparisons. For the lung sections, low power fields of each lobe of the lung and high power pictures of interstitium (4 fields) and terminal airways (4 fields) were photographed for each section using phase contrast, bright field, and dark field with a Leitz microscope.

RESULTS

TGF-β Injection Stimulates Collagen and CAT mRNA

In order to determine if the collagen promoter was stimulated in vivo by TGF-β, newborn transgenic mice, containing the 3.6 ColCAT transgene, were injected subcutaneously daily for 2 days into the nape of the neck with TGF-β (800 ng/20 μl saline) as previously described [Roberts et al., 1986]. Although the previous study reported a firm lump palpated at the injected site, this was not the case in the transgenic animals. Nevertheless, in situ hybridization was utilized in order to examine the mRNA expression of the transgene and endogenous gene in skin at the injection site. Sections were hybridized using collagen and CAT riboprobes in the sense and antisense orientation. Six sections from each animal were examined with each probe. Exposure time in the emulsion was varied from 3 to 7 days.

Both CAT and α1(I) collagen mRNAs were stimulated by subcutaneous injection with TGF-β as compared with saline controls (Fig. 1). The top 4 panels are representative photographs of sections hybridized with α1(I) collagen probe and the bottom 4 panels are serial sections hybridized with CAT probe. Each region was photographed under bright (top and bottom panels) and dark (center panels) field. Prior to photomicrography, the tissue was stained with Toluidine blue. The CAT probe did not hybridize to non-transgenic mouse sections and the sense RNA controls did not hybridize specifically to any cells (data not shown). The panels on the right represent skin samples from a site injected twice with TGF-β. The signals were clearly increased in skin injected with TGF-β as compared to the panels on the left representing skin injected with saline.

All saline injected control skin contained low levels of CAT and collagen mRNA signal located beneath the epidermal layer in the papillary dermis as judged by in situ hybridization of skin sections (Fig. 1, left column; Fig. 2A, higher magnification). There was little signal in any cells in the reticular dermal area. On the other hand, more cells expressed CAT mRNA in the saline injected control than that of α1(I) collagen mRNA (Fig. 2A). After TGF-β injection, the level of α1(I) collagen mRNA was higher than for CAT in the TGF-β injected papillary dermis (Fig. 1, right column; Fig. 2B, higher magnification). In regions where cells express CAT, cells expressed α1(I) collagen as well (Fig. 2B). However, not all cells that expressed α1(I) collagen had signal for CAT mRNA. Interestingly, the CAT probe hybridized to cells in the basal portion of the epidermis, suggesting activation of CAT mRNA expression in cells that ordinarily do not produce α1(I) collagen.

It is clear that many cells within the deep reticular dermis had increased α1(I) collagen expression after TGF-β injection, but very few cells produced CAT mRNA (Fig. 1; arrows in Fig. 2C). Little to no expression of CAT above background was found in the adipose tissue surrounding the hair follicles or in the forming granuloma region.

Endotracheal Instillation of Bleomycin Induces Higher Levels of CAT Activity in 2-Week-Old Animals

Transgenic mice were treated with endotracheal instillation of bleomycin or saline. After 2

or 3 weeks, lungs were perfused with saline, lavaged, and removed for measurement of CAT activity, and collagen hydroxyproline levels. Hydroxyproline levels were increased twofold by 3 weeks. At 2 weeks, CAT activity was increased 6–8-fold (Fig. 3A). Compilation of CAT values normalized to μg protein from 6 animals sacrificed at 2 weeks are shown in Figure 3B.

In a separate time study, animals were sacrificed at different days (3, 7, 10, 15, 18, and 21 days) after bleomycin or saline treatment. There were 4 animals in each bleomycin installation group. The CAT levels began increasing at 7 days, peaked between 15 and 18 days, and were decreased at 21 days post bleomycin treatment. There was no change in CAT activity of the saline controls.

In situ hybridization was performed on the lungs in order to locate cells that were expressing CAT and collagen mRNA. Lungs prepared from 4 animals in each treatment group were sacrificed at 2 weeks post endotracheal instillation. The lungs were inflation-fixed and serial sections were hybridized to α1(I) collagen or CAT probes. Unstained tissue was examined with phase contrast microscopy in order to visualize the grains over the tissue as well as with dark field microscopy. Hematoxylin and eosin staining was also performed on a separate serial section in order to identify pulmonary structures. Obvious differences were observed between bleomycin and saline instilled lungs in all sections examined. Figure 4 contains low power photomicrographs of serial sections of lung in dark field and stained sections. It is clear that the level of mRNA for both CAT and α1(I) collagen increased in the bleomycin treated lungs as compared to the saline treated control. Focal regions were present especially in the subpleural region. The level of activity was higher for α1(I) collagen than for CAT. As found in skin, certain regions contain cells that expressed α1(I) collagen but did not express CAT (Fig. 5, short arrows). It is evident that CAT was being expressed specifically by certain cells in both the interstitium and the parenchyma (Fig. 5, long arrows), but not as strongly as α1(I) collagen. Both CAT and α1(I) collagen, however, were being expressed in areas of increased cellularity, which can be seen in the hematoxylin and eosin stained section.

Cells surrounding the large airways and associated blood vessels expressed collagen in the saline controls. There was very little expression of CAT in this region. Our previous studies on non-transgenic mice indicated that at 3 days the expression of collagen mRNA increased around the large airways [Lucey et al., 1996]. This increase became more intense at 7 days when the cells within the interstitium began to express collagen. At 10–14 days many cells throughout the fibrotic areas hybridized intensely with the collagen probes. As seen in this study, CAT expression was also increased around airways with associated blood vessels. Certain focal regions contained cells that intensely stain for CAT and collagen mRNAs (Fig. 6, long arrows). However, it is evident that other focal areas were specifically producing a lower level of CAT message than of α1(I) collagen message (Fig. 6, short arrows). At higher magnification (Fig. 7), CAT message was also increased in the adventitia of blood vessels and large airways. The highest expression was adjacent to areas with infiltrating blood cells (Fig. 7, long arrows). However, even in these regions CAT expression was less than α1(I) collagen expression. Therefore, the transgene was differentially expressed compared to endogenous α1(I) collagen.

DISCUSSION

TGF-β is a major stimulator of collagen transcription in skin after injury or in lung following bleomycin treatment. We have established that there is a cis-acting element in the collagen α1(I) promoter located 1,624 bases upstream from the start site of transcription that responds to TGF-β in transiently transfected lung fibroblasts [Ritzenthaler et al., 1991, 1993]. This element has been linked to activation of collagen synthesis by bleomycin treatment in cell culture [King et al., 1994]. Transfection studies in culture allow initial characterization of elements in certain cells, but do not determine whether the element is active in vivo or in the various cell types that produce collagen. Transfection experiments often do not predict expression in animals due to differentiation pathways [Bedalov et al., 1994]. Therefore, we examined existing transgenic animal strains which contain the same promoter construct used in transfection experiments to examine the effects of direct application of TGF-β on skin or treatment of lungs with bleomycin.

According to Roberts et al. [1986], TGF-β injected subcutaneously daily in newborn mice causes rapid activation of fibroblasts to produce collagen. The new tissue resembles granulation tissue found during wound repair. A firm lump

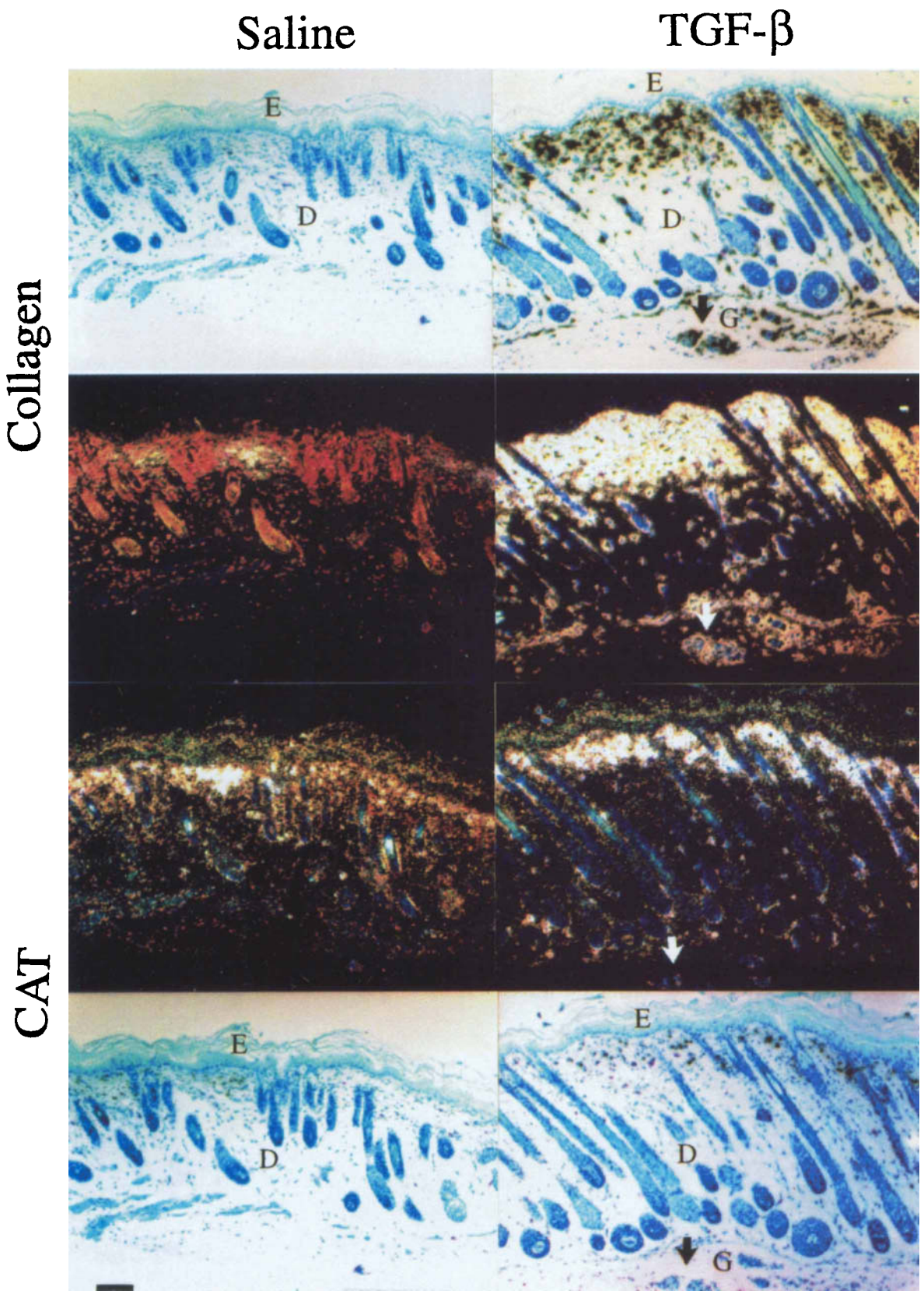

Fig. 1. Expression of collagen and CAT genes in skin from transgenic mice injected with saline or TGF-β. Newborn mice from transgenic animals containing 3.6 ColCAT gene were injected daily for 2 days with either saline or bleomycin (800 ng/20 μl saline). Skin at the injection site was prepared for microscopy and in situ hybridization as described in Materials and Methods. **Left panels:** Saline-injected skin. **Right panels:** TGF-β-injected skin. **Top four panels:** Consecutive sections hybridized with collagen probe using bright field microscopy (top panels) or dark field microscopy (middle panels). **Bottom four panels:** Consecutive sections hybridized with CAT probe using bright field (bottom panels) or dark field microscopy (middle panels). All sections were stained with Toluidine blue. Arrows indicate areas in the granuloma (G) region containing cells that express collagen with no CAT expression. E, epidermis; D, dermis; G, granuloma region. Bar, 50 μm.

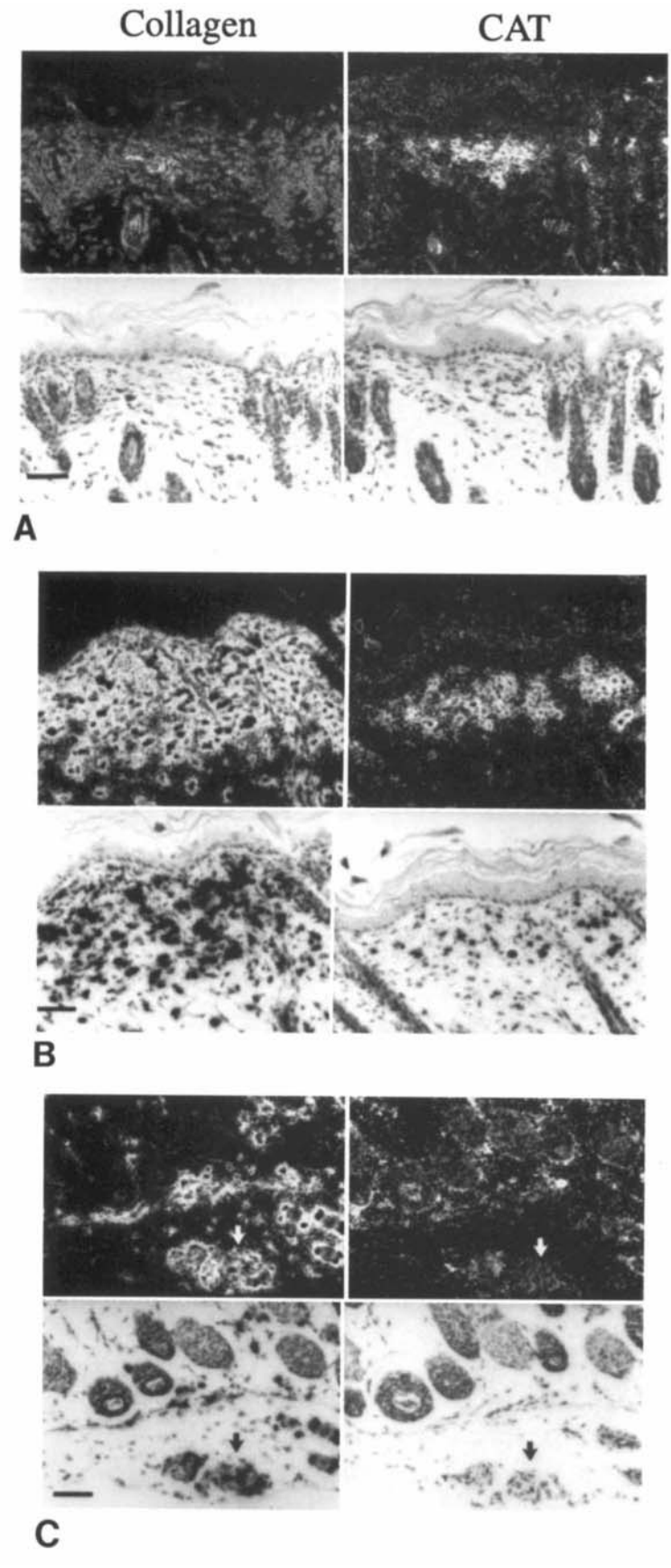

Fig. 2. Photomicrographs of papillary and reticular dermis of skin from transgenic mice injected twice with saline or TGF-β. **A:** Papillary dermis from saline-injected skin samples. Expression of collagen (left) and CAT (right) are similar. **B:** Papillary dermis from TGF-β-injected skin. Collagen expression (left) is significantly greater than CAT expression (right). **C:** Reticular dermis from TGF-β-injected skin. Collagen expression (left) is again significantly greater than CAT expression (right) especially in the granulation tissue (arrows). Bars, 50 μm.

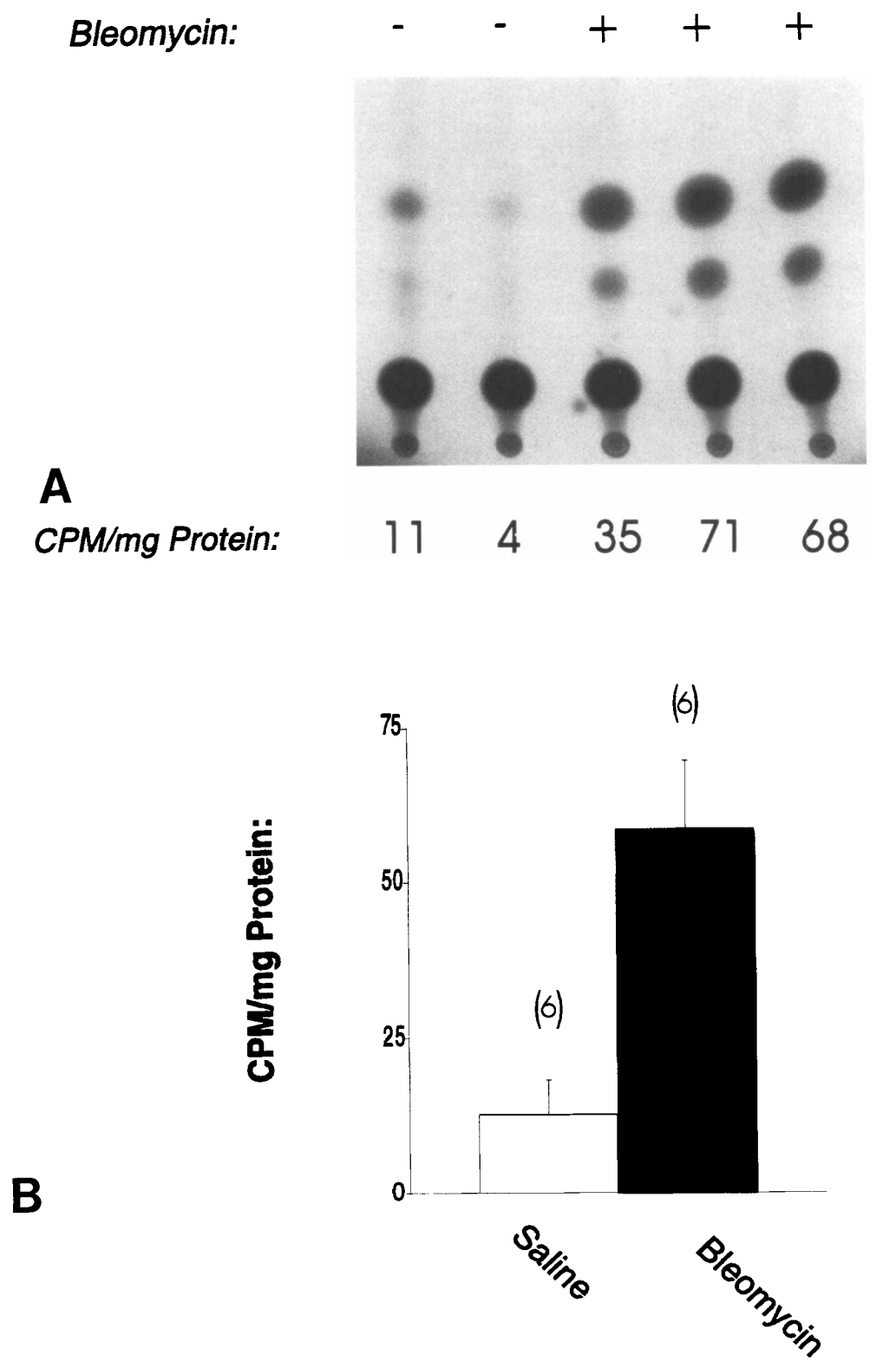

Fig. 3. CAT enzyme activity in lungs from transgenic animals treated with saline or bleomycin 14 days before sacrifice. **A:** Representative CAT assay from saline lungs (−). Numbers below represent acetylated chloramphenicol CPM/mg protein normalized to β-Gal activity. **B:** Graph of CAT assay for six separate animals with standard error bars.

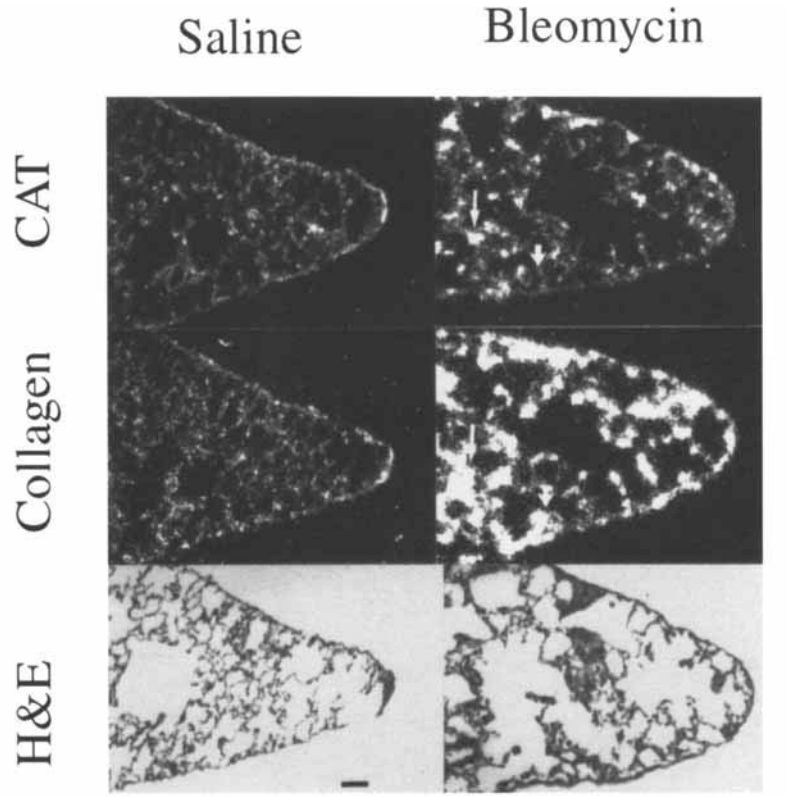

Fig. 4. Expression of CAT and collagen genes in tips of lung from transgenic mice treated with saline or bleomycin 14 days before sacrifice. **Left panels:** Saline instilled. **Right panels:** Bleomycin instilled. Consecutive sections hybridized with CAT probe (top panels) or collagen probe (middle panels). Note that certain regions (long arrow) express both CAT and collagen whereas other regions (short arrow) express only collagen. Bottom panels are consecutive sections stained with hematoxylin and eosin to demonstrate morphology difference between normal and fibrotic lungs. Bar, 50 μm.

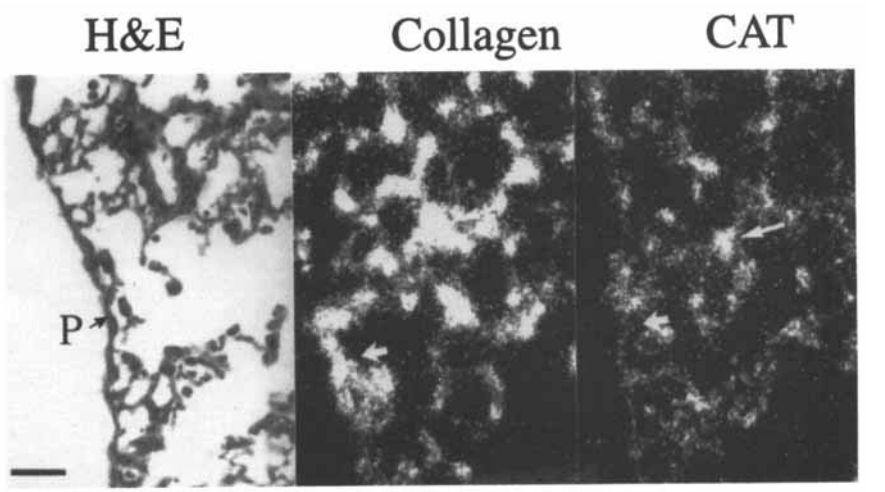

Fig. 5. Photomicrograph of the pleura (P) and sub-pleural area of a transgenic mouse treated with bleomycin 14 days before sacrifice. Consecutive sections were hybridized to CAT probe (right), collagen probe (middle), or stained with hematoxylin and eosin (left). Note that certain regions (long arrows) express both CAT and collagen whereas other regions (short arrows) express only collagen. Bar, 50 μm.

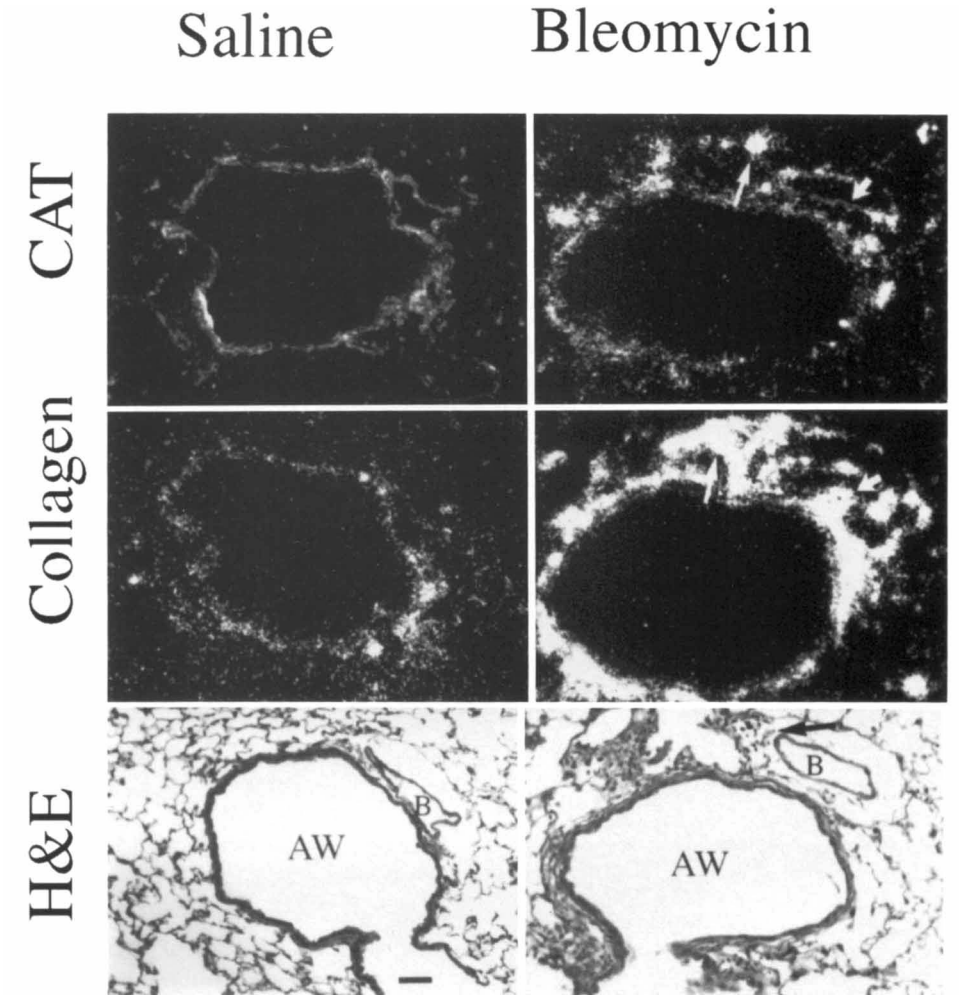

Fig. 6. Expression of CAT and collagen genes in airways (AW) of lungs from transgenic mice treated with saline or bleomycin 14 days before sacrifice. **Left panels:** Saline instilled. **Right panels:** Bleomycin instilled. Consecutive sections hybridized with CAT probe (top panels) or collagen probe (middle panels). Note that certain regions (long arrows) express both CAT and collagen whereas other regions (short arrows) express only collagen. Bottom panels are consecutive sections stained with hematoxylin and eosin to demonstrate morphology difference between normal and fibrotic lungs. A region with increased cellular infiltration from an associated blood vessel is shown by a black arrow. AW, large airway; B, blood vessel. Bar, 50 μm.

formed 48 h after TGF-β injection. In our first experiments in this paper, similar concentrations of TGF-β were injected daily subcutaneously in newborn transgenic mice. There was no obvious lump formation at the site of injection. In the earlier experiments, TGF-β was purified from human platelets whereas in this experiment recombinant TGF-β was used. It is possible that there was a contaminant in the earlier purified TGF-β preparation which enhanced granulation formation with angiogenesis or the recombinant TGF-β is not as active as the native growth factor. In addition, there may be differences in granulation tissue formation based on species differences of the mice.

Nevertheless, after microscopic observation of the tissue, it was clear that some granulation tissue did form after TGF-β injection (Fig. 1). TGF-β stimulated α1(I) collagen mRNA expression throughout the dermis whereas the CAT mRNA expression was stimulated primarily in the upper papillary dermal layer, not in the deeper reticular dermis or granuloma tissue. There are known differences in fibroblasts isolated from papillary and reticular dermis including differences in their response to TGF-β and other growth factors [Feldman et al., 1993]. Therefore, these cells may contain different trans-acting factors following TGF-β stimulation or there could be alternate stimulation path-

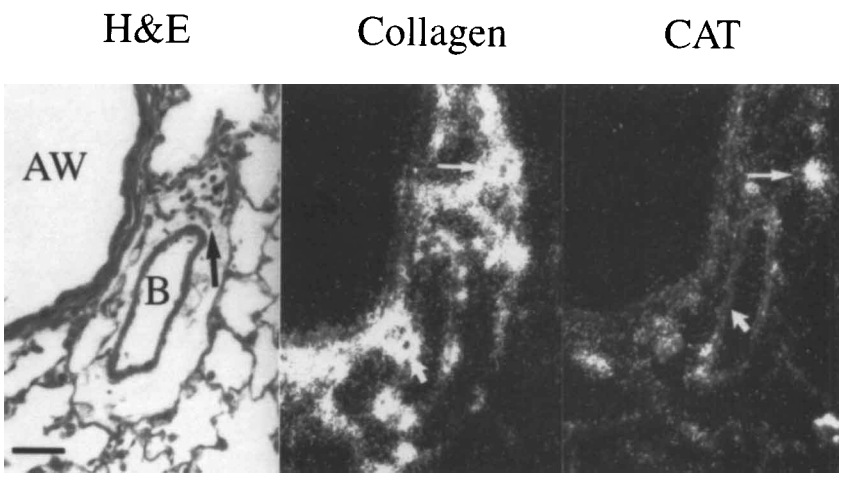

Fig. 7. Photomicrograph of large airway (AW) with associated blood vessel (B) from a transgenic mouse treated with bleomycin 14 days before sacrifice. Consecutive sections were hybridized to CAT probe (right), collagen probe (middle), or stained with hematoxylin and eosin (left). Note that certain regions (long arrows) express both CAT and collagen whereas other regions (short arrows) express only collagen. Bar, 50 μm.

ways used by heterogeneous fibroblast populations.

The transgenic mice carrying the 3.6 ColCAT chimeric gene used in this study were characterized previously [Pavlin et al., 1992; Bedalov et al., 1994; Bogdanovic et al., 1994]. The reporter gene is expressed at highest levels in tooth, bone, and tendon, lower levels in skin and lungs, and very low levels in brain, liver, and aorta. Cis-acting sequences in the collagen promoter mediate expression in bone and tendon fibroblasts. A bone specific element is located within a region close to the TGF-β response element [Ritzenthaler et al., 1991, 1993] approximately 1,624 bases upstream of the transcription start site [Krebsbach et al., 1993]. However, expression of the transgene is not detected in aorta or vascular smooth muscle cells from transgenic animals with or without the collagen first intron even though endogenous collagen expression is high in these cells [Bedalov et al., 1994]. On the other hand, a human mini-gene is expressed at the same levels as the endogenous gene in aortas suggesting that cis-acting elements necessary for collagen α1(I) gene expression in smooth muscle are present in the gene and missing from the promoter and first intron sequences. Since the rat 3.6 kb collagen promoter is active in fibroblasts and bone cells, but not in smooth muscle cells, then activation in transgenic animals can be measured in fibroblasts.

Other transgenic animal studies using human or mouse collagen promoter reporter constructs are consistent with a modular arrangement of different cell specific regulatory elements [Rossert et al., 1995; Liska et al., 1994; Houglum et al., 1995]. Although no other information is available relating to pulmonary fibrosis or wound healing in adult animals, a cis-acting element within a short human collagen α1(I) promoter fragment, containing only 440 base pairs, is sufficient for increased reporter transgene expression following administration of the fibrogenic hepatotoxin carbon tetrachloride [Brenner et al., 1993; Houglum et al., 1995].

The differential expression activated in skin by TGF-β could be explained by a lack of an element in the promoter-reporter construct. It should be noted that differential gene expression between papillary and reticular dermis was demonstrated in skin from embryos (17 d.p.c.) with the human collagen promoter transgenes [Liska et al., 1994]. The presence of the collagen first intron within the transgenic construct increased expression in reticular dermis. Therefore, the first intron may contain a cell specific element for reticular dermis cells in skin.

The absence of CAT expression in the reticular dermis could also be due to the absence of a smooth muscle cell specific cis-acting element in the construct. Granulation tissue and hair follicle dermis contain myofibroblasts which are mesenchymal cells with functional and structural characteristics in common with both smooth muscle cells and fibroblasts [Darby et al., 1990; Singer et al., 1984; Jahoda et al.,

1991]. These myofibroblasts contain smooth muscle α-actin filaments and may be involved in wound contraction [Scharffetter et al., 1989; Lefebvre et al., 1994]. The animals used in these experiments do not express reporter gene in smooth muscle cells [Bedalov et al., 1994]. It may well be that certain myofibroblasts require the cis-acting element necessary for collagen expression in smooth muscle cells rendering the 3.6 α1(I) promoter fragment inactive.

CAT mRNA expression in bleomycin induced fibrosis was localized in fibrotic regions where collagen mRNA was stimulated. However, CAT expression was markedly absent in certain focal regions. Pulmonary fibrosis, like granulation tissue, is characterized by increases in the number of myofibroblasts containing contractile filaments [Low et al., 1984; Adler et al., 1986; Evans et al., 1983; Woodcock-Mitchell et al., 1984; Mitchell et al., 1989; Kuhn and McDonald, 1991]. These smooth muscle α-actin positive cells synthesize type I collagen suggesting that they play a role in the remodeling of lung in pulmonary fibrosis [Kuhn and McDonald, 1991]. In bleomycin induced injury, myofibroblasts arise that synthesize collagen as judged by in situ hybridization and express smooth muscle α-actin as judged by immunohistochemistry (Zhang et al., 1994). There is evidence that cells expressing smooth muscle α-actin also express varying types of intermediate filament proteins, either muscle specific desmin or fibroblast specific vimentin. Another subgroup of cells in the submesothelial fibrotic lesion are actin positive but desmin negative. These results indicate that fibrotic areas contain sub-populations that vary from a fibroblastic to a more smooth muscle cell-like phenotype. Myofibroblasts may arise from pericytes (vimentin and α-smooth muscle actin positive cells, desmin-negative cells, VA phenotype) or contractile interstitial cells (VD phenotype) [Leslie et al., 1992; Kapanci et al., 1992]. CAT expression would be expected to increase only in cells that retain fibroblast phenotype. It is not clear whether the cells that express increased endogenous levels of collagen without expression of CAT were derived from fibroblasts or differentiated from a smooth muscle lineage. Immunohistochemistry with cell specific markers combined with in situ hybridization will be required to resolve this issue.

Although we favor the theory that the differential expression of CAT and endogenous gene is due to differentiation of cells which require additional cis-acting elements, alternative models are possible. First, a post-transcriptional mechanism for stabilizing collagen mRNA may increase collagen mRNA steady state levels, but not CAT mRNA. Several investigators have suggested that TGF-β increases collagen synthesis by post-transcriptional mechanisms in addition to increasing transcription [Penttinen et al., 1988; Raghow et al., 1987; Fine et al., 1990]. Määttä and Penttinen [1993, 1994] have suggested that proteins, binding in the 3′ untranslated region, stabilize collagen α1(I) mRNA and react to redox potentials inside the cell. This type of mechanism could be a factor in acute injury before or in addition to transcriptional mechanisms. Second, the integration site of the promoter could be inhibiting CAT gene expression in certain cell types.

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