

# Cell-specific Expression of $\alpha 1(I)$ Collagen-hGH Minigenes in Transgenic Mice

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**Abstract.** Sequences within the first intron of the  $\alpha 1(I)$  collagen gene have been implicated in the regulation of expression of  $\alpha 1(I)$  collagen-reporter gene constructs in cultured cells. However, the physiological significance of these intronic elements has not been established. We have used in situ hybridization to examine whether a cell-specific pattern of expression of human  $\alpha 1(I)$  collagen-human growth hormone minigenes exists in transgenic mice. Our results indicate that transgenes which contained 2,300 bp of promoter/5' flanking sequence and an intact first intron were well expressed by fibroblasts in dermis and fascia, whereas transgenes lacking the intronic sequence, +292 to +1440, were not expressed in dermis and poorly expressed in fascia. Analysis of transgene expression in cultured fibroblasts obtained from dermal explants of

transgenic animals confirmed the requirement for these intronic sequences in the regulation of the  $\alpha 1(I)$  collagen gene. In contrast, transgenes with or without the intronic deletion were expressed equally well in tendon and bone, in a manner comparable to the endogenous mouse  $\alpha 1(I)$  collagen gene, and expression of neither transgene was detected in skeletal muscle or perichondrium. These data support a model in which *cis*-acting elements in the first intron, and their cognate DNA-binding proteins, mediate transcription of the  $\alpha 1(I)$  collagen gene in some cells, such as dermal fibroblasts, but not in tendon cells or osteoblasts. Moreover, regions of the gene not included in the sequence, -2300 to +1440, appear to be required for transcription in tissues such as skeletal muscle and perichondrium.

**T**YPE I collagen, a major fibrillar extracellular matrix protein, is necessary for the structural integrity of vertebrate tissues. The synthesis of type I collagen is modulated in numerous biological processes that include wound healing, tissue morphogenesis, and fibrosis (reviewed in Adams, 1989; Bornstein and Sage, 1989; de Crombrughe et al., 1991). Correct expression of type I collagen requires the cell-specific, coordinated expression of the two genes that encode the type I collagen polypeptides,  $\alpha 1(I)$  and  $\alpha 2(I)$ . Each gene is present as a single copy and is transcribed in a number of different cell types, e.g., fibroblasts in fascia, dermis and tendon, osteoblasts, odontoblasts, and skeletal and smooth muscle cells (Sandberg and Vuorio, 1987; Cheah et al., 1991; Niederreither et al., 1992). Several extracellular signaling molecules, such as TGF- $\beta$ , IL-1, TNF $\alpha$ , and vitamins D and C, modulate the synthesis of type I collagen during normal and pathological conditions (Adams, 1989; Slack et al., 1993). Although both transcriptional and posttranscriptional mechanisms are involved in the regulation of type I collagen synthesis in response to these diverse stimuli, the predominant mode of control appears to be transcriptional (Penttinen et al., 1988; Harrison

et al., 1990; de Crombrughe et al., 1991; Diaz et al., 1993; Slack et al., 1993).

Experiments designed to identify cell-specific, *cis*-acting elements in the  $\alpha 1(I)$  collagen gene have indicated that the gene is regulated in a complex manner. The basal promoter (~400 bp) of the  $\alpha 1(I)$  collagen gene contains binding activities for a number of *trans*-acting factors that include the same CAAT-binding factor as that utilized by the  $\alpha 2(I)$  collagen gene (Brenner et al., 1989; Karsenty and de Crombrughe, 1990). However, the ability of the basal promoter to direct tissue- or cell-specific expression of reporter genes remains controversial (Bornstein and McKay, 1988; Rippe et al., 1989; Boast et al., 1990). In addition, *cis*-acting DNA elements have been identified both 5' to the basal promoter and within the first intron of the  $\alpha 1(I)$  collagen gene (Bornstein et al., 1988; Rippe et al., 1989; Boast et al., 1990; Liska et al., 1990, 1992). Two orientation-dependent intronic elements have been characterized further by DNA-binding assays and by site-directed mutagenesis: an API-binding sequence that enhances transcription (Liska et al., 1990; Määttä et al., 1993), and two tandem SPI-binding sequences that have a modest inhibitory effect on transcription (Liska et al., 1992). Although these sequences appear to be functional in primary cultured fibroblasts and in some fibroblast cell lines, several studies have shown that these *cis*-acting sequences are not functional in other cell lines, such

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as NIH 3T3 cells (Bornstein et al., 1988; Rippe et al., 1989; Boast et al., 1990; Olsen et al., 1990). One hypothesis that could account for these disparate observations is that sequences within the first intron mediate expression of the  $\alpha 1(I)$  collagen gene in a cell type-specific manner.

Another model that emphasizes the complexity of regulation of the  $\alpha 1(I)$  collagen gene is the Mov13 mouse, in which the Moloney murine leukemia virus has been integrated into the first intron of the gene. In Mov13 mice, the  $\alpha 1(I)$  collagen gene is transcriptionally inactive in fibroblasts and in most other mesenchymal cells, with the exception of osteoblasts and odontoblasts (Schnieke et al., 1983; Kratochwil et al., 1989; Schwarz et al., 1990). The block in transcriptional activation is associated with the absence of a DNase I-hypersensitive (DNase HS)<sup>1</sup> site; this finding suggests that the inhibition results from an alteration in chromatin conformation at the  $\alpha 1(I)$  collagen gene locus (Breindl et al., 1984). It has been speculated that the transcriptional block is caused by proviral disruption of interactions between *cis*-acting elements that reside upstream and downstream from the initiation site of the  $\alpha 1(I)$  collagen gene (Harbers et al., 1984; Breindl et al., 1984). Taken together, these observations suggest that fibroblasts require intronic and/or other 3' sequences to be present in a spatially correct manner for expression of the  $\alpha 1(I)$  collagen gene, whereas osteoblasts rely on 5' sequences for expression of the gene. In support of these considerations, chloramphenicol acetyl transferase (CAT) reporter constructs containing only 5'-flanking  $\alpha 1(I)$  collagen as a promoter DNA are strongly expressed in cultured osteoblast cell lines and in bone and tendon of transgenic animals (Pavlin et al., 1992; Krebsbach et al., 1993).

To investigate the regulation of the  $\alpha 1(I)$  collagen gene *in vivo*, we prepared a series of transgenic animals with human  $\alpha 1(I)$ COL-human growth hormone (hGH) minigenes, in which the collagen transgene includes or lacks putative regulatory sequences within the first intron (Slack et al., 1991). We found that transgenes driven by 2,300 bp of  $\alpha 1(I)$  collagen 5'-flanking sequence, with or without the first intron, were expressed in a tissue-specific manner and, in most tissues, at levels approaching that of the endogenous mouse gene. In contrast, several lines of mice that contained only 444 bp of  $\alpha 1(I)$  5'-flanking sequence, with or without the first intron, exhibited a level of expression in most tissues that was 15- to 500-fold lower than that observed with the longer promoter. Although transgenes that contained a 444-bp promoter and the first intron retained a modest degree of tissue-specific expression, those without the first intron lacked tissue specificity and were poorly expressed in all tissues examined.

Thus, our analysis of the tissue-specific expression of the  $\alpha 1(I)$ COL-hGH transgenes showed that 5'-flanking sequences are necessary for full expression of the  $\alpha 1(I)$  collagen gene in most tissues and suggested that, under some conditions, intronic sequences are also important. However, this analysis did not clarify the role of the first intron in the expression of the  $\alpha 1(I)$  collagen gene, nor did it assess the cell type-specific regulation of expression by the various  $\alpha 1(I)$  collagen DNA sequences. To define further the role of in-

tronic sequences, we have extended our studies of these transgenic animals to investigate the cell type-specific expression of the transgenes in the presence and absence of sequences within the first intron. We found that transgenic animals carrying constructs with 2,300 bp of 5'-flanking sequence and the first intron expressed the transgene in dermal fibroblasts and in many other collagen-synthesizing cells. However, transgenes in which putative regulatory sequences in the first intron had been deleted were expressed poorly, or not at all, in dermal fibroblasts but continued to be expressed in cells such as osteoblasts and tendon cells. These data verify that sequences within the first intron are necessary for expression of the  $\alpha 1(I)$  collagen gene in dermal fibroblasts *in vitro*. Our results also support a model in which intronic, *cis*-acting sequences regulate expression of the  $\alpha 1(I)$  collagen gene in fibroblasts by an interaction between *cis*-acting elements in the promoter and first intron mediated by DNA-binding proteins, and/or by alteration of the chromatin conformation at the  $\alpha 1(I)$  collagen gene locus.

## Materials and Methods

### Transgenic Mice

The generation of transgenic mouse lines has been previously described (Slack et al., 1991). Transgenic mouse lines were maintained by outbreeding with C57BL/6 mice. Animals positive for the transgene were identified by dot-blot analysis of tail DNA with a random prime-labeled BamHI-HindIII hGH fragment from the pUC-hGH cassette (Bornstein and McKay, 1988). Day 1 of pregnancy was determined by the presence of a vaginal plug. Pregnant females were killed on the 17<sup>th</sup> d of gestation by cervical dislocation, the uterus was removed, and embryos were rapidly dissected in phosphate-buffered saline. Embryos positive for the transgene were identified by dot-blot analysis of DNA obtained from the head of the embryo. Explants of skin for cell culture and tissue samples for RNA extraction were snap-frozen in dry ice-ethanol and were stored at  $-70^{\circ}\text{C}$  until embryos positive for the transgene were identified. For *in situ* hybridization, embryos were severed at the midline, fixed in 4% paraformaldehyde for 24 h, and stored in 70% ethanol at  $4^{\circ}\text{C}$  until embryos positive for the transgene were identified.

### Cell Culture of Dermal Fibroblasts

Full-thickness sections of skin were dissected from the animals, cut into 0.5-cm<sup>2</sup> pieces, and incubated for 1 h in DME with 20% calf serum and  $10\times$  antibiotics (25 mg/ml amphotericin, 1,000 u/ml streptomycin, and 1,000 u/ml penicillin G). Sections were subsequently incubated with 3 mg/ml collagenase (Worthington Biochemical Corp., Freehold, NJ) and 0.075 mg/ml trypsin in serum-free DME containing  $10\times$  antibiotics for 1 h at  $37^{\circ}\text{C}$ . Sections were separated from the enzyme mixture by centrifugation, rinsed, and plated onto a petri dish with the dermis side down. A small amount of medium (DME, 20% calf serum,  $10\times$  antibiotics) was added to cover the entire surface of the explant. Media were changed daily until  $\sim 100$ -200 fibroblasts migrated from the edge of the explant. The explant was gently removed and the fibroblasts were propagated in DME containing 15% calf serum and  $1\times$  antibiotics. Fibroblasts were cultured a maximum of three passages, at a split ratio of 1:3, before analysis.

### Analysis of RNA Levels

Preparation of RNA from cultured cells or tissue samples, and subsequent quantification of hGH and endogenous mouse  $\alpha 1(I)$  collagen (mCOL) mRNA by RNase protection analysis, have been described (Liska et al., 1990, 1992; Slack et al., 1991). RNA concentrations were determined by measurement at 260 nm. A riboprobe corresponding to exon 5 of the hGH minigene was used to detect the transgene; no protected bands were seen after hybridization of the hGH riboprobe to RNA from nontransgenic mice (Slack et al., 1991). We used a riboprobe specific for a 310-bp fragment containing sequences from the 3' untranslated region (3' UTR) of mCOL for detection of expressed mCOL (Slack et al., 1991; Reed et al., 1993).

1. *Abbreviations used in this paper:* CAT, chloramphenicol acetyltransferase; DNase HS, DNase I-hypersensitive; hGH, human growth hormone; mCOL, mouse endogenous  $\alpha 1(I)$  collagen; 3' UTR, 3' untranslated region.

## In Situ Hybridization

Paraformaldehyde-fixed embryos were embedded in paraffin and cut into 5- $\mu$ m sections. The sections were dewaxed as described (Reed et al., 1993) and were prehybridized in hybridization buffer (0.6 M NaCl, 0.12 M Tris-Cl, pH 8.0, 0.008 M EDTA, 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.1% Pentex BSA, 500  $\mu$ g/ml salmon sperm DNA, 600  $\mu$ g/ml yeast tRNA, and 50% deionized formamide) overnight at 50°C in an humidified chamber with filter paper saturated with 4 $\times$  SSC and 50% formamide. Sections were hybridized with <sup>35</sup>S-labeled hGH or mCOL riboprobes (6  $\times$  10<sup>5</sup> cpm per slide) overnight at 50°C in hybridization buffer that contained 10% dextran sulfate, 0.1% SDS, and 10 mM DTT. The sections were washed, incubated with RNase A (40  $\mu$ g/ml; Sigma Chemical Co., St. Louis, MO) and RNase T<sub>1</sub> (Sigma Chemical Co.), washed at high stringency (0.1 $\times$  SSC for 2 h at 65°C) to assure low background, and dehydrated with a series of ethanol/0.3 M ammonium acetate solutions. Slides were coated with NTB2 nuclear emulsion (Eastman Kodak Co., Rochester, NY), exposed for 10–14 d at 4°C in light-excluded chambers, developed in Kodak D-19 developer, and fixed in GBX fixer. Sections were counterstained with methyl green, dehydrated with ethanol, cleared in xylene, and mounted with Permount. Sections were photographed on a Zeiss photomicroscope equipped with bright and darkfield condensers (Carl Zeiss, Inc., Thornwood, NY).

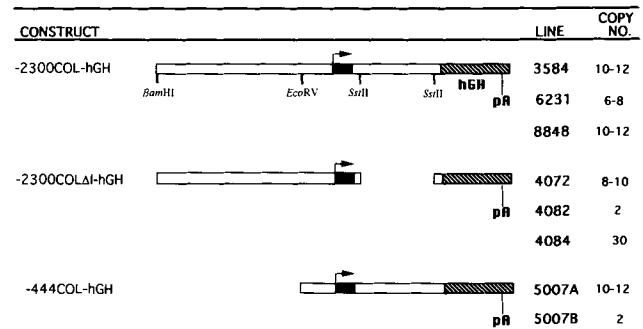
## Results

### Cell-specific Expression of the –2300COL-hGH Minigene in Transgenic Animals

To understand the regulation of expression of the  $\alpha$ 1(I) collagen gene, we had previously prepared transgenic animals carrying varying sequences of the human  $\alpha$ 1(I) collagen promoter and first intron driving an hGH minigene (Fig. 1). Transgenes were shown to be inserted into the mouse genome in tandem, head-to-tail arrays with varying copy number; no rearrangements or deletions of the transgenes were detected by Southern blot analysis (Fig. 1); (Slack et al., 1991). From these earlier studies we determined that transgenes containing –2,300 bp of 5'-flanking sequence were expressed in a highly tissue-specific, quantitatively correct manner, with maximum levels of expression in bone and tail and moderate levels in skin (Slack et al., 1991). The pattern of expression was similar, but not identical, to that seen with mCOL. Surprisingly, deletion of sequences within the first intron, between +292 and +1440, did not appear to affect the overall quantitative expression of constructs driven by 2,300 bp of promoter. In contrast, transgenes driven by 444 bp of promoter exhibited levels of expression that were 15- to 500-fold lower than those of the endogenous gene, and expression was not detected from transgenes driven by 444 bp of promoter and in which the intronic sequences had been deleted. These observations indicated that the –2300 to –444 region was necessary for full expression of the transgene; moreover, the first intron did not appear to be necessary when this region was present.

In our previous studies, we did not distinguish among the cell types in the tissues that transcribed the hGH transgene. In addition, expression of mCOL varied significantly with the age of the animal. Although expression of hGH was comparable to that of mCOL in animals of similar age, the overall variability and multicellular nature of the tissues did not permit a precise assessment of the expression of the transgenes. To define more clearly the role of sequences between –2300 and +1607 in regulation of  $\alpha$ 1(I) collagen gene expression, we investigated the cell type-specific expression of the transgenes by in situ hybridization.

Two mouse lines carrying –2300COL-hGH were studied

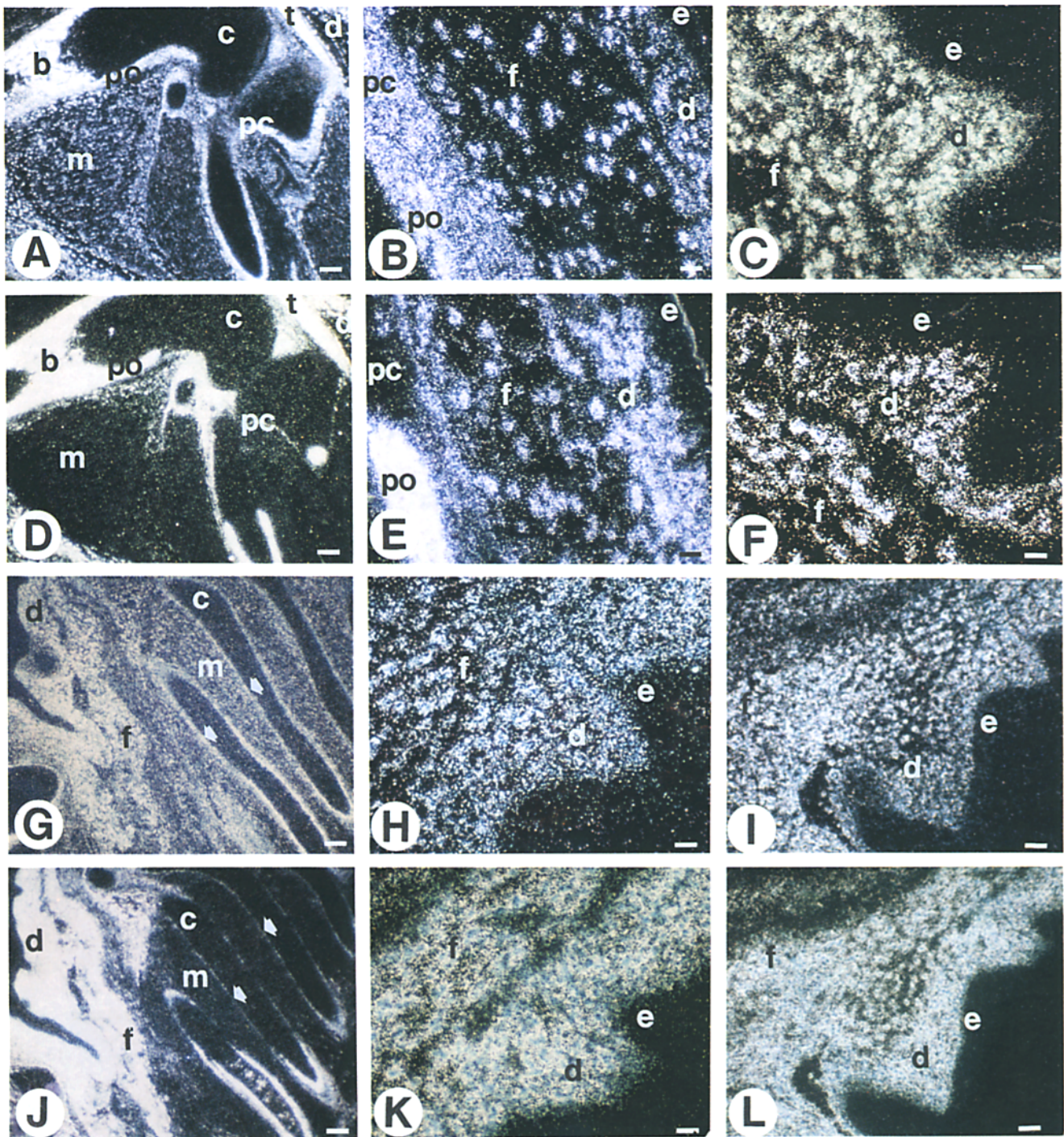


**Figure 1.** Schematic representation of human  $\alpha$ 1(I) collagen-hGH minigenes. These constructs were used for the generation of transgenic mice and cell lines (designated by the four-digit number of the original founder, with the copy number per line as shown). –2300COL-hGH contains approximately 2,300 bp of 5'-flanking sequence and extends through most of the first intron, to +1607, of the human  $\alpha$ 1(I) collagen gene, designated by the open rectangle. The  $\alpha$ 1(I) first exon is designated by the closed rectangle. The arrow indicates the start of transcription. The first intron of the  $\alpha$ 1(I) gene is fused to the third intron of the hGH cassette, designated by the hatched rectangle. The polyadenylation signal (pA) and transcription termination region are provided by the hGH cassette. The restriction endonuclease recognition sites used for deletion constructs, an EcoRV site at –444, BamHI site at –2300, and SstII sites at +292 and +1440, are also shown.

by in situ hybridization of sections from 17-d-old embryos. To compare expression of the transgene to that of the endogenous gene, we used riboprobes specific to the hGH minigene and to the mCOL 3' UTR, respectively. No hybridization with the hGH riboprobe was observed on sagittal sections of control, nontransgenic embryos (data not shown). The specificity of the mCOL riboprobe has been previously established (Slack et al., 1991; Reed et al., 1993). It should be stressed that in all of the following studies hybridization with the mCOL riboprobe was used primarily as an internal reference to compare the level of expression of different transgenes in different tissues, rather than as a measure of the extent to which the expression of a transgene mimicked that of the endogenous gene. Fig. 2 shows representative darkfield photomicrographs of sagittal sections of embryos from two separate lines of –2300COL-hGH mice, line 6231 (Fig. 2, A–F) and line 8848 (Fig. 2, G–L), after in situ hybridization with mCOL (Fig. 2, A–C and G–I) or hGH (Fig. 2, D–F and J–L) riboprobes. Fig. 2, A and G show a characteristic pattern of endogenous mCOL expression: high levels in bone, periosteum, perichondrium, tendon, fascia, and dermis. A lower level of mCOL was present in skeletal muscle, whereas no expression was observed in cartilage and epidermis. Serial sections hybridized with the riboprobe for hGH showed a similarly high level of expression of the transgene in bone, periosteum, tendon, fascia, and dermis, with no expression in cartilage or epidermis (the apparent signal in the epidermis in Fig. 2, E was not reproducible and is a common artifact of this procedure).

It was interesting that the transgene was not observed in skeletal muscle, a tissue in which the endogenous gene is clearly expressed. Furthermore, the transgene was also not detected in the perichondrium. A higher magnification of the periosteum/perichondrium (Fig. 2, B and E, see below) shows this differential expression more clearly. The endoge-





**Figure 2.** Representative photomicrographs from 17-d-old mouse embryos carrying the  $-2300\text{COL-hGH}$  transgene. Serial sagittal sections from line 6231 (A-F) and line 8848 (G-L) were hybridized with probes for either mCOL (A-C and G-I) or hGH (D-F and J-L). High magnification of skin sections are shown in B, C, E, F, H, I, K, and L. A and D show a lower magnification of the pelvic region of line 6231, and G and J show a section through the thorax of line 8848. The arrows point to perichondrium. *b*, bone; *c*, cartilage; *d*, dermis; *e*, epidermis; *f*, fascia; *m*, skeletal muscle; *pc*, perichondrium; *po*, periosteum; *t*, tendon. Bars: (A, D, G, and J) 150  $\mu\text{m}$ ; (B, C, E, F, H, I, K, and L) 30  $\mu\text{m}$ .

nous gene was expressed throughout the periosteum and perichondrium, whereas expression of the transgene was limited to the osteogenic layer of the periosteum, to the exclusion of the perichondrium. These data suggest that the sequences necessary for direction of appropriate transcription of the  $\alpha 1(\text{I})$  collagen gene to skeletal muscle and perichon-

drium are not present between  $-2300$  and  $+1607$  of the human gene.

Transcript levels for both the transgene and the endogenous gene were similar in fibroblasts of the dermis and underlying connective tissue fascia. Higher magnifications of skin sections (Fig. 2 C) indicated that, in both lines of mice,



**Table I. Summary of Tissue-specific Expression of  $\alpha 1(I)$  Collagen Transgenes in Mouse Embryos**

	Line	Bone	Tendon	Dermis	Fascia	Skeletal muscle	Perichondrium
Endogenous $\alpha 1(I)$ COL		4+	4+	3+	3+	2+	3+
-2300COL -hGH	6231	4+	4+	3+	3+	-	-
	8848	4+	4+	3+	3+	-	-
-2300COL( $\Delta I$ ) -hGH	4072	4+	4+	$\pm$	2+	-	-
	4082	4+	4+	$\pm$	$\pm$	-	-
	4084	4+	4+	-	1+	-	-

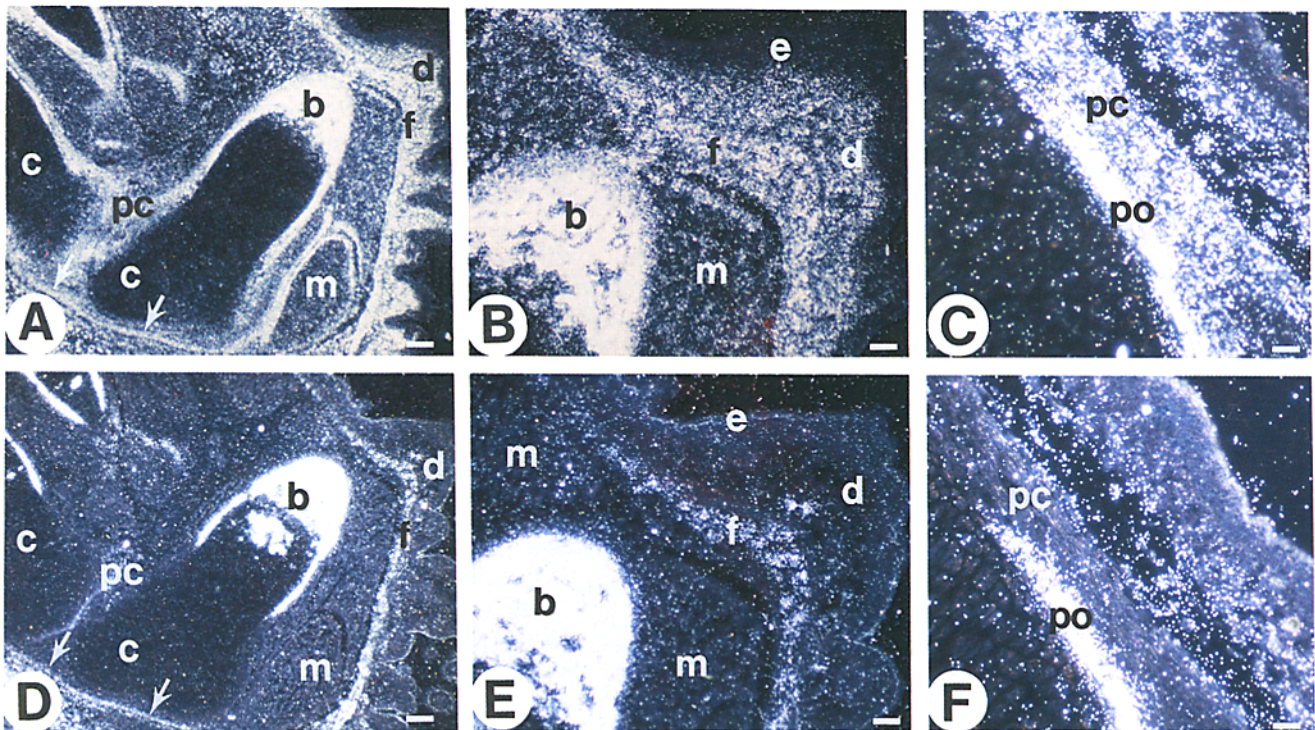
Expression of  $\alpha 1(I)$  endogenous collagen and transgene mRNAs in 17-d-old mouse embryos was assessed by in situ hybridization of  $^{35}\text{S}$ -labeled mCOL and hGH riboprobes. Degrees of expression:  $\pm$ , weak; 1+ to 4+, moderate to strong; -, no expression detected.

fibroblasts throughout the dermis and connective tissue fascia expressed the transgene in a pattern similar to that of the endogenous gene. The transgene therefore apparently contains the cell-specific information necessary for expression of  $\alpha 1(I)$  collagen by dermal and interstitial fibroblasts, as well as by tendon fibroblasts, osteoblasts, and periosteal cells. These results are summarized in Table I.

**Sequences within the First Intron Are Required for Expression of COL-hGH Minigenes by Fibroblasts, but Not by Periosteal or Tendon Cells or by Osteoblasts**

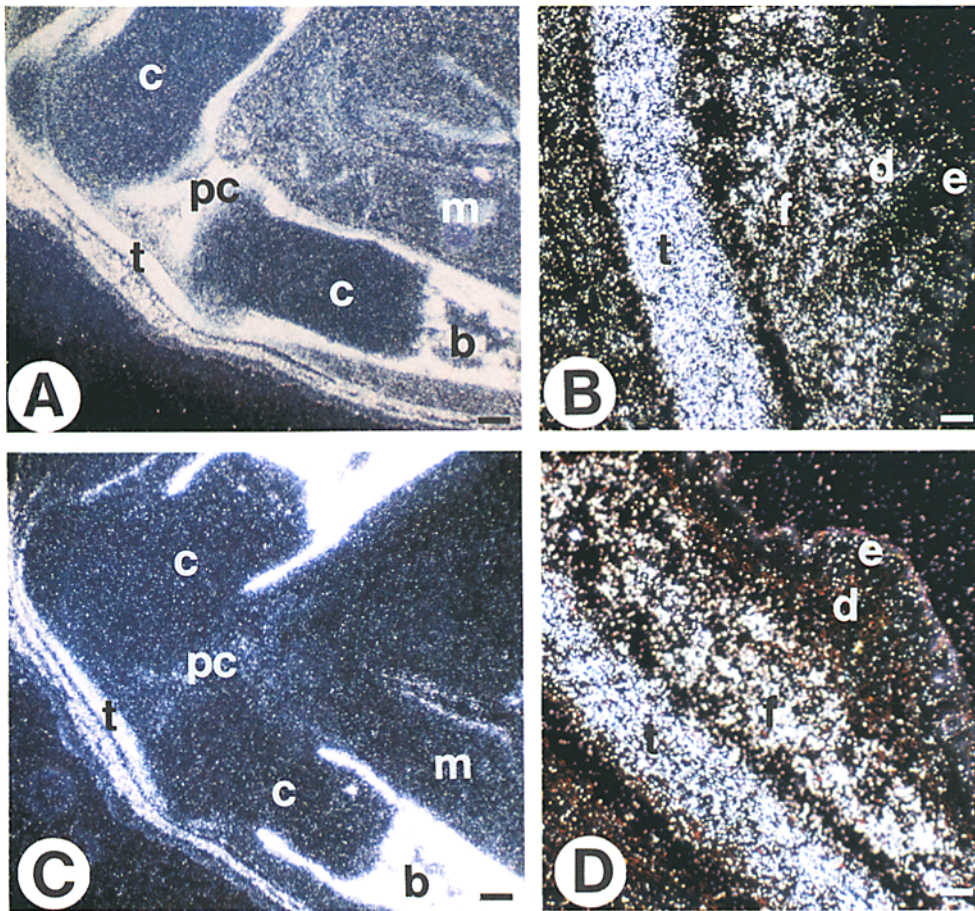
Tissues from three lines of mice containing the transgene, -2300COL( $\Delta I$ )-hGH, were examined by in situ hybridization of sagittal sections from 17-d-old embryos, concomitantly with tissues from the -2300COL-hGH transgenic

mice. Three separate experiments were performed, and sections from at least two different embryos were analyzed for each line. Figs. 3 and 4 show representative darkfield photomicrographs of sagittal sections after hybridization with the riboprobe for either mCOL (Fig. 3, A-C; Fig. 4, A and B) or hGH (Fig. 3, D-F; Fig. 4, C and D). As seen with the -2300COL-hGH transgene, high levels of signal corresponding to the intron-deleted transgene were observed in tendon, bone and periosteum, whereas expression was not apparent in skeletal muscle or perichondrium (compare Fig. 3, A and D; C and F; Fig. 4, A and C). The reduced expression of the transgene in the fibrous layer of the periosteum, previously noted in Fig. 2, B and E, was also observed in Fig. 3, C and F. However, in contrast to the -2300COL-hGH transgene, the endogenous gene and the intron-deleted



**Figure 3.** Representative photomicrographs from 17-d-old mouse embryos carrying the -2300COL( $\Delta I$ )-hGH transgenes. Serial sagittal sections from line 4084 were hybridized with probes for either mCOL (A-C) or hGH (D-F). High magnification of skin sections are shown in B, C, E, and F. Lower magnification of the pelvic regions (A and D) are shown. The arrows point to tendon. b, bone; c, cartilage; d, dermis; e, epidermis; f, fascia; m, skeletal muscle; pc, perichondrium; po, periosteum. Bars: (A and D) 150  $\mu\text{m}$ ; (B, C, E, and F) 30  $\mu\text{m}$ .





**Figure 4.** Representative photomicrographs from 17-d-old mouse embryos of line 4072 carrying the  $-2300\text{COL}(\Delta\text{I})$ -hGH transgene. Serial sagittal sections were hybridized with probes for either mCOL (A and B) or hGH (C and D). High magnifications of skin sections are shown in B and D. A lower magnification of the pelvic region (A and C) is also shown. *b*, bone; *c*, cartilage; *d*, dermis; *e*, epidermis; *f*, fascia; *pc*, perichondrium; *m*, skeletal muscle; *t*, tendon. Bars: (A and C) 150  $\mu\text{m}$ ; (B and D) 30  $\mu\text{m}$ .

transgene were differentially expressed in the dermis and fascia. For example, comparison of sections of skin from line 4084 (Fig. 3, A and D) indicated little expression in fascia and low expression in dermis of the  $-2300\text{COL}(\Delta\text{I})$ -hGH transgene. This difference in expression is more obvious in a section from dermis that is less compressed (Fig. 3, B and E). In these areas, fibroblasts of the fascia clearly express the transgene, although at an apparently lower level than that of the endogenous gene. In contrast, fibroblasts in the dermis show minimal expression of the transgene (Fig. 3 E). Similar results were obtained with sections from line 4082 (data not shown), but in this line expression in both dermis and fascia was low (Table I).

In line 4072 the  $-2300\text{COL}(\Delta\text{I})$ -hGH transgene was expressed in fascia at a relatively higher level than was the same transgene in lines 4082 and 4084. Representative photomicrographs of skin from hip (Fig. 4, B and D) showed a similar level of expression of the transgene and endogenous gene in fibroblasts of the fascia, whereas dermal fibroblasts again displayed low expression of the transgene. Thus, data from two lines of  $-2300\text{COL}$ -hGH and three lines of  $-2300\text{COL}(\Delta\text{I})$ -hGH transgenic mice indicate that some of the sequences necessary for appropriate expression of the  $\alpha\text{1(I)}$  collagen gene in dermal fibroblasts are located between +292 and +1440 (Table I).

Support for differential expression of the  $-2300\text{COL}(\Delta\text{I})$ -hGH transgene in different tissues was obtained by RNase protection analyses of whole limb and skin from 17-d-old embryos (Table II). Although there was considerable vari-

ability which can be attributed in part to small differences in age of embryos, the  $-2300\text{COL}$ -hGH and  $-2300\text{COL}(\Delta\text{I})$ -hGH transgenes were expressed at similar levels in limb, and the  $-2300\text{COL}$ -hGH transgene was expressed at similar levels in limb and skin. However, the  $-2300\text{COL}(\Delta\text{I})$ -hGH transgene was expressed at a lower level in skin than in the whole limb (Table II). This difference was particularly evident in lines 4082 and 4084 and was less apparent in line 4072, a finding which correlates with the relatively high level of expression of the intron-deleted transgene in the subdermal fascia of line 4072 (Table I).

#### ***The First Intron Is Required for Expression of the Transgene by Cultured Dermal Fibroblasts***

To clarify the conflicting results reported with various fibroblast cell lines in vitro, we determined whether cells derived from transgenic mice would express the transgene in a manner similar to that of the endogenous gene. Dermal fibroblasts were obtained from primary cultures of skin explants from transgenic animals. Cells were determined to be fibroblastic by morphology. Cultures in which cells exhibited characteristics of transformation, such as altered morphology, increased rate of proliferation and/or loss of relative contact inhibition, were excluded. After approximately three passages, RNA and DNA were obtained from the cells. RNA was analyzed for expression of both the hGH minigene and the endogenous mCOL gene by RNase protection assay. The results, presented as molecules of mCOL or hGH RNA per

**Table II. Expression of Collagen-hGH Transgenes in Tissues of 17-d-old Embryonic Mice**

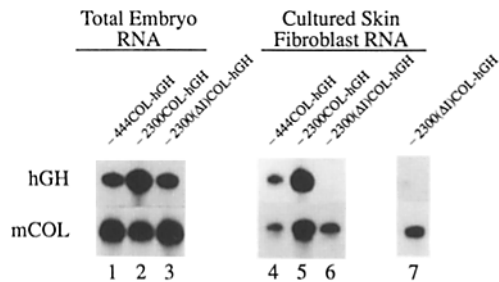
Construct	Line	hGH Expression*	
		Limb	Skin
-2300COL-hGH	3584	149 ± 83 (6)	89 ± 42 (6)
	6231	80 ± 69 (3)	74 ± 31 (3)
	8848	190 ± 82 (3)	160 ± 55 (3)
-2300COL(ΔI)-hGH	4072	132 ± 53 (3)	43 ± 22 (4)
	4082	24 <sup>‡</sup>	0.5 <sup>‡</sup> ; 0.2
	4084	105 <sup>‡</sup>	6; 9

\*Values are reported as the mean ± SEM of molecules of hGH RNA (10<sup>-7</sup>) per μg of tissue RNA. The number of analyses of independently derived samples is shown in parentheses.

<sup>‡</sup>Tissues from two or three animals were pooled prior to analysis.

microgram of total cellular RNA, were quantitated by comparison with a standard curve prepared with sense-strand RNA. Fig. 5 shows a representative RNase protection assay of hGH and mCOL. Fibroblasts obtained from the dermis of -2300COL(ΔI)-hGH transgenic mice did not contain detectable RNA for the hGH transgene, whereas endogenous mCOL RNA was readily apparent (Fig. 5, lane 6). In contrast, hGH RNA was detected in dermal fibroblasts from mice that contained either the -2300COL-hGH or the -444COL-hGH transgene (Fig. 5, lanes 4 and 5). Analysis of DNA from the same cells by dot-blot hybridization indicated that the lack of expression of the transgene in the absence of intronic sequences did not result from the loss of the transgenic DNA during the culture process (data not shown).

The results shown in Fig. 5 were reproducible when cells were obtained from animals of different ages as well as from all the lines that we tested (Table III). We observed that the transgene was expressed at lower levels than the endogenous mCOL gene in all cases; we have also observed levels of



**Figure 5.** RNase protection assay comparing expression of human  $\alpha 1(I)$  collagen-hGH transgenes and the endogenous mouse  $\alpha 1(I)$  collagen gene in 17-d-old embryos (lanes 1-3) and in fibroblasts cultured from 17-d-old embryonic skin (lanes 4-6). Lanes 1 and 4; -444COL-hGH line 5007A; lanes 2 and 5, -2300COL-hGH line 3584; lanes 3 and 6, -2300COL(ΔI)-hGH, line 4082. Lanes 1-3: 10 μg of RNA was used for analysis of hGH and 2 μg of RNA was analyzed for expression of endogenous  $\alpha 1(I)$  collagen. Lanes 4-6: 5 μg of RNA was analyzed for expression of hGH and 0.5 μg of RNA was analyzed for endogenous  $\alpha 1(I)$  collagen (RNA). Lane 7, expression of hGH as compared to expression of endogenous  $\alpha 1(I)$  collagen in dermal fibroblasts cultured from a 2-mo-old mouse from line 4084 containing the -2300COL(ΔI)-hGH transgene; 8 μg and 3.5 μg of RNA were used for analysis of hGH and endogenous  $\alpha 1(I)$  collagen, respectively. All autoradiograms were exposed for 4 h.

**Table III. Expression of Collagen-hGH Transgenes in Cultured Dermal Fibroblasts**

Construct	Line	Age	Expression*		Ratio of hGH/mCOL	
			hGH <sup>‡</sup>	mCol		
-2300COL-hGH	3584	17 d	12	31	0.4	
		24 mo	4	18	0.24	
	6231	17 d	4.2	24	0.18	
		2 mo	5	73	0.07	
	8848	17 d	8	35	0.28	
		1 mo	1	11	0.1	
-2300COLΔI-hGH	4072	15 mo	1.5	2.2	0.65	
		17 d	0	77	0	
	4082	17 d	0	11	0	
		13 mo	0	11	0	
	4084	23 mo	0	107	0.	
		17 d	0	3	0	
		2 mo	0	26	0	
		24 mo	0	12	0	
	-444COL-hGH	5007A	17 d	7	13	0.11
			12 mo	8	47	0.17
5007B		17 d	3.3	28	0.12	
		3 mo	7	7	1.15	
		12 mo	2	3	0.73	
		24 mo	99	122	0.81	

\*Values are reported as molecules of hGH or mCOL RNA (10<sup>-7</sup>) per microgram of total cellular RNA. See text for details.

<sup>‡</sup>The presence of transgenic DNA was verified in all 17 d cultured cells by dot-blot analysis. In addition, transgenic DNA was demonstrated in cultured cells from line 4084 (2 mo) and line 4084 (24 mo).

transgene expression within an order of magnitude of that of the endogenous gene in tissue homogenates of adult and 17-d-old embryo bone and tail (data not shown; Slack et al., 1991). Although the transcripts of both the hGH transgenes and the endogenous mCOL gene are relatively stable, they might differ in stability since they contain different 3' UTRs; this consideration, and the effect of multiple copies of the transgene, preclude an accurate comparison of the levels of expression of the transgenes with that of the endogenous gene.

## Discussion

Our laboratory is interested in the molecular mechanisms involved in the regulation of expression of the  $\alpha 1(I)$  collagen gene. We have established that the -2300COL-hGH transgene, which contains 2,300 bp of the promoter and the first intron of the human  $\alpha 1(I)$  collagen gene driving an hGH minigene, is expressed in most tissues of 17-d-old embryos that express the endogenous gene, with the exception of skeletal muscle and perichondrium (summarized in Table I). Transgenes that contain a deletion of sequences between +292 and +1440 of the  $\alpha 1(I)$  collagen first intron are also expressed by osteoblasts, tendon fibroblasts, and osteogenic cells. However, these transgenes are expressed poorly, or not at all, in dermal fibroblasts, both in vivo and in cell culture. These data indicate that the  $\alpha 1(I)$  collagen gene utilizes multiple and sometimes different DNA sequences to direct quantitative and cell-specific expression.

Type I collagen genes are transcribed in a large number of tissues by most cell types of mesenchymal origin, but the levels of expression of type I collagen vary greatly. It is there-

fore not surprising that the  $\alpha 1(I)$  collagen gene appears to rely on mechanisms that utilize *cis*-acting sequences located in different regions of the gene and flanking DNA for the direction of tissue- and cell-specific expression. Our study has identified at least three separate categories of cells involved in the cell-specific expression of  $\alpha 1(I)$  collagen: (a) osteoblasts, osteogenic cells, and tendon fibroblasts, which require 5'-flanking sequences and do not rely on intronic sequences; (b) fibroblasts, which require sequences within the first intron for full expression; and (c) skeletal muscle, perichondrial cells, and cells within the fibrous layer of the periosteum, which require sequences either 5' or 3' to the -2300 to +1607 region of the human  $\alpha 1(I)$  collagen gene. In addition, in our earlier studies, all the transgenes, including a -444COL( $\Delta I$ )-hGH transgene in which deletions had been made in both the 5'-flanking and intronic sequences, continued to be expressed at levels that were comparable to that of the endogenous gene in lung (Slack et al., 1991). The latter findings indicate that some cell types may require only a minimal  $\alpha 1(I)$  collagen promoter to direct appropriate expression of the gene.

The first suggestion that the  $\alpha 1(I)$  collagen gene was under differential, tissue-specific regulation was made from studies of Mov13 mice, in which germline integration of a retrovirus into the first intron of the gene abolished transcription in all mesenchymal by derived cells except osteoblasts and odontoblasts (Schnieke et al., 1983; Kratochwil et al., 1989; Schwarz et al., 1990). This finding indicated that fibroblasts required intronic and/or other 3' sequences to be present in a spatially correct manner for expression of the  $\alpha 1(I)$  collagen gene. In support of this observation, an  $\alpha 1(I)$ -CAT transgene, which contained only the promoter and 5'-flanking sequence of the rat  $\alpha 1(I)$  collagen gene, was expressed at high levels in bone, teeth, and tendon, but showed little or no expression in skin (Pavlin et al., 1992). Furthermore,  $\alpha 1(I)$  collagen-reporter gene constructs that included sequences from the first intron displayed a marked enhancement of expression over intron-deleted constructs after transient transfection into certain fibroblastic cells (Bornstein et al., 1988; Rippe et al., 1989; Boast et al., 1990; Liska et al., 1990, 1992). These observations, taken together with the present study, indicate that the first intron is required for the fibroblast-specific expression of the  $\alpha 1(I)$  collagen gene. Intronic sequences could mediate transcription of this gene by two distinct, but not necessarily exclusive, mechanisms: (a) *cis*-acting enhancer element(s) within the first intron might interact indirectly with promoter elements through specific DNA-binding proteins; or (b) sequences within the first intron might be involved in the establishment of a transcriptionally active chromatin conformation at the gene locus.

Only a few protein-DNA binding elements have been characterized in the first intron of the  $\alpha 1(I)$  collagen gene: an AP1-binding sequence that enhances transcription (Liska et al., 1990), and two tandem SP1-binding sequences (Liska et al., 1992). Mutation of the AP1 element abolished both the specific protein-DNA binding activity and the enhancement observed by the first intron in transiently transfected fetal fibroblasts and stromal cells (Liska et al., 1990), as well as in dedifferentiated chondrocytes (Mättä et al., 1993). Since 27 of the 29 bases of the AP1 element are identical in the rat and human  $\alpha 1(I)$  collagen introns, the AP1 element could play an important role in the regulation of  $\alpha 1(I)$  colla-

gen. The SP1-binding sequences are also conserved between the rat and human genes. However, site-directed mutagenesis of the SP1-binding sequences did not compromise the ability of the first intron to enhance transcription, but it was associated with a modest increase in expression of collagen-reporter gene constructs in transient transfection assays (Liska et al., 1992). These data indicate that the activity of the first intron, observed in assays *in vitro*, is mediated in part through the AP1 element and support a model in which the AP1 element stimulates transcription by transactivation.

In the second model we propose that sequences within the first intron might be involved in the formation of a transcriptionally active chromatin conformation at the transcription start site. Although the two models are not mutually exclusive, the latter predicts that specific sequences within the first intron mediate nucleosome formation and, subsequently, accessibility of the transcription complex to the transcriptional start site (reviewed by Felsenfeld, 1992). It is interesting that the SP1-binding sequences in the first intron reside within a DNase HS site (Barsh et al., 1984); these elements might therefore function in the modulation of chromatin conformation. Since the lack of  $\alpha 1(I)$  collagen gene transcription in Mov13 mice is associated with the absence of a DNase HS site at the promoter of the gene (Breindl et al., 1984), the first intron is likely to be involved in the establishment of an active chromatin conformation. However, in Mov13 mice, the  $\alpha 1(I)$  collagen gene is transcriptionally silent in all cells of mesenchymal origin except odontoblasts and osteoblasts. In our study, the deletion of sequences within the first intron inhibited expression of the transgene in dermal fibroblasts and, to a lesser extent, in fibroblasts of the fascia (Table I). It is possible that the low level of transgene expression observed in fascial fibroblasts was due to elements within the remaining intronic sequences (between +223 and +291, and/or +1440 and +1607) which were not deleted from our constructs.

While we favor one of the above models, or a combination of the two, we cannot completely exclude other explanations for the results we have observed in transgenic animals. Since the size of the splicable first intron differs in the intact and intron-deleted transgenes, it is possible that altered efficiency of splicing, with attendant consequences for efficient mRNA production, could influence transgene expression. The results reported in Table II argue against such an effect in all tissues since the -2300COL-hGH and -2300COL( $\Delta I$ )-hGH transgenes were expressed at approximately similar levels in tissues of the limb among the six lines that were studied. Although introns have been shown to facilitate the expression of genes in transgenic mice (Brinster et al., 1988), there was no obvious correlation between expression and intron length when a heterologous intron was placed between the first and second exons of an intronless gene (Palmiter et al., 1991). Indeed, introns of the approximate length of the first intron in -2300COL( $\Delta I$ )-hGH (260 bp) were effective in the study of Palmiter et al. (1991) and are naturally spliced in many genes.

In our study, deletion of intronic sequences did not alter expression of the transgenes in osteoblasts or in tendon fibroblasts. Similarly, high levels of expression of a rat  $\alpha 1(I)$ -CAT transgene, in which a sequence from -3521 to +115 of the rat  $\alpha 1(I)$  collagen gene was used as a promoter DNA, was observed in bone, developing tooth germ, and tendon



(Pavlin et al., 1992). Furthermore, Krebsbach et al. (1993) have shown that a 624-bp region of the rat  $\alpha 1(I)$  collagen gene, located between -2296 and -1672, is required for high level expression of rat  $\alpha 1(I)$ -CAT constructs in intact bone. That the rat and human  $\alpha 1(I)$  collagen genes exhibit considerable spatial and sequence conservation in this region (J. L. Slack, personal communication) indicates that the sequences necessary for high, osteoblast-specific expression of the human  $\alpha 1(I)$  collagen gene might also lie in the 5' flanking region between -2300 to -1600. Consonant with this notion, in situ hybridization and qualitative RNase protection analyses showed that the -444COL-hGH transgene was expressed at levels significantly lower than those observed with the -2300COL-hGH transgene in bone (Slack et al., 1991; data not shown). However, the low level of expression of the -444COL-hGH transgene would predict that the basal  $\alpha 1(I)$  collagen promoter contains some osteoblast-specific information. A thorough understanding of the *cis*-acting elements necessary for osteoblast-specific gene expression will require assessment of the ability of the -2300 to -1600 region to direct osteoblast-specific expression of constructs containing heterologous promoters.

Our analyses of transgene expression in vivo, by in situ hybridization, and in cultured dermal fibroblasts from transgenic animals clearly implicates *cis*-acting sequences within the first intron of the  $\alpha 1(I)$  collagen gene in the regulation of the gene in fibroblasts. We note that the effect of the intronic deletion was much more evident when transgene expression was assessed in dermal fibroblasts as opposed to fibroblasts of the fascia. However, in at least two of the three intron-deleted transgenic mouse lines, transgene expression was also compromised in fascial fibroblasts, in comparison to the expression of the endogenous gene. Several studies have indicated that fibroblasts of different tissues are heterogeneous in their reactivity, synthetic functions, and proliferative rate, although they are morphologically similar (Harper and Grove, 1978; Fleischmajer et al., 1981; Kähäri et al., 1988; Peltonen et al., 1990). Consistent with these observations, our results indicate that the  $\alpha 1(I)$  collagen gene is regulated differently in fibroblasts of the dermis than in those of the fascia. In addition, we found that some dermal fibroblasts, specifically those closest to the fascia, did express the intron-deleted transgene. This difference is interesting since the human dermis contains two structurally distinct layers, the papillary (upper) layer, and the reticular (lower) layer. Diseases of the skin rarely affect both dermal layers but are manifested in either the papillary or the reticular layer (Meigel et al., 1977; Harper and Grove, 1978). A clarification of the mechanisms that regulate the fibroblast-specific expression of the  $\alpha 1(I)$  collagen gene should further our understanding of the distribution of pathology in dermatological disorders and may suggest ways in which to correct aberrant collagen synthesis.

In the final analysis, future studies of the cell-specific regulation of the  $\alpha 1(I)$  collagen gene will have to be performed within the context of the endogenous gene in order to preserve the influence of chromatin conformation on gene expression. Targeted deletions and mutations of specific elements in mice, by a double replacement homologous recombination approach such as the "tag-and-exchange" strategy described by Askew et al. (1993), may be required to define unambiguously the mechanisms that govern cell-specific ex-

pression of the  $\alpha 1(I)$  collagen gene. Cell lines derived from such transgenic animals could also serve as useful models for investigation of the influence of collagen synthesis on the behavior of dermal fibroblasts and other cells.

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