

# Differential Utilization of Regulatory Domains Within the $\alpha 1(I)$ Collagen Promoter in Osseous and Fibroblastic Cells

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**Abstract.** Type I collagen is expressed in a variety of connective tissue cells and its transcriptional regulation is highly complex because of the influence of numerous developmental, environmental, and hormonal factors. To investigate the molecular basis for one aspect of this complex regulation, the expression of  $\alpha 1(I)$  collagen (COL1A1) gene in osseous tissues, we fused a 3.6-kb DNA fragment between bases -3,521 and +115 of the rat COL1A1 promoter, and three deletion mutants, to the chloramphenicol acetyltransferase (CAT) marker gene. The expression of these ColCAT transgenes was measured in stably transfected osteoblastic cell lines ROS 17/2.8, Py-1a, and MC3T3-E1 and three fibroblastic lines NIH-3T3, Rat-1, and EL2. Deletion of the distal 1.2-kb fragment of the full-length ColCAT 3.6 construct reduced the promoter activity 7- to 30-fold in the osteoblastic cell lines, twofold in EL2 and had no effect in NIH-3T3 and Rat-1 cells. To begin to assess the function of COL1A1 upstream regulatory elements in intact animals, we established transgenic mouse lines and

examined the activity of the ColCAT3.6 construct in various tissues of newborn animals. The expression of this construct followed the expected distribution between the high and low collagen-producing tissues: high levels of CAT activity in calvarial bone, tooth, and tendon, a low level in skin, and no detectable activity in liver and brain. Furthermore, CAT activity in calvarial bone was three- to fourfold higher than that in the adjacent periosteal layer. Immunostaining for CAT protein in calvaria and developing tooth germ of ColCAT3.6 mice also confirmed the preferred expression of the transgene in differentiated osteoblasts and odontoblasts compared to fibroblast-like cells of periosteum and dental papilla. This study suggests that the 3.6-kb DNA fragment confers the strong expression of COL1A1 gene in high collagen producing tissues of intact animals and that the 5' flanking promoter sequence between -3,521 and -2,295 bp contains one or more stimulatory elements which are preferentially active in osteoblastic cells.

**T**YPE I collagen is the predominant component of the extracellular matrix and is synthesized by a variety of connective tissue cells. The protein is encoded by two genes,  $\alpha 1(I)$  (COL1A1)<sup>1</sup> and  $\alpha 2(I)$  collagen (COL1A2), which are expressed and regulated in a coordinated fashion. Although the regulation of type I collagen genes is not as dramatic as other highly inducible genes, the control of their expression is extremely complex. Both are single copy non-housekeeping genes which are active in distinctly different connective tissue cells (Ramirez and Di Liberto, 1990) including: interstitial cells that produce skin, tendon, and the framework of all organs and connective tissues of the body; smooth muscle cells of blood vessels and other viscera; cartilage cells, in which a different RNA start site is utilized

(Bennett and Adams, 1990); activated fibroblastic cells that are involved in tissue repair and fibrosis; and osteoblasts and odontoblasts which form a mineralized matrix. Each cell type demonstrates characteristic regulation of these genes under a variety of different circumstances. Examples include activation of type I collagen genes at a specific time during development; modulation of type I collagen production during growth or in response to mechanical force; reduction of collagen synthesis in bone cells during periods of mineral deprivation through the actions of calcitrophic hormones 1,25-dihydroxyvitamin D3 (vitamin D) and parathyroid hormone (PTH)<sup>1</sup>; and activation of the gene during inflammation and repair in all cell types.

Most published experiments concerning the transcriptional control of transfected type I collagen promoter constructs have used fibroblastic cells. These studies mapped the regions responsible for regulation of COL1A1 and COL1A2 promoters by local growth factors (Choe et al., 1987; Rossi

1. *Abbreviations used in this paper:* CAT, chloramphenicol acetyltransferase; COL1A1,  $\alpha 1(I)$  collagen; COL1A2,  $\alpha 2(I)$  collagen; PTH, parathyroid hormone; TK, thymidine kinase; vitamin D, 1,25-dihydroxyvitamin D3.

and de Crombrughe, 1988), systemic hormones (Walsh et al., 1987) and viral transformation (Liau et al., 1985). A number of *cis*-acting elements from the 5' promoter region and *trans*-acting factors involved in transcriptional regulation of type I collagen genes have been described (for review see Ramirez and Di Liberto, 1990; Vuorio and de Crombrughe, 1990). These include the COL1A1 and COL1A2 promoter sequences which control the tissue-specific expression in transfected cells (Boast et al., 1990; Karsenty and de Crombrughe, 1990; Rippe et al., 1989; Schmidt et al., 1986) and in transgenic animals (Khillan et al., 1986; Slack et al., 1991), inhibition in oncogene-transformed fibroblasts (Schmidt et al., 1985), nuclear factor 1-binding sequence which mediates stimulation by transforming growth factor- $\beta$  (Rossi and Crombrughe, 1988) and CCAAT box-binding nuclear factor which was identified and purified from fibroblastic cells (Hatamochi et al., 1986; Maity et al., 1988). Furthermore, regulatory elements have been identified within the first intron which can have either strong stimulatory, inhibitory or neutral effect on the activity of COL1A1 and COL1A2 promoters (Bornstein and McKay, 1988; Rippe et al., 1989; Rossi and de Crombrughe, 1987; Rossouw et al., 1987; Sherwood and Bornstein, 1990; Slack et al., 1991).

In addition to those control mechanisms which are assumed to operate in many fibroblastic cell lines, there is growing evidence that there must be other regulatory mechanisms which are unique for the cells producing osseous tissue, osteoblasts, and odontoblasts. First, osteoblasts have a higher basal rate of collagen synthesis than fibroblastic cells. This conclusion is based on the rates of collagen synthesis measured in embryonic tissues (Diegelmann and Peterkofsky, 1971; Moen et al., 1979), primary cell cultures (Luben et al., 1976; McCarthy et al., 1988; Wong, 1982), and fetal rat calvariae in organ culture (Canalis, 1980; Rowe and Kream, 1982). Second, fully differentiated osteoblasts produce almost exclusively type I collagen, while fibroblasts make a variety of collagen types (I, III, IV, V, and VI) (Fessler et al., 1981; Liau et al., 1985; Olsen et al., 1989; Pihlajaniemi et al., 1989). Third, the synthesis of type I collagen in osteoblasts from fetal rat calvariae and cultured osteosarcoma cells is regulated by several hormones such as vitamin D, PTH (Kream et al., 1980; Lichtler et al., 1989; Rowe and Kream, 1982), insulin (Kream et al., 1985), and insulin-like growth factor-I (Canalis, 1980), whereas periosteal fibroblasts do not respond to these hormones. Finally, in the MOV13 mouse, a retroviral insertion in the first intron of the COL1A1 gene completely blocks its transcription in fibroblasts but does not inhibit its activity in odontoblasts and osteoblasts (Kratochwil et al., 1989). This observation suggests that odontoblasts and osteoblasts use *cis*-active DNA sequences within the COL1A1 gene that are different from those used in fibroblasts and which are capable of overriding the inhibitory effect of retroviral insertion.

We have recently isolated an upstream 3.6-kb rat COL1A1 promoter fragment and studied its expression and hormonal regulation in clonal rat osteosarcoma cell line ROS 17/2.8 and EL2 fibroblasts (Lichtler et al., 1989). In the present study we used several transfected osteoblastic and fibroblastic cell lines to identify regions within the COL1A1 3.6-kb promoter fragment involved in transcriptional regulation of this gene in osseous tissue. The results indicate that the sequence between -3,521 and -1,672 of the COL1A1 promoter has a greater stimulatory effect on transcription of the

transgene when expressed in osteoblastic versus fibroblastic cell lines and suggests the presence of transcription control elements in the COL1A1 promoter 5' flanking sequence that are differentially used in osteoblasts compared to fibroblasts. When introduced into transgenic animals, the regulatory elements within the full length ColCAT3.6 chimeric gene were sufficient for augmented expression in high type I collagen-producing tissues.

## Materials and Methods

### Transfection of Osteoblastic and Fibroblastic Cell Lines

A variety of immortalized osteoblastic cell lines were selected for transfection studies because they each express some of the characteristics of the fully differentiated osteoblast. The clonal rat osteosarcoma cell line ROS 17/2.8 (Majeska et al., 1978) synthesizes type I collagen, osteocalcin, and alkaline phosphatase, has vitamin D and PTH receptors and produces mineralized tumors in vivo. MC3T3-E1 is a mouse calvaria osteoblastic cell line which mineralizes in culture and has a PTH-responsive adenylate cyclase (Suda et al., 1983). PylA cells (Lichtler, A. C., B. E. Kream, D. W. Rowe, G. Carmichael, and R. Majeska, 1987. *J. Bone Min. Res.* 2S:125) synthesize type I collagen and alkaline phosphatase and respond to cortisol, insulin-like growth factor-I, and vitamin D (B. E. Kream, unpublished data). The fibroblastic lines NIH3T3, Rat-1 (Zarbl et al., 1987), and E12 (Liboi et al., 1984) were used as a nonosseous cell source. All cells were maintained in F-12 medium supplemented with nonessential amino acids, 4 mM glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 10% FCS in a humidified atmosphere of 5 or 7.5% CO<sub>2</sub>.

To produce stably transfected lines, cells were plated at 3-4  $\times$  10<sup>4</sup> cells/cm<sup>2</sup> in either 60- or 100-mm tissue culture dishes and cotransfected the next day with 4-6  $\mu$ g of one of the ColCAT DNA constructs (described below) and one tenth that amount of pSV-2neo (Gorman et al., 1983), using either the calcium phosphate precipitation or Lipofectin (Bethesda Research Laboratories, Gaithersburg, MD) method. 6 h after addition of the DNA/calcium phosphate complex, the cells were shocked with 1.5 ml of 15% glycerol in 1 $\times$  Hepes-buffered saline for 1 min. The cells were subsequently washed in PBS and placed in F-12 medium containing 10% FCS. The Lipofectin-mediated transfections were carried out in serum-free F-12 medium, which was replaced 14-18 h later with F-12 containing 10% FCS. With either method, the cells were split at a 1:3 ratio the following day and placed under selection with 400  $\mu$ g/ml of the G-418 (Geneticin; Gibco Laboratories, Grand Island, NY). 3 to 5 wks later 50-900 colonies of cells carrying the same construct were pooled, expanded, and assayed for CAT activity or collagen synthesis.

### Collagen Promoter Constructs

ColCAT3.6 contains a 3.6-kb fragment of the rat COL1A1 gene between bases -3,521 and +115, fused to the CAT marker gene (Lichtler et al., 1989). Deletion mutants of this parental construct (see Fig. 2) were cloned by using naturally occurring restriction sites. ColCAT2.3 and ColCAT1.7 deletion mutants, extending from -2,295 and -1,672 bp upstream of the RNA start site, were cloned after cutting ColCAT3.6 with HindIII and PstI, respectively, and self ligating the ends. To clone the ColCAT0.9 mutant (-944 to +115 bp), the 1,062-bp PvuII-XbaI fragment was first cloned into the XbaI/HincII site of Puc19 from which the XbaI/HindIII fragment was then ligated into pUCCAT 13 (Lichtler et al., 1989).

To test for a potential transcriptional activity of COL1A1 upstream regulatory elements on a heterologous promoter, a 2112 bp XbaI/ApaI genomic fragment was inserted upstream of the herpes simplex virus thymidine kinase (TK) minimal promoter (bases -109 to +151) within the plasmid pTE1 (Edlund et al., 1985). The XbaI/ApaI fragment spans between bases -3,521 and -1,408 and thus includes all of the sequences of the XbaI/PstI fragment. Before insertion, the 598-bp spacer between the BamHI site and NruI site was removed from pTE1, bringing the polylinker closer to the TK promoter.

### Assays of Collagen Synthesis and CAT Activity

Either two 60-mm plates or one 100-mm plate was used per assay point. Cells were grown to confluency and fresh medium containing 5  $\mu$ Ci/ml [<sup>5</sup>-<sup>3</sup>H]proline and 50  $\mu$ g/ml ascorbic acid was added to the culture medium

24 h before harvesting. Radiolabeled collagen and noncollagenous proteins were assayed (Diegelmann and Peterkofsky, 1971) from the culture medium (Kream et al., 1986) as a measure of endogenous collagen gene expression. The percent collagen in the medium was corrected for the relative abundance of proline in collagen compared to noncollagen protein.

The cell layer was harvested for the CAT assay by scraping in buffer containing 0.04 M Tris-HCl, pH 7.4, 1 mM EDTA, and 0.15 M NaCl, centrifuged, and by resuspending the cell pellet in 100  $\mu$ l of 0.25 M Tris-HCl, pH 7.8. After disrupting the cells by three cycles of freezing and thawing, aliquots of the cell extract containing the indicated amounts of protein (Bradford, 1976) or DNA (Brunk et al., 1979) were assayed for CAT activity as described previously (Gorman et al., 1982). The assay mixture contained 0.46 M Tris, pH 7.8, and 50  $\mu$ Ci/mmol [1,2- $^{14}$ C] chloramphenicol in a final reaction volume of 150  $\mu$ l. The volume of extract was selected to produce conversion within the linear range of the CAT assay. The reaction was started by addition of 4 mM acetyl-CoA and carried out at 37°C for 4–6 h. The acetylated chloramphenicol products were separated by thin layer chromatography on a silica gel plate (Baker-Flex IB2; J. T. Baker Inc., Phillipsburg, NJ), visualized by autoradiography, and the intensity of the spots was quantitated by either scintillation spectrometry or by using Betagen Betascope 603 (Betagen Corporation, Waltham, MA) blot analyzer. The diffusion CAT assay was performed as described (Sambrook et al., 1989).

### Southern Blot Analysis

Genomic DNA was prepared from stably transfected cell populations or tissues from transgenic animals as described previously (Padula et al., 1988). DNA was restricted with EcoRI or PvuII, separated on a 1% agarose gel, transferred to a nylon membrane (Zetabind; Cund Inc., Meriden, CT), and hybridized as described (Singh and Jones, 1984), except for the following modifications: the transfer was done by capillary blotting and the hybridization contained as additional components 10% dextran sulfate, 30% formamide, 0.1% Sarkosyl instead of sodium dodecyl sulfate, and 0.1 mg/ml salmon sperm DNA. DNA fragments were labeled by the random primer method (Feinberg and Vogelstein, 1983). Two approaches were used to determine the copy number of the transgene incorporated in stably transfected cells or transgenic animals. In the first, a 1.4-kb EcoRI fragment encoding the CAT and SV-40 sequences present in the transfected vectors was hybridized to EcoRI-digested genomic DNA and the copy number was calculated by comparison to cloned CAT-containing DNA standards. The second approach was designed to detect both the transfected and endogenous collagen gene by hybridizing the PvuII-XbaI promoter fragment (–944 to +115 bp) to PvuII-digested genomic DNA. Hybridization to the endogenous gene produced a band of 2.6 kb, while the transfected construct produced a 1.2-kb band. The amount of radioactivity in the hybridized bands was quantitated using a Betagen Betascope 603 blot analyzer.

### Analysis of Transgenic Mice

Transgenic mice were produced by microinjection (Hogan et al., 1986) of ColCAT3.6 construct linearized by FspI (line 1), or DNA fragment obtained by digestion with HaeII of ColCAT3.6 and purified by agarose gel electrophoresis. Each HaeII restriction fragment contained the ColCAT hybrid gene and about 200 bp of flanking vector sequence. The transgenic founder animals, as well as subsequent breeders, were identified by dot blot hybridization for the CAT gene. Genomic DNA was denatured in 0.2 M NaOH, neutralized with HCl and 6 $\times$  SSC, spotted on nylon membrane (Zetabind; Cund Inc.) by using dot blot apparatus (Schleicher and Schuell, Inc., Keene, NH), hybridized with the same 1.4-kb CAT probe, and quantitated as described above.

To prepare tissue extracts for CAT assays, various tissues from 3- to 5-d-old progeny of either heterozygote or homozygote breeders were homogenized using a Polytron in 0.5 ml of buffer (250 mM Tris-HCl, 1 mM EDTA, and 1 mM PMSF), and sonicated for 20 s (setting 4). At this point, an aliquot of each sample was taken for DNA content analysis and the remainder of sample was centrifuged at 12,000 *g* for 30 min at 4°C. The supernatant of each tissue extract was then assayed for the expression of CAT reporter gene. In indicated experiments, an aliquot of each sample was used for protein assay. In some experiments, dissected calvarial halves were incubated in the collagen cross-linking inhibitor  $\beta$ -aminopropionitrile and 24 h later the periosteum was stripped from the central bone using a fine scalpel blade (Rowe and Kream, 1982). In other experiments, mandibular first molar tooth germs were dissected from 4- or 5-d-old mice and homogenized as described above.

### Light Microscopy and Immunofluorescence

Calvaria and tooth germs were fixed for 1 h at 4°C in 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, postfixed with 1% OsO<sub>4</sub>, dehydrated in a graded series of ethanols followed by propylene oxide and embedding in Polybed (Polysciences Inc., Warrington, PA). Thick sections (1–2  $\mu$ m) were cut with a glass knife and stained with 0.01% toluidine blue and 1% sodium borate. For immunofluorescence, calvariae were fixed with 5% paraformaldehyde and 2% sucrose in 0.1 M cacodylate buffer, pH 7.4, for 1.5 h on ice, washed extensively in 2% sucrose in the same buffer, drained, and frozen in liquid nitrogen. Tooth germs were fixed overnight in 95% ethanol at 4°C, fully dehydrated, and embedded in paraffin. Cryostat sections (calvariae) or deparaffinized sections (tooth germs) were reacted with a 1:20 dilution of rabbit antibody against CAT (5 Prime - 3 Prime Inc., West Chester, PA) for 1 h, rinsed with PBS, and incubated with a 1:100 dilution of rhodamine- or fluorescein-conjugated goat anti-rabbit IgG (Chemicon, El Segundo, CA) for 1 h, and washed. Control sections were only treated with the second antibody. Sections were mounted in 2.5% *n*-propyl gallate in PBS:glycerol and observed with an Optiphot fluorescence microscope (Nikon, Inc., Garden City, NY).

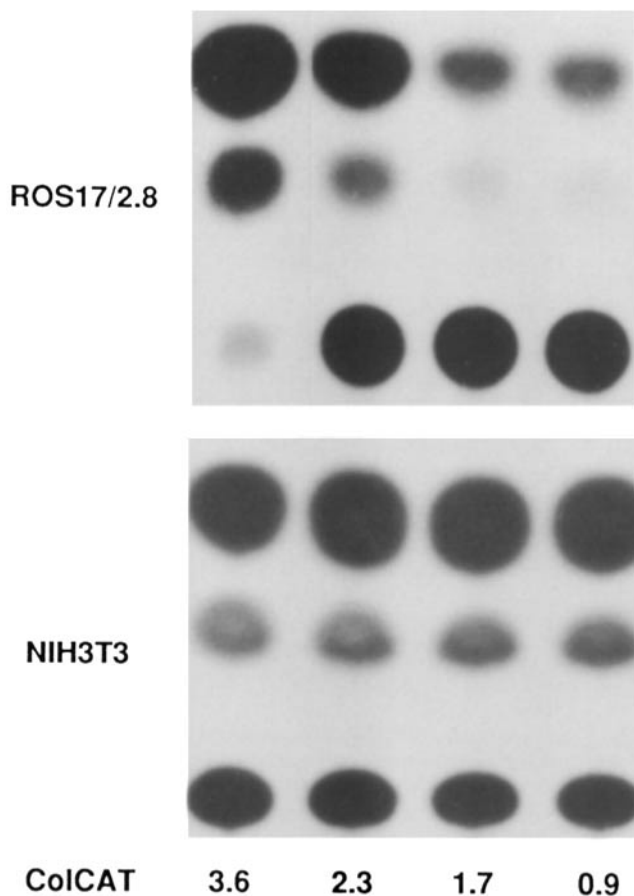
### RNase Protection

Isolation of total RNA was performed as described (Chomczynski and Sacchi, 1987). The RNase protection probe was a TaqI-EcoRI fragment of ColCAT3.6 which extends from –269 bp in the collagen promoter, includes 115 bp of COL1A1 mRNA, to +256 bp in the CAT gene (see Fig. 8). Hybridization of this probe to collagen CAT mRNA produced by the transfected DNA protects a 371-bp band, while hybridization to the endogenous collagen mRNA protects a 115-bp band. This fragment was cloned into pBS+ and transcribed with T7 RNA polymerase in the presence of  $\alpha$ - $^{32}$ P UTP to produce a uniformly labeled antisense probe. The probe was mixed with 5  $\mu$ g total cellular RNA, precipitated with ethanol, hybridized in 80% formamide, 0.4 M NaCl, 40 mM Hepes, pH 7.6, and 2 mM EDTA at 50°C overnight, and digested as described (Hart et al., 1985), except that we used a crude preparation of T2 and T1 ribonucleases which we have found to give equivalent results to purified T2 (Lichtler et al., 1991). Total volume of each reaction mixture was phenol/chloroform extracted, ethanol precipitated, electrophoresed on an 8% polyacrylamide gel containing 8 M urea, and bands were revealed by autoradiography.

### Results

To determine if the 5' flanking sequences of the rat COL1A1 promoter are preferentially active in osteoblastic versus fibroblastic cell lines, four ColCAT constructs and the neomycin resistance plasmid pSV2neo were cotransfected into three osteoblastic (ROS 17/2.8, Py-1a, and MC3T3-E1) and three fibroblastic cell lines (NIH-3T3, EL2, and Rat-1). The cell populations which emerged from the antibiotic selection, containing either stably integrated ColCAT3.6 or one of the deletion mutants, maintained their osteoblastic or fibroblastic phenotype as judged by morphological criteria, and in the case of the osteoblastic cells, the response to dexamethasone (Hodge and Kream, 1988), PTH, and vitamin D (Kream et al., 1986; data not shown). A representative CAT assay, shown in Fig. 1, compares the activity of the ColCAT3.6 construct and three deletion mutants in ROS17/2.8 and NIH-3T3 cells. The strength of expression of ColCAT3.6, as compared to ColCAT2.3, is not fully appreciated in ROS 17/2.8 line, because the activity is beyond the linear range of the CAT assay.

The quantitative results of CAT assays from a number of experiments and the percent collagen synthesized by the cell population are summarized in Fig. 2. In ROS 17/2.8 cells, the deletion of sequences upstream of the HindIII site (–2,295 bp) caused a sevenfold decrease in CAT activity. Deletion of the next fragment (between –2,295 and –1,672 bp) resulted in another sevenfold decrease in activity. Thus, there was a 50-fold difference in activity between the most



**Figure 1.** Representative assay for CAT activity of the four COL1A1 promoter constructs in stably transfected cells ROS 17/2.8 and NIH-3T3. Equal amounts of protein from the cell extracts (85  $\mu$ g from ROS 17/2.8 and 70  $\mu$ g from NIH-3T3) were added to each reaction. The expression of the full-length ColCAT3.6 construct in ROS 17/2.8 cells was beyond the linear range of the CAT assay.

active and the least active construct in ROS 17/2.8 cells. In contrast, the NIH-3T3 and Rat-1 cells showed no significant difference in the CAT expression between the four constructs, while in EL2 cells deletion from  $-3,521$  to  $-2,295$  bp produced only a twofold decrease in activity. The lack of a stimulatory element in the 5' upstream sequences of the COL1A1 gene in NIH-3T3 cells, which we found here, has been observed by two other research teams (Rippe et al., 1989; Stacey et al., 1988). The results from two other osteoblastic cell lines, Py-1a, and MC3T3-E1 (Fig. 3), which were obtained by using diffusion CAT assay, showed that the CAT activity of the ColCAT3.6 was reduced 10 and 27-fold in Py-1a and MC3T3-E1, respectively, after deletion of sequences upstream to  $-2,295$  bp. Unlike in ROS 17/2.8, all of the stimulatory activity was located between  $-2,295$  and  $-3,521$  bp in both the Py-1a and MC3T3-E1 cells. In summary, deletion of sequences between  $-3,521$  and  $-1,672$  bp had a significant effect on CAT activity in all three osteoblastic cell lines, but had no comparable effect on activity of fibroblastic lines.

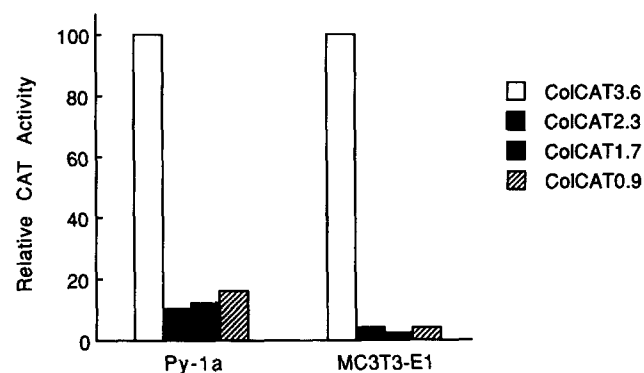
To ensure that the changes in the activity of the deletion constructs did not reflect a difference in the ability of the cells to transcribe the collagen promoter brought on by the transfection and cell selection process, the level of endogenous

ColCAT construct	Ros17/2.8		NIH 3T3		Rat-1		EL2	
	CAT	% col	CAT	% col	CAT	% col	CAT	% col
3.6	100	12	13	9	3	5	28	4
2.3	14	14	10	9	2	4	13	4
1.7	2	10	14	10	2	6	9	3
0.9	3	12	11	10	3	6	10	4

**Figure 2.** Summary of the results of CAT expression and endogenous collagen production by the cell lines stably transfected with four COL1A1 promoter constructs. The CAT activity was assayed by thin layer chromatography, as described in Materials and Methods, and expressed as the percentage of total chloramphenicol converted to acetylated derivatives per mg protein from the cell extract. Each value is the mean from 3 to 20 CAT assay points from 1 to 4 different cell populations, as shown in Table I. The values are normalized to the expression of ColCAT3.6 in ROS 17/2.8 cells, which was taken as 100%. Collagen synthesis (% col) is expressed as the percent of total radiolabeled protein secreted into the culture medium.

collagen synthesis was measured in most of the cell lines. The percent collagen in the medium (Fig. 2) was similar for the cell populations of the same cell line, regardless of the transgene carried in the genome. Furthermore, the copy number of the chimeric gene in the stably transfected cell populations was analyzed by Southern blots in most of the cell populations. Comparison of average CAT expression in any particular cell population with the transgene copy number is shown in Table I. These data indicate that there was no correlation between the expression and the copy number of the transgene in these stably expressing cells.

There was little correlation between the relative levels of expression of the endogenous gene and the transgene between the cell types. This was particularly true for ROS 17/2.8 and NIH-3T3 lines. Radiolabeled collagen accumulated in the culture media was comparable (10–14% ROS 17/2.8, 9–10% NIH-3T3). However, the full-length transgene was expressed seven times higher in ROS 17/2.8, while ex-



**Figure 3.** CAT expression in two osteoblastic cell lines stably transfected with four COL1A1 promoter constructs, measured by the diffusion CAT assay. The CAT activity (expressed as cpm/ $\mu$ g protein/h) was normalized to the expression of the ColCAT3.6 construct in each cell type, which was taken as 100%. The level of endogenous collagen synthesis, which was determined by measuring the percent collagen in the culture medium, did not vary within the cell lines of the same phenotype containing different ColCAT constructs (not shown).

**Table I. CAT Activity and Copy Number of the Transgene in Stably Transfected Cell Populations**

Transgene	ROS 17/2.8			EL2			NIH-3T3			Py-1a			MC3T3			Rat1		
	Tx*	CAT†	Copy No.	Tx	CAT	Copy No.	Tx	CAT	Copy No.	Tx	CAT	Copy No.	Tx	CAT	Copy No.	Tx	CAT	Copy No.
ColCAT3.6	18/1	168	48	22L/1	35	5	23L/1	15	9	Py3.6	22	11	MC3.6	12	64	29/1	3	9
	18/2	123	23	22/1	46	nd												
	19/1	60	36	22/2	47	nd												
	19/2	46	4															
ColCAT2.3	18/3	21	8	22L/3	8	16	23L/3	14	12	Py2.3	2.1	21	MC2.3	0.5	43	29/4	2	9
	18/4	2	2	22L/4	8	8	23L/4	11	17						29/5	2	14	
	19/3	26	9	22/3	17	nd									29/6	2	15	
ColCAT1.7	18/5	2	9	22L/5	10	nd	23L/5	16	25	Py1.7	3	25	MC1.7	0.2	6	29/7	2	16
	19/2	1	3	22/5	31	nd												
ColCAT0.9	18/6	3	17	22L/6	2	nd	23L/6	14	33	Py0.9	4	13	MC0.9	0.5	9	29/8	3	11
	18/7	3	nd	22L/7	0.5	nd	23L/7	9	29						29/9	4	17	
				22/6	29	nd												
				22/7	33	nd												

\* Stably transfected cell population derived from independent transfection.

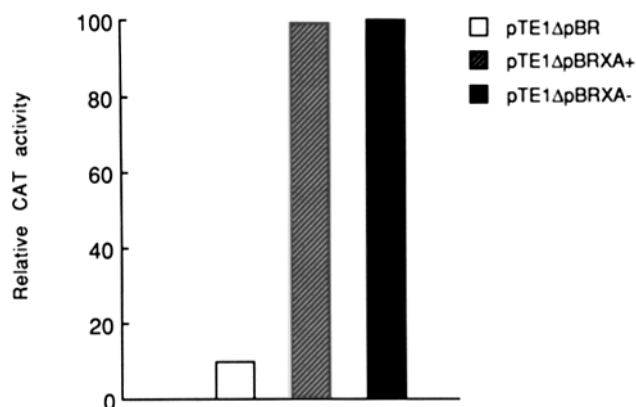
† CAT activity is expressed as the percent of chloramphenicol conversion per 10 µg of protein from the cell extract.

‡ Not done.

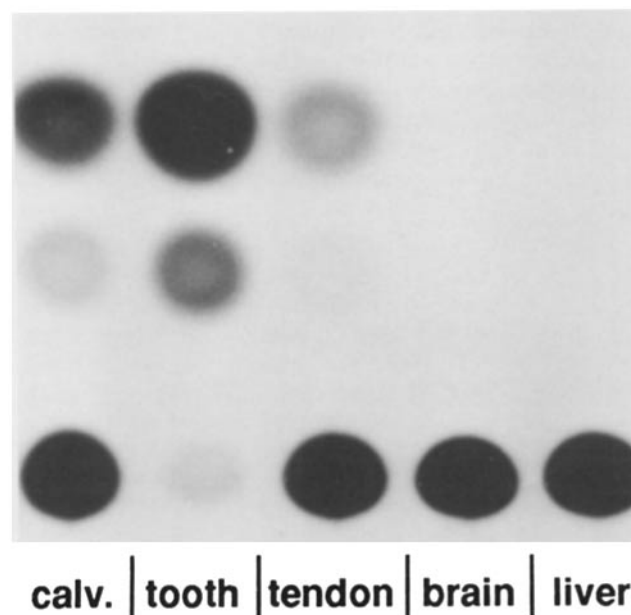
pression of the two shortest promoter fragments was four- to fivefold higher in NIH-3T3 than in ROS 17/2.8. In the Rat-1 and EL2 cells, which accumulated substantially less collagen in culture medium, the expression of the full-length transgene was similar to the activity of the two shortest promoter fragments in ROS 17/2.8 cells.

We next examined if the stimulatory elements located between bases -3,521 and -1,672 are active when fused to a heterologous promoter. The XbaI-ApaI fragment from the ColCAT3.6 was inserted in both positive and negative orientation upstream of the minimal TK promoter within the pTE1 vector, after removal of 598-bp fragment from pBR322 between the TK promoter and polylinker cloning sites. Previ-

ous studies with the TK promoter region between -109 and +151 bp showed higher stimulation by a heterologous enhancer when the pBR322 spacer was removed (Edlund et al., 1985). Fig. 4 shows that the XbaI-ApaI fragment stimulated the activity of the TK promoter 10-fold in ROS 17/2.8 cells in an orientation independent manner, which is typical for most enhancer elements.



**Figure 4.** Stimulation of a heterologous promoter by COL1A1 upstream regulatory elements. Three stably transfected populations of ROS 17/2.8 cells were produced as described in methods. The CAT activity, expressed as in Fig. 2 and normalized to construct pTE1ΔpBRXA-, represents an average expression from ~350 (pTE1ΔpBR), 900 (pTE1ΔpBRXA+), and 800 (pTE1ΔpBRXA-) pooled colonies of transfected cells. XbaI/ApaI genomic fragment from COL1A1 was inserted into the plasmid pTE1ΔpBR in positive (pTE1ΔpBRXA+) or negative (pTE1ΔpBRXA-) orientation.



**Figure 5.** The CAT activity in tissues of transgenic mice from line 2 carrying the ColCAT3.6 chimeric gene. 2 µg of protein from tissue extract was applied to each reaction. The calvarial sample in this experiment contained both central calvarial bone and its adjacent periosteum. In subsequent experiments, shown in Fig. 5 and Table I, calvarial bone, and periosteum were assayed for CAT as separate samples.

**Table II. CAT Activity in Tissues of Transgenic Mice Carrying ColCAT 3.6 Construct**

Line	Copy No.	Litter	n*	Calvaria		Tooth	Tend.	Skin	Liver	Brain
				Bone	Periost.					
1	6	Tg3	7	127 <sup>‡</sup>	nd <sup>§</sup>	387	59	nd	0	0
		Tg4	13	124	34	nd	nd	nd	nd	nd
2	15	Tg7	7	45	17	nd	11	2	0	0
		Tg12	7	63	nd	21	25	2	0	0

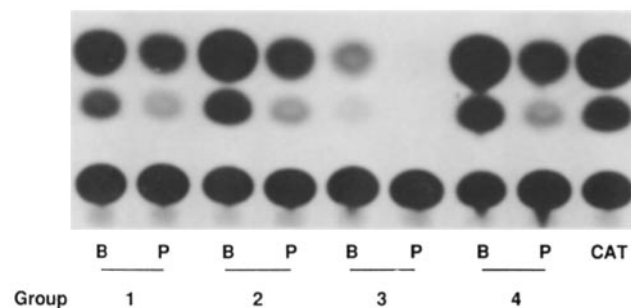
\* Number of animals per litter.

<sup>‡</sup> CAT activity is expressed as the percent of chloramphenicol conversion per 100 ng DNA and each number represents the mean value of measurements from individual animals or pools of animals. The litters from line 1 were from heterozygous transgenic parent and extracts from each tissue were pooled in one (Tg3) or four groups of three to four animals each (Tg4), with unknown ratio of transgenic versus wild type animals in each group. Both litters from line 2 were from homozygous transgenic parent and, therefore, contained only transgenic animals.

<sup>§</sup> Not done.

Although cultured bone cells provide a useful model system for analyzing tissue-specific expression, these cells are not fully differentiated osteoblasts and thus may not demonstrate the complete spectrum of gene regulation found in osteoblasts of an intact animal. Therefore, to examine the expression of the transgene in various animal tissues, two separate transgenic mouse lines were established carrying the ColCAT3.6 construct. A representative experiment comparing the CAT expression in various tissues of transgenic mice from line 2 is shown in Fig. 5. High levels of expression were found in COL1A1-producing tissues of whole calvaria, tooth germ, and tendon, while there was no detectable activity in liver and brain. Quantitative data from several experiments using both mouse strains are summarized in Table II. The high level of CAT expression was found in calvarial bone, tooth, and tendon, with significantly lower activity in skin and no activity in liver and brain.

Since osteoblasts of the rat calvariae produce three- to fourfold more collagen than adjacent periosteal tissue (Rowe and Kream, 1982), CAT activity in central bone osteoblasts, and periosteum was compared. A similar difference should also exist in the activity of the full-length ColCAT3.6 con-



**Figure 6.** The CAT activity in central calvarial bone and the adjacent periosteum of the transgenic mice line 2. An aliquot of the sample containing 0.4  $\mu$ g of DNA was assayed. The litter was randomly divided in four groups of three to four pups. Since the breeder was heterozygous for the CAT transgene, the proportion of transgenic animals in each pool varied, hence the differences in CAT expression between the groups. B, central bone; P, periosteum.

struct in the transgenic mice if the transgene contains the control elements responsible for osseous tissue-preferred transcription. As shown in Fig. 6 and Table II, the CAT expression in central bone from 5-d-old mouse calvariae from the transgenic lines 1 and 2 was three- to fourfold higher as compared to that in calvarial periosteum.

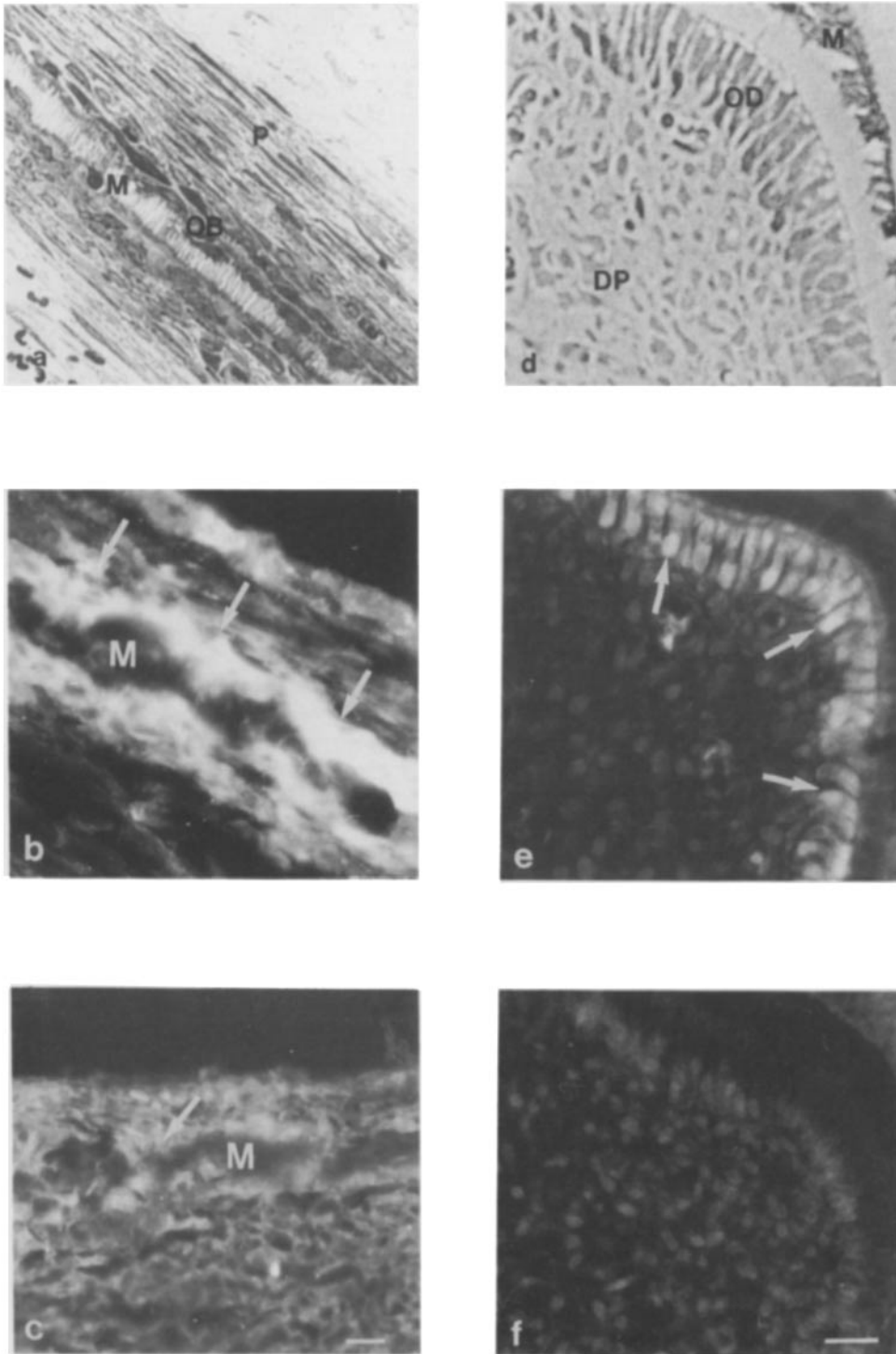
The differential expression of the ColCAT3.6 transgene in osseous versus fibroblastic cells was also detected in calvariae from the transgenic mouse line 2 stained with an antibody against CAT (Fig. 7). Although immunoreactivity was seen in periosteal fibroblasts, osteoblasts lining the newly formed osteoid surface demonstrated considerably higher staining intensity (Fig. 7 b). The developing tooth germ is a highly polarized structure composed of undifferentiated precursor cells of dental papilla and polygonal, highly differentiated odontoblasts which underlie the mineralizing dentin (Fig. 7 d). Immunostaining was considerably higher in odontoblasts as compared with the cells of the dental papilla (Fig. 7 e).

RNAse protection experiments were performed to ensure that the mRNA transcripts of the CAT gene are initiated at the proper start site. The cRNA probe used is expected to protect the fragments of 371 nt and 115 nt when hybridized to the transcripts of transfected chimeric gene and endogenous collagen gene, respectively. As shown in a representative experiment in Fig. 8, the transcripts of the ColCAT chimeric gene were correctly initiated in both stably transfected cell populations and in transgenic mice. Although both extracts contained equivalent amounts of collagen mRNA, the level of transgene mRNA was higher in transgenic animals than in transfected cells.

## Discussion

Our approach to identify *cis*-active elements which control type I collagen genes in bone was to characterize the activity of collagen promoter DNA constructs in bone-derived and fibroblastic cell lines. Multiple cell lines representative of osseous and nonosseous cells were examined because of the heterogeneity inherent to various lines. Subsequently, the ability of the longest promoter construct to confer differential regulation in low and high collagen producing cells was confirmed in intact tissues of transgenic animals.

The results of our functional analysis of deletion mutants of ColCAT3.6 construct suggest the presence of transcription stimulatory DNA sequences that are preferentially used by osteoblasts and odontoblasts and located between -3,521 and -1,672 bp in the rat COL1A1 promoter. In osteoblastic cell lines ROS17/2.8, Py-1a, and MC3T3-E1, a 7-25-fold difference in expression between the ColCAT3.6 and ColCAT2.3 constructs was observed (Figs. 2 and 3). The same deletions did not cause any changes in the promoter activity in NIH3T3 or Rat-1 cells and caused only twofold decrease in EL2 cells. This suggests that a sequence between -3,521 and -1,672 bp is active primarily in osteoblastic cells. The lack of the effect of deletions on promoter activity in NIH-3T3 and Rat-1 cells is consistent with the results from other laboratories. Transient transfection studies in NIH-3T3 fibroblasts (Rippe et al., 1989) revealed only negative regulatory elements upstream from -222 bp in the mouse COL1A1 promoter. Studies using stably transfected mouse fibroblasts showed that sequences upstream of -1,000 bp in the mouse



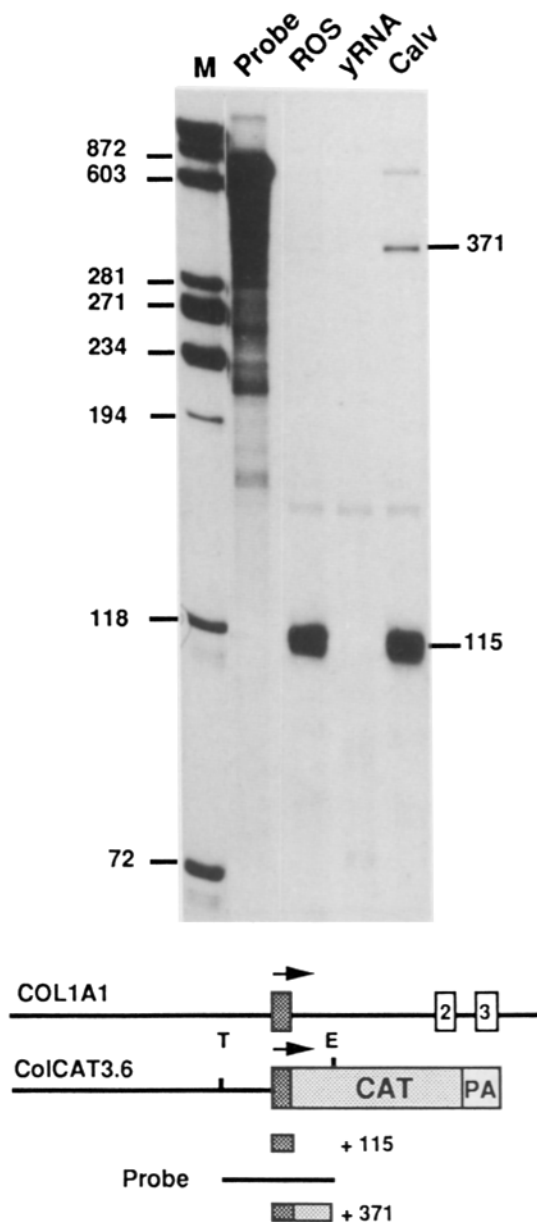
**Figure 7.** Visualization of CAT immunoreactivity in differentiated osteoblasts and odontoblasts from 5-d-old transgenic mice carrying the ColCAT-3.6 chimeric gene. (a and d) Light micrographs of a section through calvaria (a) and developing mandibular first molar (d). Osteoblasts (OB) and odontoblasts (OD) are observed adjacent to a layer of mineralized matrix (M). (P) Periosteum; (DP) dental papilla. (b and e) Immunofluorescence micrographs of similar sections from calvaria (b) and tooth (e). Specific immunoreactivity (arrows) is visible in osteoblasts and odontoblasts. (c and f) Negative controls (second antibody only) of similar sections demonstrating lack of specific immunoreactivity. Bar, 20  $\mu$ m.

COL1A1 promoter have no effect on transcription (Stacey et al., 1988). We decided to use stably transfected cell populations because our initial results from transiently transfected ROS17/2.8 and fibroblastic lines, although suggesting bone-preferred stimulatory sequences in the upstream promoter, were quite variable. We believe that the variability resulted from the competing requirements of rapidly dividing cells needed for high transfection efficiency and slower dividing higher density cells for expression of the osteoblastic phenotype (Owen et al., 1990). Once the permanently transfected

cell populations were established, transgene activity could be examined repeatedly, even after a cycle of cryopreservation, without a significant change in transgene activity.

The enhancing activity was distributed between the two distal deletion fragments in ROS 17/2.8, while in the other two osteoblastic cell lines it resided only within the most distal fragment. This may be a result of the presence of one or more enhancer element within the HindIII-PstI fragment (-2,295 to -1,672), which is active in ROS17/2.8 but not in any other of the cell lines we examined. We have evidence





**Figure 8.** RNase protection of endogenous COL1A1 mRNA and transgene mRNA. The 640 nt probe between the Taq I (T) and EcoRI (E) restriction sites was prepared and labeled as described in Materials and Methods. The entire reaction mixture containing 5  $\mu$ g of total cellular RNA was applied to the gel. The expected bands of 115 and 371 nt, corresponding to the protected fragments from the endogenous and transgene mRNAs respectively, were observed when the probe was hybridized to RNA from ROS 17/2.8 cells (ROS) stably transfected with the ColCAT3.6 hybrid gene and RNA from the calvaria (Calv) of the transgenic mice line 2, bearing the ColCAT3.6 gene. These bands were not present when the same probe was hybridized to total yeast RNA (yRNA). The 640-nt band, observed in this experiment in calvarial sample, may represent either undigested probe or a minor upstream transcript. The latter possibility is unlikely because this band has been an inconsistent finding in other experiments using this and other cell and transgenic lines. (M) DNA size markers; (2,3) COL1A1 exons; (PA) polyadenylation sequence.

that the HindIII-PstI fragment contains the domain that mediates the inhibitory effect of vitamin D on the COL1A1 promoter activity (Pavlin, D., A. C. Lichtler, J. Angily, B. E. Kream, and D. W. Rowe. 1989. *J. Bone Min. Res.* 4S:412). Analyses of other steroid hormone elements with down-regulating activity indicate that in the absence of the ligand, the element could act as a stimulator of the gene (Levine and Manley, 1989). Addition of the steroid hormone results in inhibition of the gene by repressing the element and reducing the activity to the baseline level. Therefore, the vitamin D response element may account for some of the enhancing activity of the HindIII-PstI fragment in ROS 17/2.8 cells.

The full-length ColCAT3.6 construct was expressed sevenfold higher in ROS 17/2.8 than in NIH-3T3 cells, despite the fact that the endogenous collagen synthesis was similar between the two cell types. This may be because the ColCAT3.6 lacks some of the sequences necessary for maximum expression in NIH 3T3 cells. The relative activity of the transgenes was reversed with the two shortest constructs, ColCAT1.7 and ColCAT0.9, in which the CAT activity was four- to fivefold lower in ROS 17/2.8 than in NIH-3T3 cells (Fig. 2). One possible explanation for this is that, although NIH-3T3 cells cannot use the upstream regulatory domains, they use promoter elements near the transcription start site more effectively than ROS 17/2.8 cells.

In transgenic mice the expression of the full-length ColCAT3.6 construct followed the expected distribution between the high and low collagen-producing tissues. CAT expression in the osteoblast-rich layer of bone was 20- to 30-fold higher than that in skin and three- to fourfold higher than that in adjacent periosteum (Table II). Not surprisingly, high CAT activity was present in extracts from molar tooth germs and tendon. The former is attributed to highly differentiated dentin-producing odontoblasts, which represent 10 to 20 percent of the cells in dental papillae of dissected tooth germs, and the latter to uniquely specialized tendon fibroblasts which produce large amounts of type I collagen. Accurate quantitation of CAT activity in the high collagen producing cells of calvaria and tooth germ, however, is not possible because they contain a mixture of osseous and fibroblastic cells. Similarly, skin is a mixture of dermal fibroblasts and noncollagen producing epithelial cells. However, an appreciation of the preferred expression of the ColCAT3.6 construct in osseous cells versus fibroblasts was revealed by immunohistochemistry (Fig. 7). The layer of differentiated osteoblasts lining the mineralized matrix of the calvarial bone was stained for CAT much stronger than the periosteal cells, confirming the higher level of type I collagen synthesis in osteoblasts compared to periosteum (Rowe and Kream, 1982). Similarly, two distinct cell populations were observed in tooth germs: a single layer of differentiated odontoblasts, actively engaged in type I collagen synthesis (Ruch, 1985), and the adjacent population of less active precursors within the dental papilla.

While the RNase protection experiments showed the correct transcriptional initiation of the chimeric gene promoter, the relative intensities of the 115- and 371-bp bands indicate that the level of mRNA accumulation from the transgene is significantly less than the endogenous gene. The basis for this difference is likely to be multifold and very important for a full understanding of the complexity of type I collagen regulation in specialized connective tissues. Candidate sites



that could account for the observed differences include: (a) The difference in stability of COL1A1 and transgene mRNAs; (b) the presence of regulatory sequences that increase basal activity, such as those described in the first intron (Choi et al., 1991), or locus control regions which confer copy number dependence of the transgene activity (Grosveld et al., 1987); (c) other tissue-specific sequences which are still absent from the test construct. Although the level of transgene mRNAs suggests that the full complement of regulatory elements could be missing from our construct, the CAT enzyme and immunofluorescence data clearly indicate that it reflects the microheterogeneity of expression in differentiated collagen producing cells.

The present results indicate subtle inconsistencies in the effect of promoter deletions depending on which osteoblastic cell line is used to express the construct (Figs. 2 and 3). This emphasizes the dependence of transfection studies on the cell type used. Although the osteoblast-like cell lines, such as ROS 17/2.8, Py-1a, and MC3T3-E1, are useful for mapping of *cis*-active DNA elements, the results should be verified in transgenic animals, since differences in transgene activity in these two systems were observed. For example, Fig. 8 demonstrates that the transgene is expressed significantly higher in calvarial bone of transgenic mouse than in ROS 17/2.8 cells when normalized to endogenous collagen mRNA. This observation may reflect differences of the transgene chromatin structure in transfected cells versus that in transgenic animals, since the latter has the same developmental history as the endogenous gene (Palmiter et al., 1991). Appreciating the differences between the cell culture and the transgenic animal model systems is essential before embarking on studies of the DNA response elements that control differentiation, hormonal regulation or tissue repair. The cell lines and transgenic mice that have been established as part of this work will be of great value to investigators interested in the fine control of the type I collagen gene in a differentiated cellular environment.

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