Separate *cis*-acting DNA Elements of the Mouse Pro-α1(I) Collagen Promoter Direct Expression of Reporter Genes to Different Type I Collagen-producing Cells in Transgenic Mice

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Abstract. The genes coding for the two type I collagen chains, which are active selectively in osteoblasts, odontoblasts, fibroblasts, and some mesenchymal cells, constitute good models for studying the mechanisms responsible for the cell-specific activity of genes which are expressed in a small number of discrete cell types. To test whether separate genetic elements could direct the activity of the mouse pro-α1(I) collagen gene to different cell types in which it is expressed, transgenic mice were generated harboring various fragments of the proximal promoter of this gene cloned upstream of the *Escherichia coli* β-galactosidase gene. During embryonic development, X-gal staining allows for the precise identification of the different cell types in which the β-galactosidase gene is active. Transgenic mice harboring 900 bp of the pro-α1(I) proximal promoter expressed the transgene at relatively low levels almost exclusively in skin. In mice containing 2.3 kb of this proximal promoter, the transgene was also expressed at high levels in osteoblasts and odontoblasts, but not in other type I collagen-producing cells. Transgenic mice harboring 3.2 kb of the proximal promoter showed an additional high level expression of the transgene in tendon and fascia fibroblasts. The pattern of expression of the *lacZ* transgene directed by the 0.9- and 2.3-kb pro-α1(I) proximal promoters was confirmed by using the firefly luciferase gene as a reporter gene. The pattern of expression of this transgene, which can be detected even when it is active at very low levels, paralleled that of the β-galactosidase gene. These data strongly suggest a modular arrangement of separate cell-specific *cis*-acting elements that can activate the mouse pro-α(I) collagen gene in different type I collagen-producing cells. At least three different types of cell-specific elements would be located in the first 3.2 kb of the promoter: (*a*) an element that confers low level expression in dermal fibroblasts; (*b*) a second that mediates high level expression in osteoblasts and odontoblasts; and (*c*) one responsible for high level expression in tendon and fascia fibroblasts. Our data also imply that other *cis*-acting cell-specific elements which direct activity of the gene to still other type I collagen-producing cells remain to be identified.

D IFFERENT mechanisms can be envisioned to account for the cell-specific activity of a gene that is expressed in a small number of discrete cell types. A first possible mechanism is that a single type of *cis*-acting element recognized by a unique cell-specific transcription factor be present in the different cell types that are actively expressing this gene. According to another hypothesis, separate *cis*-acting elements would be present in the regulatory segments of such genes and each of these would be recognized by distinct transcription factors specific for each of the cell types in which the gene is active. The genes for the type I collagen chains are good models for testing these possibilities. Indeed, these genes are active in several discrete cell types, such as osteoblasts, odontoblasts, and various types of fibroblasts and mesenchymal cells. In case the second hypothesis were true, it would also be important to begin to identify the individual elements as a prelude to the isolation of the various lineage-specific transcription factors which would bind to these elements.

Although several laboratories have generated transgenic mice harboring various regulatory segments of the human and rat pro-α1(I) collagen genes, the precise sequences responsible for the lineage-specific expression of this gene have not yet been defined. These previous exper-

iments used either a partially deleted human pro-α1(I) collagen minigene with a 2.3-kb promoter (Sokolov et al., 1993) or the same human 2.3-kb DNA promoter segment fused to a growth hormone reporter gene (Slack et al., 1991; Liska et al., 1994) or different lengths of the rat proximal promoter fused to the CAT gene (Pavlin et al., 1992; Bedalov et al., 1994; Bogdanovic et al., 1994). In mice harboring the human minigene, the levels of RNA and polypeptides that were specified by the minigene paralleled those corresponding to the endogenous gene in tail, bone, skin, muscle, heart, lung, intestine, kidney, and eye (Sokolov et al., 1993). Hence, this minigene should contain the sequences that were needed for approximately correct levels of expression in the tissues that were tested. In mice harboring the 2.3-kb human pro-α1(I) proximal promoter linked to the human growth hormone gene, a high degree of tissue-specific expression of the reporter was seen when various tissue extracts of 4–11-wk-old animals were tested (Slack et al., 1991). High levels of growth hormone RNA were found in bone, tail, and skin, and very low levels in liver, spleen, and thymus. Expression of the transgene was, however, not identical to that of the endogenous gene. Indeed, in later experiments using embryos from the same mice, in situ hybridization assays showed no expression of the transgene in perichondrium and in skeletal muscle (Liska et al., 1994), implying that additional sequences might be needed to obtain expression of the gene in all type I collagen-producing cells. In newborn mice carrying a 3.6-kb rat pro-α1(I) proximal promoter linked to the CAT gene, high levels of transgene expression were found in extracts of bone, tooth, and tendon; lower levels in skin; and no activity in liver and brain (Pavlin et al., 1992; Bogdanovic et al., 1994). In mice with a 2.3-kb rat promoter linked to the CAT gene, CAT enzyme levels in bone extracts were similar to those in mice with the 3.6-kb promoter, whereas CAT enzyme levels in tendons were two to four times lower than in mice carrying the 3.6-kb promoter (Bogdanovic et al., 1994). CAT levels in skin extracts were also substantially lower in mice harboring the 2.3-kb promoter than in mice with the 3.6-kb promoter (Bogdanovic et al., 1994). Also, almost no transgene expression was found in periosteal fibroblasts and aorta in mice harboring the 3.6-kb promoter (Pavlin et al., 1992; Bedalov et al., 1994). These experiments were consistent with the notion that additional elements are needed for activity of the transgene in all type I collagen-producing cells.

We have used various fragments of the proximal promoter of the mouse pro-α1(I) collagen gene cloned upstream of the *E. coli* β-galactosidase gene to generate transgenic mice and test the hypothesis of whether separate elements in intact embryos could direct activity of the transgene in different cell types in which the endogenous gene is expressed. The major advantage of using the β-galactosidase reporter is that X-gal staining of individual cells can be detected and, hence, the identity of cell types in which the transgene is active can be determined. Moreover, the timing at which the transgene is activated in specific cell types during mouse embryonic development can also be accurately determined and compared to the temporal and spatial activation of the endogenous gene. Our results indicate that distinct elements of the mouse pro-

α1(I) collagen gene promoter direct expression of a reporter gene to different type I collagen-producing cells during mouse embryonic development.

Materials and Methods

Plasmid Constructions

Two expression vectors were used: placF contains the *lacZ* reporter gene, whereas pA3luc contains the firefly luciferase gene. In placF the *lacZ* gene is cloned upstream of the sequence of the mouse protamine gene between +95 and +625, which supplies an intron and a polyadenylation signal (Mercer et al., 1991). In pA3luc the firefly luciferase gene is cloned upstream of an SV40 splice site and polyadenylation signal. Moreover, a polyadenylation cassette has been cloned upstream of the luciferase gene to prevent read-through transcription in transfection experiments (Wood et al., 1989). Before inserting fragment of the pro-α1(I) collagen gene proximal promoter in these vectors, new unique restriction sites were introduced in the polylinkers located upstream of the *lacZ* gene and downstream of the mouse protamine gene in placF, by standard recombinant techniques (placH). New unique restriction sites were also introduced in the polylinker located between the polyadenylation cassette and the firefly luciferase gene in pA3luc (pluc4).

Three segments of the proximal promoter of the mouse pro-α1(I) collagen gene were used: a 3,260-bp fragment extending from a KpnI site located 3,150 bp upstream of the start-site of transcription to an XbaI site located 110 bp downstream of the start site of transcription, just 5′ of the translation initiation codon; a 2,420-bp fragment extending from a HindIII site located 2,310 bp upstream of the start-site of transcription to the XbaI site; and a 1,015-bp fragment extending from a PvuII site located 905 bp upstream of the start-site of transcription to the XbaI site. The 3,260-bp fragment was derived from pI (Harbers et al., 1984), whereas the 2,420- and the 1,015-bp fragments were derived from pG70 (Karsenty et al., 1990). Each of these three fragments was cloned in the polylinker region of placH (3200*lacZ*, 2300*lacZ*, and 900*lacZ*, respectively), and of pluc4 (3200lucif, 2300lucif, and 900lucif, respectively), by standard recombinant techniques. A 2,954-bp matrix attachment region of the chicken lysozyme locus (Phi-Van and Strätling, 1988; McKnight et al., 1992) has been cloned upstream of the pro-α(I) promoter fragment in 2300*lacZ*, 900*lacZ*, 2300lucif, and 900lucif (2300*lacZ*MAR, 900*lacZ*MAR, 2300lucifMAR, 900lucifMAR, respectively) by standard recombinant techniques.

Generation of Transgenic Mice

Before injection, the constructs were digested with restriction enzymes cutting immediately downstream of the polyadenylation signal and upstream of either the pro-α1(I) collagen gene sequences or the chicken lysozyme matrix attachment region locus. The inserts were separated from the vectors by electrophoresis on agarose gel, and recovered by electroelution. The solution containing the DNA was extracted twice with phenol/chloroform/isoamylalcohol, and once with chloroform/isoamylalcohol. After ethanol precipitation and two 70% ethanol washes, the DNA was redissolved in 5 mM Tris-HCl (pH 7.4), 0.1 mM EDTA (pH 8.0), 5 mM NaCl. When *lacZ* constructs were injected alone, the DNA was dissolved at a concentration of 2 ng/μl. When both *lacZ* containing and luciferase gene containing constructs were coinjected, each DNA was dissolved at a concentration of 1.5 ng/μl. DNAs were injected into pronuclei of mouse $B_6 \times D_2F_2$ fertilized eggs by standard procedures (Hogan et al., 1986). The injected eggs were then transferred into CD1 foster mothers. The integration of the transgene(s) in the genome of founder mice was assessed by Southern blot analysis of either tail DNA or placenta DNA. 12 mice out of a total of 37 mice which were positive by Southern blot analysis showed no X-gal staining in whole embryos and had no luciferase activity.

Southern Blot Analysis

Genomic DNA was isolated from tail or placenta by standard procedures (Sambrook et al., 1989). 15 μg of DNA were digested overnight with EcoRI and XbaI. The DNA was then separated by electrophoresis on a 0.8% agarose gel. After denaturation, the DNA fragments were transferred to a nylon membrane (Zeta-Probe GT; Bio-Rad Laboratories, Hercules, CA), which was hybridized overnight with both a *lacZ* and a luciferase DNA probe. The filter was then washed in 2 × SSC, 0.1% SDS (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The probes used

were a 3-kb *lacZ* fragment (obtained by digestion of pLacH with EcoRI), and a 1.1-kb luciferase fragment (obtained by digestion of pluc4 with EcoRI and XbaI). Each probe was radiolabeled with [^{32}P]αdCTP using a random primed DNA labeling kit (Boehringer Mannheim, Mannheim, Germany).

β-Galactosidase Expression

β-galactosidase activity was assessed on embryos and newborn mice as previously described (Mercer et al., 1991). Briefly, embryos were fixed for 30–40 min in 0.1 M NaPO$_4$ (pH 7.3), 2% formalin, 0.2% glutaraldehyde, 5 mM EGTA, and 2 mM MgCl$_2$. After washing, they were stained overnight with X-gal (1 mg/ml) in PBS containing 2 mM MgCl$_2$, 4 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.2% (vol/vol) NP-40, and 0.1% sodium deoxycholate. For embryos older than 16.5 d post conception (p.c.),[1] the skin was removed before fixation. After staining, the embryos were washed in PBS and photographed. Some 18.5 d p.c. embryos were partially clarified in potassium hydroxide (McLeod, 1980) before being photographed. For histology, embryos were dehydrated in ethanol, and embedded in paraffin. 8-μm-thick sections were sliced on a microtome and counterstained with eosin.

Luciferase Expression

Luciferase activity was assessed in 4-wk-old mice. Organs were homogenized in 500 μl of solution A containing 0.1 M KPO$_4$ (pH 7.8), 1 mM DTT,

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1. *Abbreviation used in this paper:* p.c., post conception.

and then submitted to a freeze/thaw procedure. After centrifugation, 100 μl of the supernatant were diluted with 250 μl of solution B containing 0.1 M KPO$_4$ (pH 7.8), 15 mM MgSO$_4$, 5 mM ATP, 1 mM DTT. The luciferase activity was measured in this 350-μl sample in the presence of an excess of D luciferin (0.07 mg/ml) by using a luminometer (Monolight-2010; Analytical Luminescence Laboratory, San Diego, CA). Protein levels were measured by the Bradford assay (Bradford et al., 1976). Results were expressed as luciferase light units per 1 mg of protein. The amounts of protein contained in tissue samples homogenized in solution A were slightly variable from one experiment to the other, but average amounts (mean ± SEM) were: 4.5 ± 0.2 mg/ml for tail, 6.2 ± 0.4 mg/ml for bone, 0.07 ± 0.01 mg/ml for tooth, 3.4 ± 0.2 mg/ml for skin, 0.8 ± 0.08 mg/ml for tendon, 20.8 ± 3.8 mg/ml for muscle, 28.3 ± 4.6 mg/ml for heart, 7.7 ± 0.6 mg/ml for lung, 10.1 ± 0.7 mg/ml for peritoneum, 1.0 ± 0.2 mg/ml for aorta, 7.5 ± 0.7 mg/ml for intestine, 57.4 ± 4.4 mg/ml for liver, 29.6 ± 5.3 mg/lg for spleen, 41.2 ± 4.1 mg/ml for kidney, 4.9 ± 0.8 mg/ml for thymus, 28.8 ± 4.1 mg/ml for brain. Background values, obtained by measuring 100 μl of buffer A diluted to 350 μl with buffer B were comprised between 130 and 240 light units (177 ± 11). Raw values less than 100 light units above background, were considered as not significantly different from background, and reported as undetectable.

Results

Generation of Transgenic Mice

Transgenic mice were generated by microinjecting the following constructs (Fig. 1) into fertilized oocytes: (*a*) si-
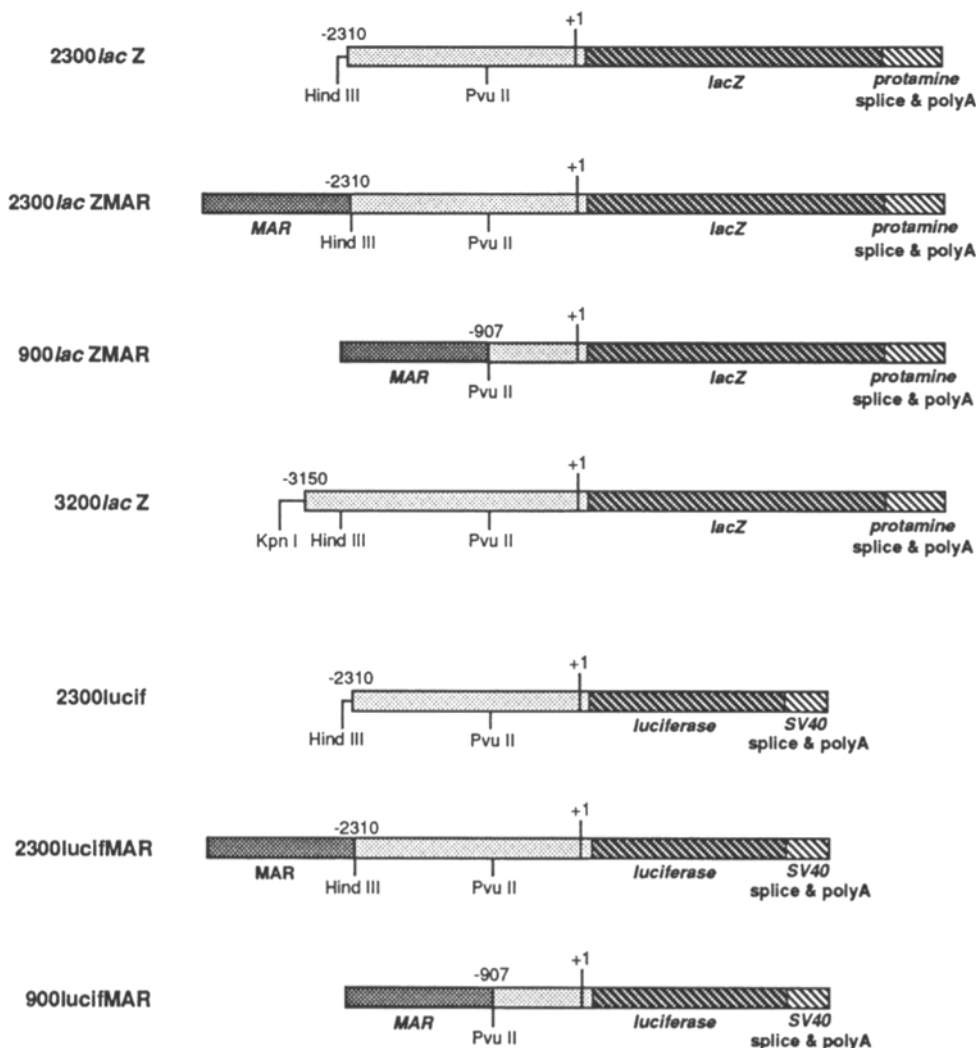

Figure 1. Schematic representation of DNA constructs used to generate transgenic mice. See Materials and Methods for details. *MAR,* matrix attachment region of the chicken lysozyme gene.

multaneous microinjection of 2300lacZ and 2300lucif; (b) simultaneous microinjection of 2300lacZMAR and 2300lucifMAR; (c) simultaneous microinjection of 900lacZMAR and 900lucifMAR; and (d) 3200lacZ. For each construct, Southern blot analyses identified the mice that had integrated the transgene(s) and the approximate number of copies of each transgene. Table I shows the number of copies of the transgenes, in all founder mice or lines expressing the transgene(s), as determined by either X-gal staining or measurement of luciferase activity.

The histochemical stain for β-galactosidase, X-gal, provides considerable detail about the type of cells in which the cloned regulatory sequences are active and about the stage of embryonic development at which these sequences become active. The luciferase reporter provides a very sensitive assay that allows quantitative determination of the product of the transgene in various tissue extracts. It was used to confirm that the absence of X-gal staining in some cell types was not the results of a lack of sensitivity of the X-gal assay.

Transgenic Mice for 2300lacZ, 2300lucif, 2300lacZ MAR, and 2300lucifMAR

We first used 2.3 kb of the proximal promoter of the mouse pro-α1(I) collagen gene cloned upstream of either the lacZ gene or the luciferase gene (2300lacZ and 2300-lucif, respectively) to generate transgenic mice. Seven transgenic mouse lines that expressed either β-galactosidase or luciferase were generated (Table I). Among these, four lines (lines 1.33, 1.35, 1.46, 1.49) had integrated both transgenes; two (lines 1.2, 1.13) had integrated only 2300-lacZ; and one (line 1.50) integrated only 2300lucif.

In all six lines expressing β-galactosidase, an identical pattern of X-gal staining was observed during embryonic development. No β-galactosidase activity could be detected until 13 d p.c. (Fig. 2 A). Thereafter, starting between 13 and 14 d p.c., a very close spatial and temporal correlation was observed between the expression of the transgene (Fig. 2, B–D) and bone ossification determined by alizarin staining (Kaufman, 1992). Histological sections of embryos at various times of development confirmed the

close spatial and temporal correlation between expression of the transgene and osteoblast differentiation (Fig. 2, E–I). Whereas osteoblasts were stained by X-gal (Fig. 2, E–H), no staining was seen in chondrocytes (Fig. 2, E, G, and I), condensed mesenchymal cells (data not shown), perichondrial or periosteal fibroblasts (Fig. 2, E, F, H, and I). In addition to osteoblast staining, histological sections performed either during the last four days of embryonic development or in newborn mice also showed a strong expression of the transgene in odontoblasts (Fig. 2 J), which synthesize high levels of type I collagen. Other dental cells that do not synthesize type I collagen, such as ameloblasts, were not stained (Fig. 2 J). Expression of the transgene in odontoblasts was also temporally regulated in parallel to expression of the endogenous gene. For example, staining of odontoblasts was detected at 16.5 d p.c. in incisors, but only 3 d later in first molars, which parallels data obtained by in situ hybridization (Schwarz et al., 1990). Finally, faint X-gal staining of the skin appeared at 16.5 d p.c. in whole-mount embryos (data not shown). This staining was, however, too faint to be detected by histology. There was no staining of the other fibroblast-rich tissues in any of the six lines, both in whole embryo and in histological sections. For example, connective tissue fascia (Fig. 2 H), tendons (Fig. 2 I), muscle fibroblasts (Fig. 2, E and F), heart valves, or organ capsules (data not shown) were all completely negative, whereas in situ hybridization using a pro-α1(I) probe showed a strong signal in these tissues (K. Niederreither, R. D'Souza, M. Metsaranta, H. Eberspaecher, E. Vuorio, B. de Crombrugghe, manuscript in preparation). With the exception of line 1.49, which showed some faint staining in the brain (data not shown), there was no promiscuous expression of the lacZ gene in tissues or cells not producing type I collagen. We conclude from these experiments that the 2,300-bp pro-α1(I) collagen proximal promoter was highly active in osteoblasts and odontoblasts, faintly active in skin, and inactive in other cell types including fibroblasts of other tissues.

In tissue extracts of five lines expressing the luciferase transgene, enzyme activity was measured in one-month-old pups (Table II). Despite the sensitivity of this assay, there was no or almost no luciferase activity in tissues

Table I. Copy Number of the Transgenes in Mouse Lines or Founder Mice Expressing β-Galactosidase and/or Luciferase

| Construct | Line | Copy no. | | Construct | Line | Copy no. | |
		lacZ	Luciferase			lacZ	Luciferase
2300lacZ	1.2	3–5	0	900lacZMar	2.10	5–7	1–3
2300lucif	1.13	15–20	0	900lucifMAR	2.13	8–10	0
	1.33	10–15	1–3		2.15	1–3	5–7
	1.35	10–15	3–5		2.23	5–7	0
	1.46	1–3	1–3		2.26	3–5	5–7
	1.49	3–5	3–5		2.30	8–10	8–10
	1.50	0	1–3		2.31	3–5	1–3
2300lacZMAR	7.9	3–5	3–5	3200lacZ	5.1*	3–5	
2300lucifMAR	7.14	1–3	1–3		5.2*	5–7	
	7.17	5–7	0		5.14*	10–15	
	7.32	0	1–3		5.30	8–10	
					5.41	1–3	
					5.43	8–10	
					5.54	10–15	

* Founder was sacrificed.

known to express type I collagen but negative by X-gal staining such as muscle, heart, lung, peritoneum, aorta, or intestine (less than 0.1% of the luciferase activity in tail). The only exception was a very low luciferase activity in the tendons of the tail in four of five lines. This activity was 100–500× lower than the luciferase activity detected in complete tails and could be a result of a low level of contamination by osteoblasts of tail vertebrae. Indeed, we never detected any X-gal staining of tendon fibroblasts in mice harboring a 2,300-bp pro-α1(I) collagen promoter. As expected from the pattern of expression of the β-galactosidase gene, high levels of luciferase activity were observed in bone, and in teeth. In all lines there was also a low level of luciferase activity in skin. Although this skin value was low compared to the luciferase value in bone, it was always at least 20× higher than the background luciferase values.

The expression of the luciferase gene was highly tissue specific. In four lines (1.33, 1.35, 1.46, 1.49), no or almost no luciferase activity could be detected in organs such as liver, spleen, kidney, and thymus, which are known to contain very little type I collagen RNA (Slack et al., 1991; Goldberg et al., 1992). Line 1.50 displayed some luciferase activity in these organs, but it was always very low, more than 5,000× lower than in tail. In all five lines, some luciferase could be detected in the brain, but it too was always extremely low. The differences in absolute luciferase values between the different mouse lines are probably due to differences in sites of integration of the transgene. Despite these differences the pattern of expression of the transgene is remarkably similar in the different lines.

In conclusion, the very sensitive luciferase measurements in 1-mo-old animals were in complete agreement with the results of X-gal staining and confirmed that the pattern of expression of the transgene was much more restricted than that of the endogenous gene.

The so-called matrix attachment region of the chicken lysozyme locus has previously been shown to increase the proportion of mice expressing a transgene, and to allow a more faithful developmental regulation of the transgene

(McKnight et al., 1992). To test whether this DNA segment might also cause expression of the transgene in type I collagen-producing cells other than osteoblasts and odontoblasts, we cloned the chicken lysozyme gene matrix attachment region upstream of the promoter of the pro-α1(I) collagen gene both in 2300*lacZ* and in 2300lucif. Because the copies of the transgene mostly integrate in the genome as tandem repeats in a head-to-tail configuration, we reasoned that all transgene copies, with the possible exception of the most 3' or 5' ones, would be separated from neighboring sequences in the genome by a matrix-attachment region. Four lines were generated that expressed β-galactosidase and/or luciferase (Table I). Two lines harbored both transgenes, one contained only 2300-*lacZ*MAR, and one contained only 2300lucifMAR.

In all cases, the pattern of expression of the *lacZ* gene was identical to the one described for 2300*lacZ* (data not shown). The transgene was expressed at high levels only by osteoblasts and odontoblasts. There was faint skin staining visible at 16.5 d p.c. No X-gal staining was detected in other tissues, including connective tissue fascia, tendons, heart valves, organ capsules, blood vessels, or muscle fibroblasts. The temporal expression of the transgene during embryonic development was identical to the one described for the lines expressing 2300*lacZ*, which did not contain the chicken lysozyme gene matrix-attachment region.

The pattern of expression of the luciferase gene was also similar to the one described for mice harboring 2300lucif (Table III). It was expressed at high levels in bone and teeth and at lower levels in skin. Although the skin luciferase values were generally higher in the three lines containing 2300lucifMAR than in the lines of Table II, these differences are not statistically significant. It was also not or almost not expressed in muscle, heart, lung, peritoneum, aorta, and intestine (at least 1,000× less than in tail). There was little or no luciferase activity in liver, spleen, kidney, thymus, and brain.

We conclude from these experiments that the presence of the matrix-attachment region from the chicken lyso-

Table II. Luciferase Activity in Tissue Extracts of One-Month-Old Transgenic Mice Harboring 2300lucif

	Line 1.33	Line 1.35	Line 1.46	Line 1.49	Line 1.50
Tail	1042 (100)*	9525 (100)	110 (100)	1430 (100)	11783 (100)
Bone	1970 (189)	10198 (107)	163 (147)	1725 (121)	14271 (121)
Tooth	1732 (166)	18621 (195)	94 (85)	2043 (143)	10478 (89)
Skin	7 (0.7)	48 (0.5)	0.8 (0.7)	42 (3)	957 (8)
Tendon	4 (0.4)	18 (0.2)	und‡	3 (0.2)	123 (1)
Muscle	und	und	und	und	7 (0.06)
Heart	0.02	und	und	und	3 (0.02)
Lung	0.1	und	und	und	0.7
Peritoneum	und	0.5	und	und	8 (0.07)
Aorta	1 (0.1)	und	und	und	6 (0.05)
Intestine	und	0.6	und	und	0.2
Liver	und	und	und	und	0.08
Spleen	und	und	und	und	1
Kidney	und	und	und	und	0.2
Thymus	0.2 (0.03)	und	und	und	2 (0.02)
Brain	1 (0.1)	0.1	0.3 (0.3)	0.1	7 (0.06)

* Luciferase activities are expressed as 10^3 light units/mg protein. Values in parentheses represent percent of the luciferase activity in tail; percent values below 0.01% are not indicated.
‡ und, undetectable (i.e., not significantly different from values obtained with buffer alone).
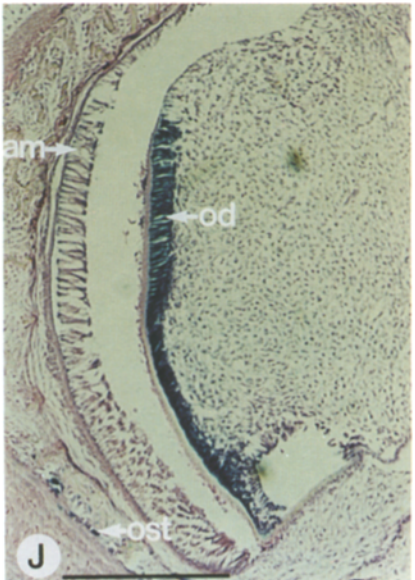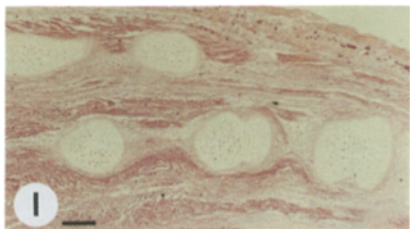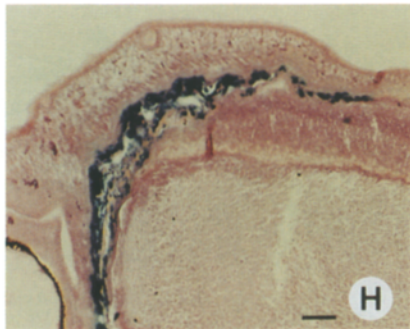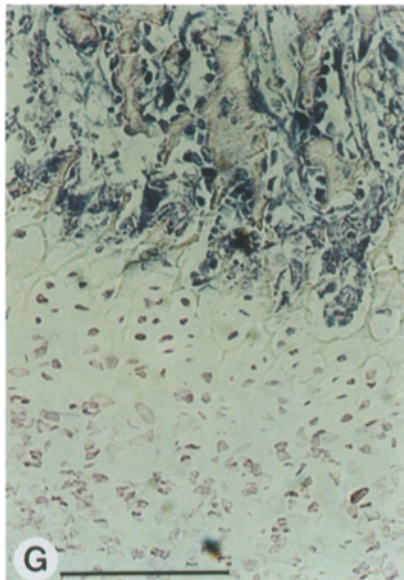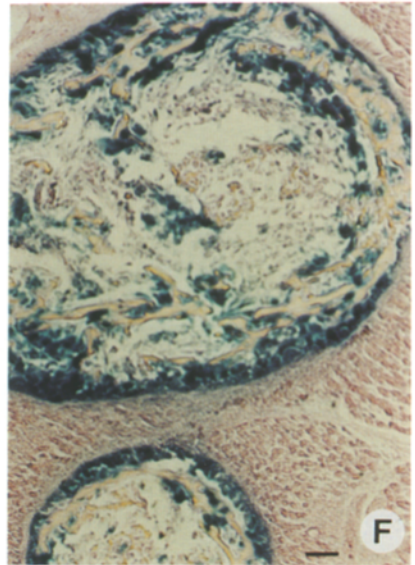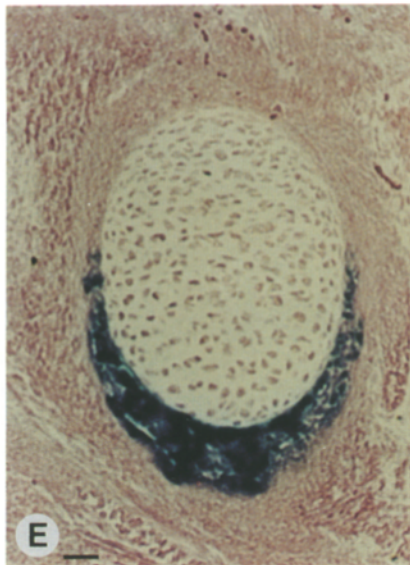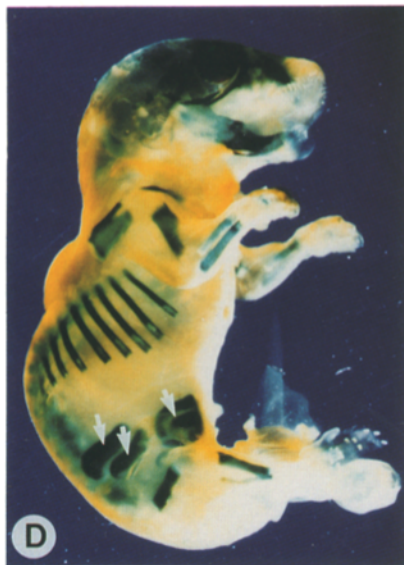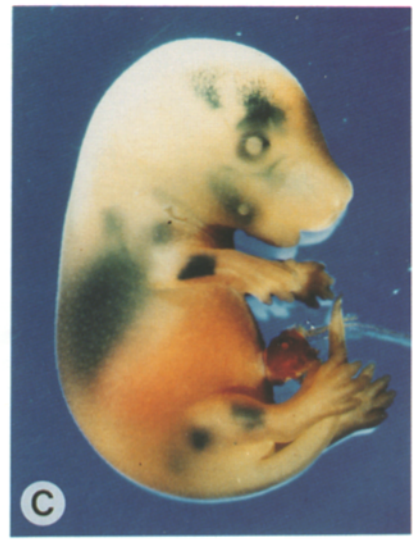
zyme gene had practically no influence on the pattern of expression of the transgene in different tissues and did not cause activation of the transgene in fibroblastic or mesenchymal cells.

Transgenic Mice for 900lacZMAR and 900lucifMAR

To further delineate the region of the pro-α1(I) promoter responsible for the pattern of expression described above, we generated transgenic mice harboring only 905 bp of the proximal pro-α1(I) promoter cloned upstream of either the *lacZ* or the luciferase genes. In each construction, the matrix attachment region of the chicken lysozyme gene was cloned upstream of the pro-α1(I) promoter. Five transgenic lines expressed both transgenes, and two expressed only 900*lacZ*MAR (Table I).

No X-gal staining was detected in lines 2.13, 2.23, 2.26, 2.30, and 2.31 before 16.5 d p.c. (data not shown). At 16.5 d p.c., a faint skin staining similar to the one observed with 2300*lacZ* and 2300*lacZ*MAR was detected in whole-mount embryos (Fig. 3, *A* and *B*). This faint X-gal staining could not, however, be detected by light microscopy after histological sectioning. No osteoblast or odontoblast staining was detected either in whole-mount embryos or by histology.

As shown in Table IV, three lines (2.26, 2.30, 2.31) displayed a similar tissue pattern of luciferase expression. The transgene was expressed in skin whereas very low levels of luciferase activity were found in bone. The raw values in skin were at least 400× higher than background values ($P = 0.01$). Low levels of luciferase were found in the tendons of the tail, and no or almost no activity was found in organs such as muscle, heart, lung, peritoneum, aorta, or intestine (at least 1,000× less than in tail). There was also almost no luciferase activity in organs expressing the pro-α1(I) gene at very low levels such as liver, spleen, kidney, thymus, or brain.

Two of the lines that contained both β-galactosidase and luciferase transgenes (lines 2.10 and 2.15) showed widely promiscuous expression of these reporter genes (data not shown). Hence, the presence of the matrix attachment region DNA segment in the transgenic constructions did not prevent promiscuous expression of the transgene.

It thus appears that the 905-bp segment of the proximal pro-α1(I) promoter was able to direct tissue-specific expression of the transgenes. Indeed, both transgenes were expressed in skin but not in bone or teeth. Together with the pattern of expression of mice carrying a 2,300-bp promoter fragment, these results suggest that different regions of the pro-α1(I) promoter can direct expression of reporter genes in osteoblasts and in skin fibroblasts.

Table III. Luciferase Activity in Tissue Extracts of One-Month-Old Transgenic Mice Harboring 2300lucifMAR

	Line 7.9	Line 7.14	Line 7.32
Tail	408 (100)*	1437 (100)	11136 (100)
Bone	654 (160)	2440 (170)	46859 (420)
Tooth	1021 (250)	2808 (195)	31175 (280)
Skin	143 (35)	273 (19)	612 (5)
Tendon	7 (2)	69 (5)	133 (1)
Muscle	und‡	und	4 (0.03)
Heart	und	0.06	2 (0.02)
Lung	und	und	und
Peritoneum	und	und	2 (0.02)
Aorta	und	1 (0.07)	9 (0.08)
Intestine	0.6 (0.1)	und	1
Liver	und	und	und
Spleen	und	und	0.1
Kidney	0.02	und	0.04
Thymus	1 (0.2)	und	4 (0.03)
Brain	0.2 (0.05)	0.4 (0.03)	3 (0.03)

* Luciferase activities are expressed as 103 light units/mg protein. Values in parentheses represent percent of the luciferase activity in tail; percent values below 0.01% are not indicated.

‡ und, undetectable (i.e., not significantly different from values obtained with buffer alone).

Transgenic Mice for 3200lacZ

To determine if additional tissue-specific elements were located upstream of the first 2.3 kb of the promoter, we cloned 3150 bp of the promoter in the *lacZ* vector (3200-*lacZ*) to generate transgenic mice, extending the promoter by 850 bp. Seven transgenic mice expressing β-galactosidase were obtained (Table I). Three were sacrificed and stained at 15.5 d of embryonic development, and four were used to generate transgenic lines.

As for mice harboring 2,300 bp of the pro-α1(I) proximal promoter, we observed X-gal staining of ossification centers in whole-mount embryos in all cases (Fig. 4, *A*, *C*, and *D*). This staining could be detected after a shorter incubation with X-gal than for the lines transgenic for 2300*lacZ*, and 2300*lacZ*MAR (a few hours compared to overnight). Histological sections confirmed osteoblast staining (Fig. 4, *E–G*) and also showed odontoblast staining during the last four days of embryonic development (data not shown). The staining of these tissues was more intense than for the mice harboring only 2,300 bp of the promoter (compare Fig. 4 *F* with Fig. 2 *F*) suggesting the presence of an enhancing element which increases expression in osteoblasts and odontoblasts located between −2,300 and −3,200 bp. There was also a faint skin staining in whole-mount embryos, appearing at 16.5 d p.c. (data not shown), but as for the other constructs, this skin staining could not be detected by histology.

Figure 2. Expression of the *lacZ* gene in embryos or newborn mice transgenic for 2300*lacZ*, as indicated by blue X-gal stain. (*A*) Absence of X-gal staining in a 13 d p.c. whole-mount embryo (line 1.35). (*B–D*) 14.5, 15.5, 18.5 d p.c. whole-mount embryos, respectively, showing staining of ossification centers (line 1.35); in (*D*), the embryo has been partially clarified with potassium hydroxide after X-gal staining; staining of intestine (*arrows*) in (*D*) is due to endogenous β-galactosidase. (*E*) Transverse section of a forelimb at 14.5 d p.c. showing expression of the *lacZ* gene in osteoblasts, but not in chondrocytes, perichondrial and muscle fibroblasts (line 1.13). (*F*) Transverse section of a hindlimb at 16.5 d p.c. showing X-gal staining of osteoblasts but not of muscle fibroblasts (line 1.35). (*G*) Longitudinal section of a growth plate at birth, with staining restricted to osteoblasts (line 1.35). (*H*) Sagittal section of a head at 15.5 d p.c. showing staining of osteoblasts (line 1.2). (*I*) On a sagittal section of tail at 16.5 d p.c. (line 1.49), there is no staining of chondrocytes, perichondrial fibroblasts, or tendons fibroblasts. (*J*) Longitudinal section of an upper incisor at birth (line 1.35) showing staining of odontoblasts (*od*) and of mandibular osteoblasts (*ost*), but not of ameloblasts (*am*). Bars, 200 μm.
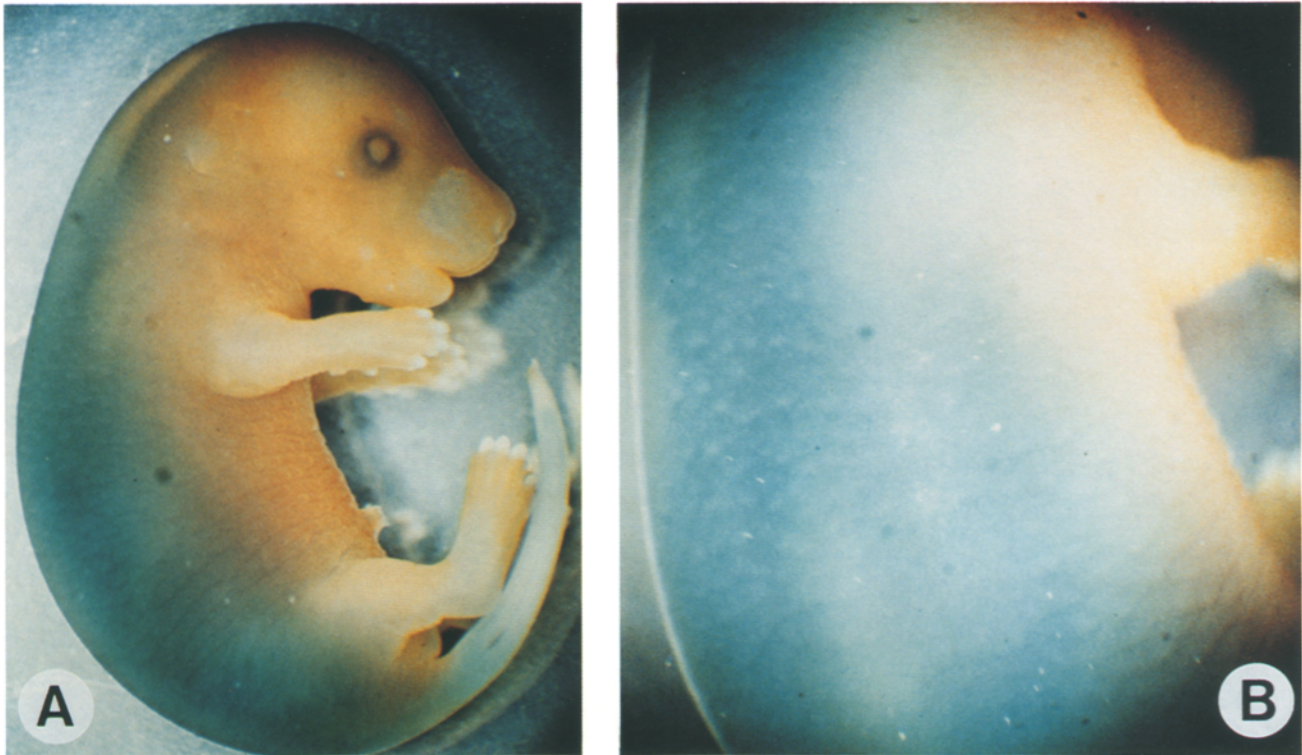
Figure 3. (*A*) β-galactosidase staining of a 16.5 d p.c. whole-mount embryo transgenic for 900*lacZ* (line 2.26). Blue staining, which indicates expression of the transgene, is faint and restricted to the skin. (*B*) Higher magnification of the skin.

In addition to this expected pattern of expression of the transgene, and as suspected from whole-mount embryos (Fig. 4, *A–D*), histological sections showed a staining of ligaments and tendons in all cases (Fig. 4, *G–I*). This staining was detected beginning at 14.5 d p.c. in tendons of the limbs (Fig. 4 *A*) and two days later in tendons of the tail (Fig. 4 *B*). Finally, histological sections showed staining of fibroblasts forming the connective tissue fascia (Fig. 4, *E* and *J*), appearing at 13 d p.c. No X-gal staining was seen, however, in tissues like organ capsules (Fig. 4 *J*), lung (Fig. 4 *J*), muscle fibroblasts (Fig. 4 *F*), or cardiac valves. There was also no staining of the perichondrium (Fig. 4, *G–I*). These data suggest that an element located between 2,300 and 3,200 bp upstream of the start site of transcription is important for activating expression of the *lacZ* transgene specifically in tendon and fascia fibroblasts.

Discussion

The experiments presented here strongly suggest the notion that different *cis*-acting cell-specific elements exist in the pro-α1(I) collagen promoter which control the expression of this gene in different type I collagen-producing cells during embryonic development. This model is based on the high degree of specificity of X-gal staining, which allows clear identification of the specific cell types in which the transgene is active, and on identical spatial and temporal patterns of X-gal staining at various stages during embryonic development in several mice strains for each construction. The very sensitive measurements of lu-

ciferase enzyme activities assayed in extracts of tissues on one-month-old transgenic mice supported similar conclusions.

Transgenic mice harboring 900 bp of the pro-α1(I) proximal promoter expressed low levels of the *lacZ* and lu-

Table IV. Luciferase Activity in Tissue Extracts of One-Month-Old Transgenic Mice Harboring 900lucifMAR

	Line 2.26	Line 2.30	Line 2.31
Tail	209 (100)*	101 (100)	85 (100)
Bone‡	1 (0.5)	2 (2)	1 (1)
Tooth	und§	und	und
Skin	632 (302)	384 (384)	181 (213)
Tendon‡	1 (0.5)	14 (14)	8 (9)
Muscle	und	und	0.1 (0.1)
Heart	und	und	und
Lung	0.3 (0.1)	und	und
Peritoneum‡	und	0.1 (0.1)	0.1 (0.1)
Aorta	und	und	und
Intestine	und	und	und
Liver‡	0.05 (0.02)	0.05 (0.05)	0.03 (0.03)
Spleen	und	und	und
Kidney	und	0.02 (0.02)	und
Thymus‡	0.3 (0.1)	0.4 (0.4)	und
Brain‡	0.1 (0.05)	0.07 (0.07)	2 (2)

* Luciferase activities are expressed as 10^3 light units/mg protein. Values in parentheses represent percent of the luciferase activity in tail.
‡ Values statistically significantly lower than skin values ($P < 0.05$, with unpaired Student's t test).
§ und, undetectable (i.e., not significantly different from values obtained with buffer alone).

ciferase reporter genes almost exclusively in skin. Transgenic mice containing 2.3 kb of the proximal promoter expressed the transgene at high levels in osteoblasts and odontoblasts, at low levels in skin, but not in other type I collagen-producing cells. In these mice, activation of the transgene in osteoblasts started around day 13.5 p.c. and coincided with the developmental appearance of osteoblasts in various ossification centers. Similarly, activation of the transgene in odontoblasts followed the same developmental pattern as that of odontoblasts themselves during the formation of different teeth organs. Transgenic mice harboring 3.2 kb of the pro-α1(I) proximal promoter expressed the *lacZ* reporter gene at high levels in osteoblasts and odontoblasts as well as in tendon and fascia fibroblasts and at low levels in skin (see Fig. 5). Taken together, these data strongly suggest a modular arrangement of separate cell-specific *cis*-acting elements that can activate the pro-α1(I) collagen gene in different type I collagen-producing cells. At least three different types of cell-specific elements would be located in the first 3.2 kb of the pro-α1(I) promoter: (*a*) an element the confers a low level expression of the transgene in dermal fibroblasts, located in the proximal 900 bp of the promoter; (*b*) an element that mediates high level expression of the gene in osteoblasts and odontoblasts, located between −2,300 and −900 bp; and (*c*) an element needed for high level expression of the gene in tendon and fascia fibroblasts, located between −3,200 and −2,300 bp.

We have recently further delineated the osteoblast element to a much smaller 120-bp fragment which in conjunction with a 220-bp proα1(I) promoter confers high level osteoblast specificity (J. Rossert, unpublished observation). The segment between −3,200 and −2,300 bp also appears to increase promoter activity in osteoblasts and odontoblasts above the level observed with the −2,300 bp promoter. This could be due to the presence of an additional osteoblast/odontoblast-specific element in this sequence. Alternatively, since the tendon enhancer might be composed of both DNA binding sites for ubiquitous transcription factors and for a tendon-specific element, it is possible that the activity of the osteoblast/odontoblast element between −900 and −2,300 bp could be increased by ubiquitous factors binding more upstream.

Our results could also be consistent with the existence of elements within the 2.3-kb proximal promoter that would cooperate with the tendon element between −3.2 and −2.3 kb to confer high level transgene activity in tendon fibroblasts. Our results suggest that if such elements exist within the 2.3-kb segment, they are unable by themselves to direct expression in tendon fibroblasts.

Our data further suggest that still other *cis*-acting cell-specific elements remain to be identified. For example, in none of our mice was there expression of the transgenes in perichondrium, skeletal muscle, aorta, or organ capsules. Our data are also consistent with the possibility that elements similar to those observed here are present in other parts of the gene that were not examined in this study.

Among the eight lines harboring the 2.3-kb pro-α1(I) collagen gene proximal promoter cloned upstream of the luciferase gene, six lines showed levels of expression of the reporter gene in tail between 10^6 and 12×10^6 luciferase light units per mg of protein. Two lines had lower levels of

luciferase activity (10^5 and 4×10^5 luciferase light units per mg of protein for lines 1.46 and 7.9, respectively). This variability in the levels of expression reflects a very poorly understood phenomenon known as site of integration effect. Interestingly, however, the patterns of expression of the reporter genes were the same for the two low expressing lines as for the other six.

Previous data obtained with transgenic mice for the human and the rat pro-α1(I) collagen gene were also consistent with such a modular arrangement of different cell-specific regulatory elements. With the human gene, 2.3 kb of the proximal pro-α1(I) promoter directed high levels of expression of a reporter gene in bone and tendon but not in perichondrial fibroblasts or skeletal muscle (Liska et al., 1994). With the rat gene, mice harboring a 3.6-kb pro-α1(I) proximal promoter cloned upstream of the CAT gene expressed the transgene at high levels in bone, teeth and tendons but not in aorta or in perichondrial fibroblasts (Pavlin et al., 1992; Bedalov et al., 1994; Bogdanovic et al., 1994). In all three species, the element(s) responsible for high level expression of the transgene in bone appears to be located within the first 2.3 kb of the promoter (Slack et al., 1991; Bogdanovic et al., 1994; Liska et al., 1994). The *cis*-acting segments responsible for high level expression of the gene in tendon fibroblasts are located within the first 2.3 kb of the human proximal promoter (Liska et al., 1994). In mice our results show that an element important for the expression of the pro-α1(I) gene in tendon fibroblasts is located between −3.2 and −2.3 kb, but we cannot rule out that this element cooperates with other ones located downstream of −2.3 kb. Results using the rat promoter are consistent with the notion that elements located between −1.7 and −2.3 kb and between −2.3 and −3.6 kb are involved in the expression of the pro-α1(I) gene in tendon fibroblasts in this species (Bogdanovic et al., 1994). Alternatively, it is possible that measurements of the transgene-encoded CAT enzyme in tendons might have been contaminated with CAT enzyme from osteoblasts, as would likely occur in extracts of tail, and that in rats an important *cis*-acting element that directs activity of the pro-α1(I) gene in tendons has the same location as in mice. The location of an element responsible for expression of the transgene in skin does not seem to be the same in rat and in mouse. In the rat promoter, such an element appears to be located upstream of −1.7 kb (Bogdanovic et al., 1994), whereas in the mouse promoter, one such element is located within the proximal 0.9 kb. These discrepancies suggest the possibility of different arrangements of regulatory sequences within the pro-α1(I) promoter in different species.

In our experiments the transgenes were always expressed at low levels in skin, suggesting that a cooperativity with other elements is required to obtain high levels of expression in skin fibroblasts. Such elements could be located in the first intron since deletion of most of the first intron from a construction containing the human 2.3-kb pro-α1(I) promoter strongly decreased expression of a growth hormone reporter gene in dermal and fascial fibroblasts of 17-d p.c. embryos as assayed by in situ hybridization (Liska et al., 1994). However, this difference was not clearly observed when measurements of growth hormone mRNA were performed in extracts of full thickness skin
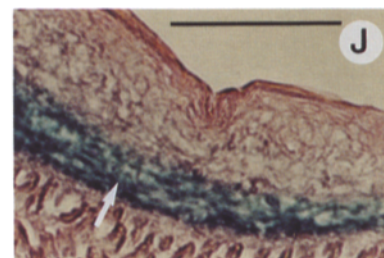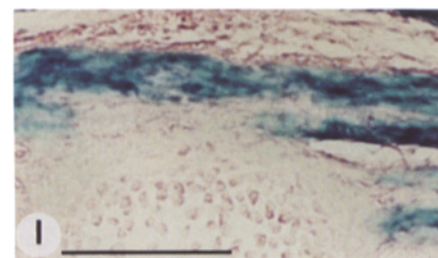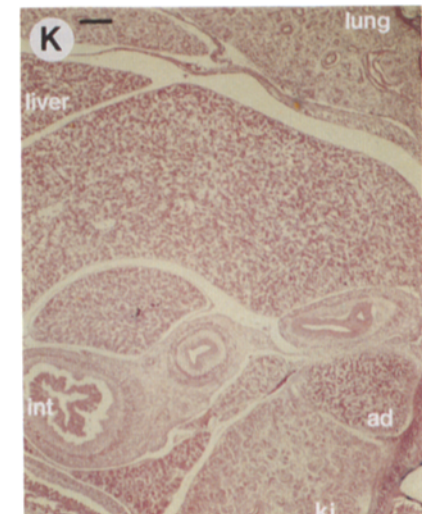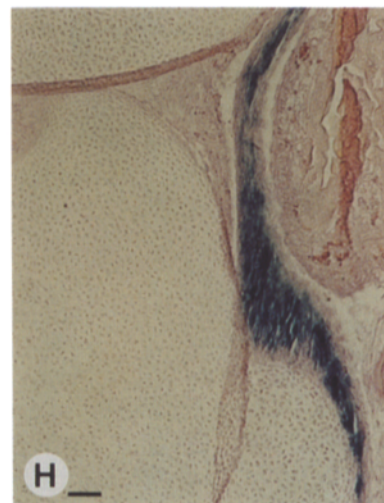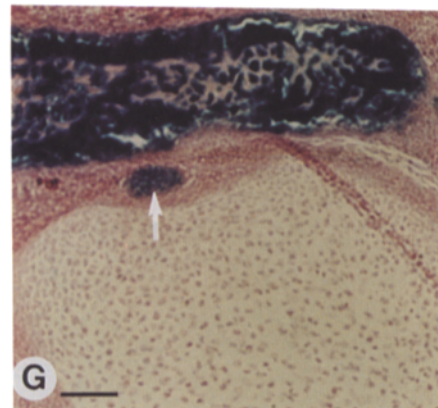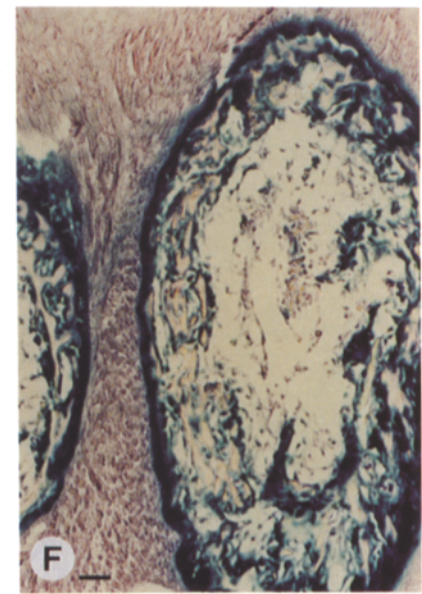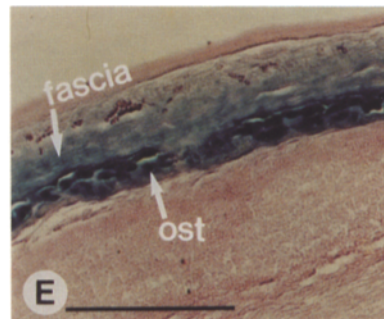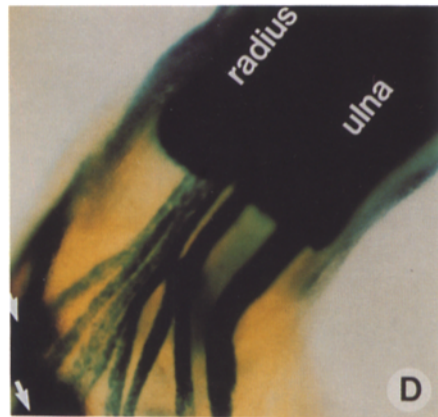
COL1A1 PROXIMAL PROMOTER

EXPRESSION

	Skin Fibroblasts	Osteoblasts Odontoblasts	Tendon, Fascia Fibroblasts
-900	+	−	−
-2300	+	+++	−
-3200	+	++++	+++

Figure 5. Summary of the tissue-specific expression of the different pro-α1(I) transgenes. See text for details.

biopsies of the same animals at 4 to 11 weeks of age, using a method that was less discriminating than in situ hybridization (Slack et al., 1991). Another report showed that, in mice harboring a human pro-α(I) procollagen minigene, the pattern of expression of the transgene was not different from those carrying the same minigene with most of the first intron deleted (Sokolov et al., 1993). Also, the tissue-specific expression of a CAT reporter gene linked to the 3.6-kb rat proximal promoter was identical, with or without most of the first intron (Bedalov et al., 1994). Hence it appears that the effect of the first intron in transgenic mice is complex, might be species dependent, and might need methods providing greater histological detail to be detected.

The Mov13 mouse strain is characterized by an insertion of a 9-kb Moloney murine leukemia retroviral sequence in the first intron of the pro-α1(I) collagen gene (Harbers et al., 1984). This retroviral DNA insertion has been shown to prevent the initiation of transcription of the pro-α1(I) procollagen gene in fibroblastic cells (Hartung et al., 1986) but did not prevent the presence of pro-α1(I) RNA in osteoblasts nor in odontoblasts (Kratochwil et al., 1989; Schwarz et al., 1990). This observation was consistent with the idea that transcription of the pro-α1(I) collagen gene was controlled by different cis-regulatory sequences in fibroblasts and in osteoblasts and odontoblasts. Our results support this hypothesis.

Evidence exists that the coordinate synthesis in a 2:1 ratio of the α1 and α2 chains of type I collagen occurs at the level of transcription (Bornstein and Sage, 1989), strongly suggesting that the same molecular mechanisms activate both genes. Studies in transgenic mice have indicated that the 2,000-bp sequence upstream of the start of transcription of the mouse pro-α2(I) collagen gene contains elements that are sufficient for expression of a β-galactosi-

dase transgene in most type I collagen-containing cells (Niederreither et al., 1992). Similarly, a 350-bp proximal promoter was also active in many of the same cells (Niederreither et al., 1992), although this 350-bp promoter appeared to be considerably weaker than the 2,000-bp promoter, and its expression in osteoblasts barely detectable; this could suggest the possibility that the segment between −350 and −2,000 bp in the pro-α2(I) promoter contains an osteoblast-specific element. These results and those presented here suggest that the arrangement of cell-specific regulatory elements is different in the two type I collagen genes. We also believe that other elements outside the 2,000-bp proximal promoter in the pro-α2(I) gene are needed for fully activity of this gene. Studies are in progress to identify such elements and compare them with similar elements in the pro-α1(I) collagen gene.

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Figure 4. Expression of the *lacZ* gene in embryos transgenic for 3200*lacZ*, as indicated by blue X-gal staining. (*A*) Whole-mount 15 d p.c. embryo (line 5.30) showing staining of ossification centers and limb tendons (*arrows*). (*B*) Hindlimb and tail of a 16.5 d p.c. embryo (line 5.30) showing staining of tendons (*arrows*). (*C* and *D*) Hindlimb and forelimb, respectively, of an 18.5 d p.c. embryo (line 5.30) showing staining of bones (*fib*, fibula; *arrows*, metacarpal bones), and tendons. The embryo was partially clarified in potassium hydroxide after X-gal staining. (*E*) Sagittal section of a head at 15.5 d p.c. (line 5.41) showing staining of osteoblasts (*ost*) and of surrounding connective tissue fascia. (*F*) Transversal section of a hindlimb at 16.5 d p.c. (line 5.1) showing staining of osteoblasts. (*G*) Section of a shoulder at 15.5 d p.c. (line 5.30) showing staining of clavicle osteoblasts and tendon fibroblasts (*arrow*) but not of chondrocytes or perichondrial fibroblasts. (*H*) Sagittal section of an ankle at 16.5 d p.c. (line 5.14) with staining of Achilles tendon but not of chondrocytes or perichondrial fibroblasts. (*I*) Sagittal section of the tail of a 16.5 d p.c. embryo (line 5.30) with staining of tendons but not of chondrocytes or perichondrial fibroblasts. (*J*) Fascia staining (*arrow*) at 16.5 d p.c. (line 5.30). (*K*) Sagittal section of a 15.5 d p.c. embryo (line 5.30) showing absence of staining of lung fibroblasts, of the capsules of liver, kidney (*ki*), and adrenal gland (*ad*), and of the peritoneum surrounding intestine (*int*). Bars, 200 μm.

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