Tissue- and Development-Specific Expression in Transgenic Mice of a Type I Procollagen (COL1A1) Minigene Construct with 2.3 kb of the Promoter Region and 2 kb of the 3′-Flanking Region. Specificity Is Independent of the Putative Regulatory Sequences in the First Intron†

Boris P. Sokolov, Peter K. Mays,‡ Jaspal S. Khillan, and Darwin J. Prockop*

Department of Biochemistry and Molecular Biology, Jefferson Institute of Molecular Medicine, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Received January 19, 1993; Revised Manuscript Received June 18, 1993®

ABSTRACT: Previous reports have provided inconsistent data as to the cis-regulatory elements that are essential for correct expression of the gene for the proα1(I) chain of type I procollagen (COL1A1) in the many tissues in which the protein is synthesized. Here, two internally deleted minigene versions of the human COL1A1 gene were used to prepare transgenic mice. The constructs made it possible to test regulatory sequences in the normal context of the gene. Also, in contrast to the reporter genes used in previous experiments, the constructs made it possible to assay quantitatively expression of the exogenous genes relative to expression of the endogenous COL1A1 gene, both as mRNA and as protein. The average level of expression of the minigenes varied among three transgenic lines, but the ratio of expression of the minigenes to expression of the endogenous gene was the same in all transgenic mice of a given line. Within the same line, the ratio of expression was essentially the same in nine or more tissues in which expression of the endogenous gene varied widely. Also, the ratio of expression within a given line was the same in 15-day-old embryos and in mice ranging in age from 4 days to 4 months. In addition, the ratio remained constant during repair of a surgical wound. The results demonstrated, therefore, that the minigene constructs with about 2.3 kb of the promoter region and about 2 kb of the 3′-flanking region contained all of the sequences necessary for correct expression of the genes in a tissue-specific and development-specific manner. Assays of a line of transgenic mice expressing the minigene with the deletion in the first intron demonstrated that the first intron of the COL1A1 gene had no discernible effect on the specificity of expression. Therefore, the results did not support previous data suggesting that the relatively large first intron of the gene contains important regulatory sequences.

Type I collagen is the most abundant structural protein in vertebrates, and it is synthesized in a tissue- and development-specific manner (Prockop & Kivirikko, 1984; Martin et al., 1985; Sandell & Boyd, 1990). Expression of genes for the proα1(I) and proα2(I) chains of the precursor procollagen (COL1A1 and COL1A2) is regulated by a variety of growth factors, hormones, and other agents (Rosenbloom et al., 1984; Choe et al., 1987; Ignotz et al., 1987; Pierce et al., 1987; Raghow et al., 1987a,b, 1989; Walsh et al., 1987; Fine & Goldstein, 1987; Solis-Herruzo et al., 1988; Penttinen et al., 1988; Rossi et al., 1988; Fine et al., 1989; Bornstein & Sage, 1989; Ritzenthaler et al., 1991), but the regulatory elements have been difficult to define. For example, there are conflicting reports as to the size of the promoter region of the COL1A1 gene necessary for tissue-specific expression and as to whether or not the promoter contains all of the important cis-regulatory elements.

In transient transfection experiments, Bornstein and McKay (1988) observed that a CAT[1] construct containing 331 bp of the promoter region of the human COL1A1 gene was transiently expressed in chick tendon fibroblasts synthesizing type I collagen at levels that were 30–100-fold higher than those in baby hamster kidney cells and HeLa cells that do not synthesize type I collagen. Similarly, Rippe et al. (1989) reported that a CAT construct containing only 222 bp of the promoter region of the mouse COL1A1 gene was more actively expressed in NIH 3T3 fibroblasts than in cells that synthesized little or no collagen. In contrast, Boast et al. (1990) reported that a CAT construct containing 2500 bp of the promoter region of the human COL1A1 gene did not show cell specificity in similar transient expression assays. More recently, Pavlin et al. (1992) reported that a region between 3521 and 2295 bp of the rat COL1A1 gene was required for high levels of transient expression of a CAT construct in three lines of osteoblasts, but deletion of the region had no effect on expression in two lines of fibroblasts.

In experiments with transgenic mice, Pavlin et al. (1992) demonstrated that the CAT construct containing 3600 bp of the rat COL1A1 promoter was expressed in several tissues that synthesized high levels of type I procollagen. In similar studies in transgenic mice, Slack et al. (1991) reported that a growth hormone–reporter gene construct containing 2300 bp of the human COL1A1 gene was expressed at levels approaching those of the endogenous mouse COL1A1 gene in most tissues but at anomalously high levels in lung and low levels in muscle. In addition, there have been conflicting reports as to whether or not the first intron of the COL1A1 gene contains important cis-regulatory elements (Barsh et al., 1984; Harbers et al., 1984; Bornstein et al., 1987; Rossouw

† The work was supported in part by NIH Grants AR38188 and AR39740, a grant from the Lucille P. Markey Charitable Trust, and a grant from the March of Dimes–Birth Defects Foundation. P.K.M. is the recipient of a Wellcome Trust (U.K.) Travel Award.

* Author to whom correspondence should be addressed.

‡ Present address: Organogenesis, Inc., 150 Dan Road, Canton, MA 02021.

® Abstract published in *Advance ACS Abstracts,* August 15, 1993.

[1] Abbreviations: CAT, chloramphenicol acetyltransferase.

0006-2960/93/0432-9242$04.00/0

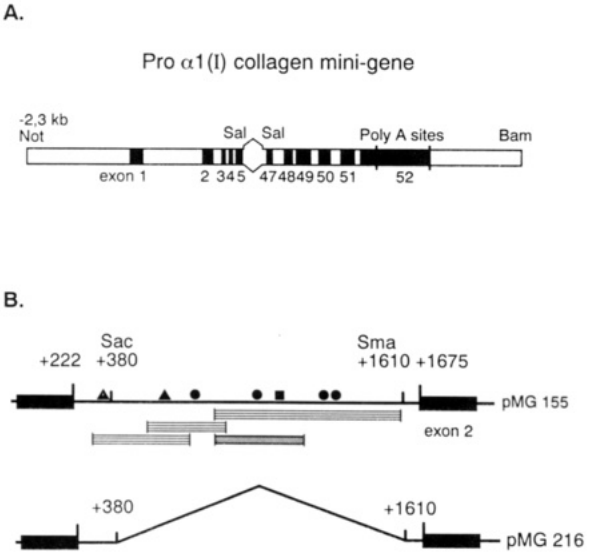

FIGURE 1: (A) Minigene construct of the human COL1A1 gene (Olsen et al., 1991). (B) Schematic diagram of the first intron in the intact minigene (pMG155) and the portion removed in the deleted version (pMG216). Symbols: ▲, Ap1 motif; ●, Sp1 motif; ■, enhancer "core" motif [from Rossouw et al., (1987)]; horizontal lined bars, sequences reported to enhance expression of an α1-globin reporter gene (Rossouw et al., 1987), CAT reporter gene (Bornstein & McKay, 1988), and human growth hormone reporter gene (Liska et al., 1990) in transiently transfected cells; horizontal dotted bar, sequence reported to inhibit expression of a CAT reporter gene in transiently transfected cells (Bornstein & McKay, 1988).

et al., 1987; Bornstein & McKay, 1988; Kratochwill et al., 1989; Boast et al., 1990; Sherwood & Bornstein, 1990; Liska et al., 1990; Schwarz et al., 1990; Slack et al., 1991).

Here we have employed two internally deleted minigene versions of the human COL1A1 gene, which contained most of the sequence context of the normal gene and which allowed quantitative assay in transgenic mice of expression of the constructs relative to expression of the endogenous COL1A1 gene.

MATERIALS AND METHODS

Gene Constructs. The proα1(I) minigene, pMG155, consisted of 11 kb of the human COL1A1 gene that contained the first five exons and introns joined to the last six exons and introns (Olsen et al., 1991). The construct (Figure 1A) also contained 2300 bp of the 5′-flanking sequence and about 2000 bp of the 3′-flanking sequence beyond the second polyadenylation site. The junction between the 5′- and 3′-halves was made in an intron so as to preserve all of the known consensus sequences required for correct RNA splicing. The first half of the encoded protein consisted of 23 amino acids of the signal peptide, 85 amino acids of the nonhelical domain of the NH_2-propeptide, and 48 amino acids of the Gly-Xaa-Yaa sequence from the triple-helical domain of the NH_2-propeptide followed by a single glycine. The second half of the encoded protein consisted of the last 69 amino acids of the Gly-Xaa-Yaa sequence of the triple-helical domain, the COOH-telopeptide, and the complete COOH-propeptide. The minigene with a deletion in the first intron (Olsen et al., 1991), pMG216, was produced by eliminating sequences between the *Sac*I site at +380 and the *Sma*I site at +1610 (Figure 1B). The deletion removed most of the putative regulatory sequences of the first intron.

Preparation of Transgenic Mice. For injection into fertilized eggs, the minigene insert was cleaved from the plasmid with *Bam*HI and *Not*I. The insert was isolated by sucrose gradient centrifugation and dialyzed against 10 mM Tris-HCl buffer (pH 7.5) and 1 mM EDTA. The DNA was used directly for microinjection in a concentration of 2 μg/mL and about 600 copies per embryo. Alternatively, the insert was separated by agarose gel electrophoresis. The band with the insert was cut out and separated by electrophoresis in a second agarose gel. After electroelution of the band from the second gel, the solution of DNA was extracted by an equal volume of isoamyl alcohol. The DNA was purified and concentrated on an ion exchange column (ELUTIP, Schleicher and Schuell). The DNA was further concentrated by ethanol precipitation and redissolved at a concentration of 2 μg/mL for microinjection. One-cell zygotes were obtained from the mating of inbred FVB/N males and females. Inbred CD1 females were used as the pseudopregnant recipients, and founder mice were identified by Southern blot analysis of tail extracts (Khillan et al., 1991).

mRNA Assay. For mRNA assays, total cellular RNA was isolated from tissues by extraction with guanidine thiocyanate, extraction with acidic phenol–chloroform, and precipitation with isopropyl alcohol (Chomczynski & Sacchi, 1987). The ratio of mRNA for human minigene proα1(I) chains to mRNA for mouse proα1(I) chains was measured by reverse transcriptase followed by PCR. Five micrograms of total cellular RNA was reverse transcribed in 20 μL of reaction mixture using primer BS33 (5′-ACTAAGTTTGA-3′) (200 pmol) and a commercial preamplification system for first strand cDNA synthesis (SuperScript, GIBCO, BRL). After RNaseH treatment, the cDNA was amplified by PCR (GeneAmp PCR reagent kit, Perkin-Elmer Cetus) with primers BS31 (5′-TTGGCCCTGTCTGCTT-3′) and BS32 (5′-TGAATG-CAAAGGAAAAAAAT-3′) at concentrations of 4 pmol per 100 μL of reaction mixture. PCR conditions were 1 min 20 s at 94 °C, 1 min at 47 °C, and 20 s at 72 °C for 15 cycles. One of the primers used in PCR was 5′-labeled with ^{32}P using a 5′-DNA-terminus labeling system (GIBCO, BRL). After PCR, 10 μL of reaction mixture was treated with 2 units of *Bst*N1 for 1 h at 60 °C. Three microliters of the product was heat denatured and separated by 15% PAGE containing 6 M urea. The gel was fixed, dried, and exposed to X-ray film. The relative intensities of the appropriate bands were measured with a laser densitometer (Ultroscan XL, KLB).

Protein Assays. For assays of expression of the minigene as protein, 50–200 mg of tissue was crushed to a powder after cooling in liquid nitrogen, and 10–40 mg of the powder was homogenized in 0.5 mL of buffer that contained 50 mM Tris-HCl (pH 6.8), 2% SDS, 6 M urea, 0.0015% bromophenol blue, 5% 2-mercaptoethanol, 25 mM EDTA, 10 mM ethylmaleimide, 1 mM phenylmethanesulfonyl fluoride, and 0.01% NaN_3. The homogenate was shaken at 4 °C for 2 h, heated at 100 °C for 5 min, and centrifuged for 5 min at 12000*g*. The supernatant (2–15 μL, 0.03–0.4 μg of protein) was electrophoresed in a 4–15% polyacrylamide gel in a minigel apparatus (Protein II, Bio-Rad). The protein was electroeluted onto a filter (PVDF membrane, product number 71925, U.S. Biochemical). The filter was then reacted with polyclonal antibodies that reacted with the COOH-propeptide of the proα1(I) chain from both human and mouse type I procollagens [see Olsen et al. (1991)]. The polyclonal antibodies were kindly provided by Dr. Larry Fisher of the National Institute of Dental Research (National Institutes of Health, Bethesda, MD). The secondary antibodies were anti-rabbit IgG coupled to alkaline phosphatase (Promega Biotek).

After reaction with secondary antibodies, blots were developed using a chemiluminescence assay based on a phenyl

phosphate-substituted 1,2-dioxetane that produces light by reaction with alkaline phosphatase (Protein Images, U.S. Biochemical). The light emitted was detected by exposure to X-ray film, and the film was scanned with a laser densitometer (Ultroscan XL, LKB). Preliminary experiments were carried out with varying exposure times and different amounts of tissue extracts to establish the linear range of response for the assay. Most of the experiments were carried out with a single preparation of primary antibodies that gave reproducible results over many months. A few experiments were carried out with a second preparation that gave higher values for the ratio of minigene proα1(I) chains to endogenous proα1(I) chains in the same samples, and values were normalized to make the data comparable.

Assays of Gene Copy Number. Gene copy number in the transgenic mice was assayed by Southern blotting of genomic DNA from tail samples. The DNA was digested with *Eco*RI and probed with the intact minigene construct labeled with [^{32}P]dCTP by random primer extension with a commercial kit (Amersham). The minigene constructs contained a single *Eco*RI site. Therefore, two bands of construct plus flanking sequences were detected in line 85, which had a single copy of the exogenous gene, three bands of about equal intensity in line 75-1, which had 2–4 copies, and a very intense band in line 73, which had multiple copies of the gene.

RESULTS

Transgenic Mice. The minigene constructs employed here were designed so as to contain about 2.3 kb of the promoter region, the first five exons, and the last six exons of the human COL1A1 gene (Figure 1). The 5th exon ended in a complete codon, and the 47th exon began with a complete codon; therefore, the constructs encoded a shortened proα1(I) mRNA that was in-frame in terms of coding sequences and was translated into a shortened proα1(I) chain (Olsen et al., 1991).

In previous experiments (Khillan et al., 1991), the construct containing the intact minigene (Figure 1A) was injected into over 3000 fertilized embryos that were transferred into over 150 pseudopregnant females. Only six F_0 founders that transmitted the gene to the F_1 generation were obtained. Of the six lines, two lines had normal phenotypes and expressed the transgene at levels that were too low for accurate assay relative to expression of the endogenous COL1A1 gene. Three of the remaining four lines expressed higher levels of the transgene but had a phenotype that was consistently lethal in the F_1 generation. The remaining sixth line expressed intermediate levels of the minigene and had a normal phenotype in the F_0 founder female. Transgenic F_1 males from the line were bred to wild-type females to generate progeny for analysis. (The sixth line was previously referred to as line V and here is referred to as line 73.) In further experiments, the same construct containing the intact minigene was injected into over 1000 additional fertilized embryos that were transferred to over 50 pseudopregnant females. One line expressing moderate levels of the transgene (line 75-1) was obtained and analyzed.

The construct containing the minigene with a deletion of most of the putative regulatory sequences of the first intron (Figure 1B) was also injected into over 1000 fertilized embryos that were transferred to over 50 pseudopregnant females. One breeding line of transgenic mice expressing moderate levels of the transgene (line 85) was obtained.

The low yield of transgenic mice obtained with the two constructs was probably explained by previous observations indicating that high levels of expression of mutated genes for fibrillar collagens produce lethal phenotypes [see Stacey et al. (1988), Khillan et al. (1991), and Vandenberg et al. (1991)]. Generation of lethal phenotypes was not apparent from the size of litters that varied from 7 to 12. In parallel experiments, however, the same operators in the same laboratory obtained a yield of 10 lines of transgenic mice expressing a CAT construct containing an elastin promoter by injecting the construct into only 400 fertilized embryos that were implanted into 20 pseudopregnant recipients (J. S. Khillan and J. J. Uitto, manuscript in preparation).

Assay Procedure for Expression of the Minigenes as mRNA. To assay expression of the minigenes as mRNA in the transgenic mice, Northern blot analysis and primer extension analysis were used in initial experiments. Northern blot assays were successful with cultured cells [see Olsen et al. (1991)], but were not consistently satisfactory with tissues from the transgenic mice, apparently because of the difficulty in extracting high-quality RNA from many collagen-rich tissues of older mice. In the primer extension assays, the high degree of homology between sequences in the 5′-ends of the human and mouse mRNAs made it difficult to design primers that generated easily distinguishable bands. Accordingly, a new assay was developed on the basis of reverse transcription of the mRNA and then PCR amplification. The sequences of the 3′-end of the human and mouse mRNAs for proα1(I) chains are highly conserved but contain several differences (Mooslehner & Harbers, 1988; Bernard et al., 1983; Westerhausen et al., 1991). The primer for reverse transcription and the different primers for PCR were designed so as to be complementary to identical sequences in human and mouse proα1(I) mRNAs (Figure 2) and thereby ensure that the efficiency of both the reverse transcriptase reaction and PCR was the same for both templates. The human and mouse PCR products were 176 and 177 bp, respectively, and therefore not distinguishable on the basis of size. However, fragments of different sizes were obtained after cleavage with *Bst*NI (Figure 3). Experiments with mixtures of total human and mouse RNA from fibroblasts demonstrated that the assay was linear over a broad range (Figure 4). Also, the values for the ratio remained the same with 15–25 cycles of PCR (not shown).

Expression of Minigenes as mRNA. The selective assay for mRNA from the minigenes and the endogenous gene was used to examine various tissues from two lines of transgenic mice: one expressing the intact minigene (line 73) and one the minigene with a deletion in the first intron (line 85). With transgenic mice from each line, the ratio of the two mRNAs did not show any significant differences among the values from different tissues (Table I).

Examination of tissues from mice at different stages of development also did not show any significant differences. With mice expressing the intact minigene, the ratio of the two mRNAs was essentially the same for mice ranging in age from 4 to 120 days (Figure 5A). The only exception was perhaps a slight upward trend in the values for skin. With mice expressing the minigene with the deletion in the first intron, the value of the ratio also remained constant over the same range of ages (Figure 5B).

Expression of the Minigenes as proα1(I) Chains. In further experiments, expression of the minigenes was assayed by Western blotting with antibodies that reacted with the COOH-propeptides of both the human proα1(I) chain and the mouse proα1(I) chain (Olsen et al., 1991). The two gene products were readily distinguishable because the proα1(I) chains from the human minigene were shorter than the normal proα1(I) chains from the mouse gene (Figure 6). The assay by Western

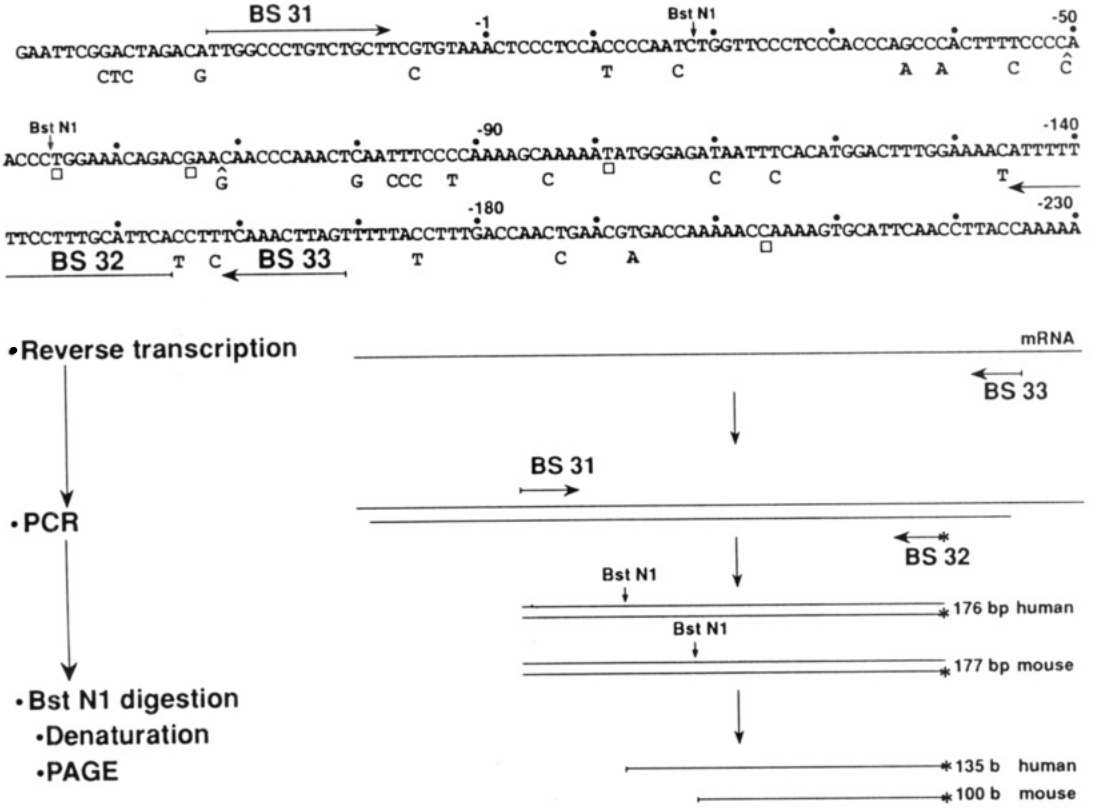

FIGURE 2: Schematic description of the reverse transcription–PCR assay of the relative levels of human proα1(I) mRNA and endogenous mouse proα1(I) mRNA. The mouse sequence is shown (Mooslehner & Harbers, 1988). Bases below the mouse sequence indicate substitutions, insertions (→), and deletions (□) found in the corresponding human sequences (Bernard et al., 1983; Westerhausen et al., 1991). An asterisk indicates a ^{32}P-labeled primer.

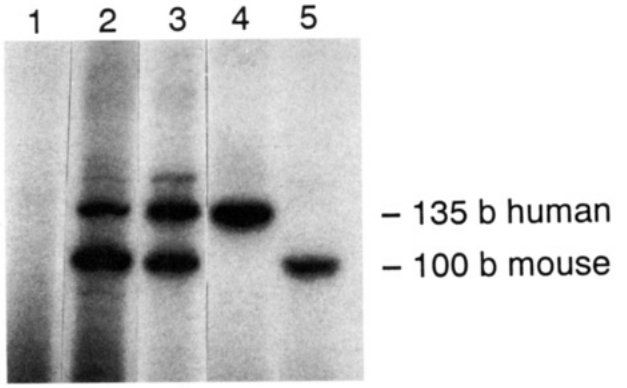

FIGURE 3: Assay of human and mouse proα1(I) mRNAs. Reverse transcriptase–PCR was performed as described in Materials and Methods. Lane 1: Control of total RNA from the tail of 4-month-old transgenic mice (line 73 with intact minigene) not treated with reverse transcriptase. Lanes 2 and 3: Total RNA from 4-month-old transgenic mice of line 85 with deletion in first intron and line 73 intact minigene, respectively. Lanes 4 and 5: Total mRNA from human and mouse cultured fibroblasts, respectively.

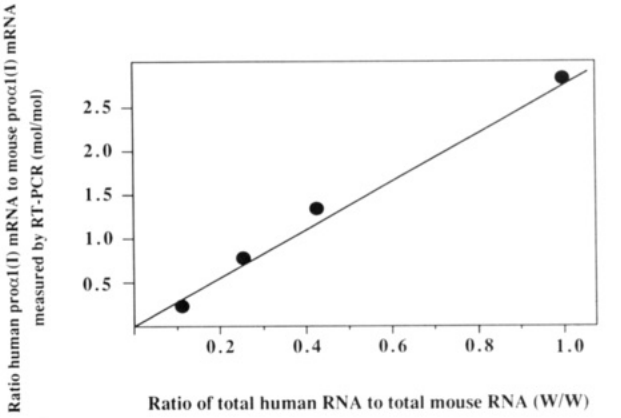

FIGURE 4: Linearity of the mRNA assay. Total RNA from human fibroblasts was mixed at different ratios with total RNA from mouse fibroblasts. The ratio of human proα1(I) mRNA to mouse proα1(I) mRNA was measured by reverse transcriptase–PCR as described in Materials and Methods. The total amount of RNA in each tube was 3 μg.

blotting for the ratios of the two proα1(I) chains was linear with varying ratios of the two chains (Figure 7). Assays of heterozygotes from the three transgenic lines indicated that there were no significant differences among tissues from the same line (Table II). For example, all of the values of line 73 expressing the intact minigene were within 1 standard deviation of the mean value for all tissues (Table III), except for the somewhat higher value for aorta (Table II). Similarly, all of the values for line 85 expressing the minigene lacking the first intron were within 1 standard deviation of the mean for all tissues, except for the somewhat lower value in liver and the higher single values for kidney and brain. Also, there were no significant differences in the ratio in tissues from mice ranging in age from 15-day-old embryos to 120-day-old mice (Figure 8). As expected, the assay gave values for ratios of the two chains that were twice as high with skin and cultured fibroblasts from homozygous mice compared to heterozygous mice (Table IV).

Gene Copy Number and Phenotype. The gene copy number in the three lines varied from 1 to over 50, but there was no apparent relationship between the gene copy number and the level of expression (Table III). However, the values for levels of expression in the three lines appeared to parallel the severity of the phenotype. Line 85 with the lowest level of expression had no discernible phenotype. Line 73 had an intermediate

Table I: Tissue Specificity of Human Mini-proα1(I) mRNA Expression in Transgenic Mice

tissue	ratio of mini-proα1(I) to proα1(I) mRNA	
	line 73[a] (intact intron)	line 85 (deleted intron)
skin	0.66 ± 0.19 (6)[b]	0.38 ± 0.12 (7)
lung	0.73 ± 0.18 (3)	0.59 ± 0.26 (3)
intestine	0.84 ± 0.26 (3)	0.51 ± 0.30 (3)
bone	0.51 ± 0.23 (8)	0.43 ± 0.26 (3)
muscle	0.66 ± 0.16 (5)	0.39 ± 0.29 (3)
tail	0.67 ± 0.24 (5)	0.31 ± 0.09 (4)
heart	0.41 ± 0.27 (3)	0.52
kidney	0.77	0.63
eye	0.55	

[a] Line 73 was developed previously and referred to as line V (Khillan et al., 1991). [b] Values are mean and standard deviation. The numbers of mice assayed are indicated in parentheses.

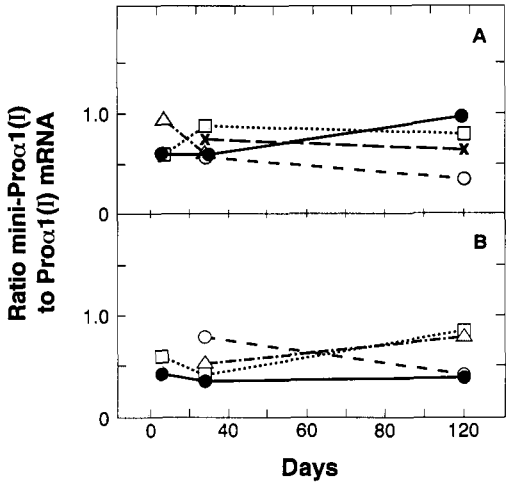

FIGURE 5: Age dependence of expression of the minigenes as mRNA: (A) line 73 with intact minigene; (B) line 85 with deletion in the first intron. Values are the means of 2–6 independent assays from the tissues of 1–4 transgenic mice. Symbols: ●, skin; ○, bone; □, lung; △, intestine; ×, tail.

level of expression, and about 6% of the transgenic mice had a lethal phenotype (Khillan et al., 1991; Pereira et al., 1993). Line 75-1 had the highest level of expression. The F_0 founder of line 75 (Khillan et al., 1991) and the F_1 male used to generate line 75-1 mice analyzed here had no apparent phenotype, but about 90% of the transgenic mice from subsequent generations had the same lethal phenotype of brittle bones described previously for other transgenic lines expressing high levels of the same minigene construct [see Khillan et al. (1991) and Pereira et al. (1993)].

Expression of the Minigenes during Wound Healing. Increased synthesis of type I collagen by fibroblasts is a major feature of wound repair (Barnes et al., 1975; Deigelmann et al., 1975; Clark, 1988; McPherson & Piez, 1988). In further experiments here, we examined expression of the two minigenes during repair of a surgical wound to the tails of the transgenic mice. To create a wound, a piece of tail of about 1.5 cm was cut from 2–5-month-old transgenic mice. Immediately thereafter, an adjacent second section of about 1.0 mm was cut for assay as a zero-time control. After 1, 5, 6, or 10 days, another 1.0-mm piece of tail was cut from different mice for assay of expression as the ratio of proα1(I) chains by Western blotting. As shown in Figure 9, the ratio of mini-proα1(I) chains to endogenous mouse proα1(I) chains did not change after injury and was constant for up to 10 days of the healing process. Similar results were obtained with mice from the line (line 73) expressing the intact minigene and the line (line 85) expressing the minigene with the deletion in the first intron (Figure 9).

DISCUSSION

The minigene constructs employed here have several important advantages for examining the expression of a collagen gene in transgenic mice. One is that the constructs made it possible to test regulatory sequences within the normal sequence context of the COL1A1 gene (Figure 1). Another advantage is that it was possible to assay quantitatively the expression of the minigenes relative to that of the normal endogenous gene in terms of the steady-state levels of both mRNA and newly synthesized protein. As a result, the specificity of expression of the exogenous gene could be assayed far more accurately than with reporter gene constructs whose level of expression is difficult to relate to endogenous genes and whose expression can frequently vary by an order of magnitude in the same tissues from different transgenic mice of the same line [see Slack et al. (1991)]. In addition, since the assays measured expression relative to expression of the endogenous gene in the same sample, the assays circumvented the considerable problem of dissecting collagen-synthesizing tissues free of other tissues. Although type I collagen accounts for one-half or more of the protein in tissues such as tendon, skin, and bone, almost every tissue contains measurable amounts of the protein either in associated blood vessels or supporting membranes and related structures [see Prockop and Kivirikko (1984)]. Also, the rate of synthesis of type I collagen varies widely in the same tissues during development, so that the bulk of type I collagen in many tissues becomes metabolically stable as animals mature, but a fraction is subject to continual turnover (Prockop & Sjoerdsma, 1961; Prockop & Kivirikko, 1984).

For these reasons, the values for expression of a type I collagen gene based on reporter gene constructs are difficult to relate in a quantitative manner to expression of the normal gene in transgenic animals. Similar problems are encountered in cell transfection experiments because the expression of type I procollagen genes in cultured fibroblasts varies widely with the state of confluency of the cells, the passage number, and other conditions of cell culture [see Hämäläinen et al. (1985) and Olsen and Prockop (1989)]. As reported previously (Olsen et al., 1991), the human minigene constructs made it possible to assay expression of the exogenous gene relative to the endogenous genes in stably transfected 3T3 in cells. As demonstrated here, similar assays were possible in tissues from transgenic mice. Most importantly, the levels of expression did not show any consistent differences among different tissues from different transgenic mice of the same line.

One disadvantage of the minigene construct of collagen genes encountered in the course of the experiments carried out here and previously (Khillan et al., 1991; Vandenberg et al., 1991; Pereira et al., 1993) is that expression of the constructs frequently produced lethal phenotypes. As a result, large numbers of fertilized embryos had to be injected to produce lines of transgenic mice that expressed the minigenes at levels that were high enough to be assayed relative to the endogenous genes but not high enough to produce lethal phenotypes. Similar problems have been encountered in generating breeding lines of transgenic mice expressing genes for fibrillar collagens with substitutions for glycine codons in the triple-helical domain of the protein and related mutations (Stacey et al., 1988; Garofalo et al., 1991; Metsäranta et al., 1992). The major effect of synthesis of mini-proα1(I) chains was probably to reduce the net amount of type I collagen in

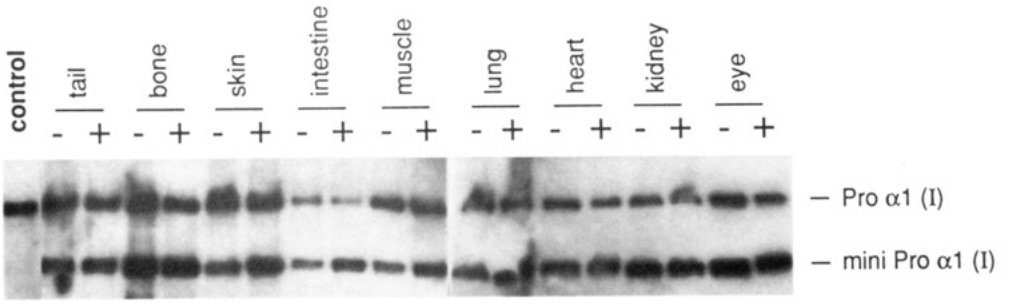

FIGURE 6: Western blot analysis of different tissues from 4-day-old transgenic mice. Symbols: +, line 73 expressing intact minigene; –, line 85 expressing minigene with the deletion in the first intron. Western blotting was carried out as described in Materials and Methods. The amount of total protein loaded on each lane was different so as to obtain approximately equal intensity of proα1(I) chains.

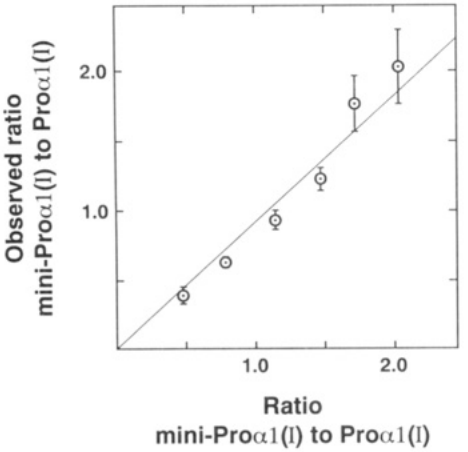

FIGURE 7: Linearity of the assay for proα1(I) chains. Protein extracts from normal mouse fibroblasts were mixed with protein extracts from fibroblasts from a homozygous transgenic mouse from line 73 to obtain the ratios of the two chains indicated on the abscissa. The ratio of mini-proα1(I) chains to normal proα1(I) chains was then assayed as indicated in Figure 6 and described in text. Values indicate mean and standard deviations (n = 4) for the same gel that was exposed to X-ray films for 2.5, 5, 10, and 16 min.

Table II: Tissue Specificity of Human Mini-proα1(I) Chain Expression in Transgenic Mice

	ratio of human mini-proα1(I) to mouse proα1(I) chains[a]		
tissue	line 73 (intact intron)	line 75-1[b] (intact intron)	line 85 (deleted intron)
skin	0.80 ± 0.16 (7)	2.36 ± 0.97 (3)	0.59 ± 0.28 (7)
intestine	1.26 ± 0.69 (4)	2.06	0.69 ± 0.37 (6)
lung	0.92 ± 0.11 (5)	0.85	0.59 ± 0.24 (5)
muscle	0.87 ± 0.20 (4)	1.98 ± 0.85 (3)	0.45 ± 0.22 (5)
tail	0.83 ± 0.33 (6)	1.10 ± 0.22 (3)	0.64 ± 0.09 (11)
liver	0.87 ± 0.41 (3)		0.37 ± 0.20 (3)
bone	1.40 ± 0.19 (6)	1.62 ± 0.28 (3)	0.97 ± 0.18 (4)
aorta	2.30 ± 0.56 (3)		1.28 ± 0.31 (3)
kidney	1.46		1.35
spleen	1.20		0.93
brain	0.85		1.85
heart	1.47		0.90

[a] Values are means and standard deviations. The numbers of mice assayed are in parentheses. [b] Assays on newborn pups with lethal phenotype.

tissues because of degradation of procollagen monomers containing shortened and normal proα chains (Khillan et al., 1991; Pereira et al., 1993). The mini-proα1(I) chains may, however, have had additional deleterious effects such as feedback inhibition from processed propeptides or toxic effects of degradation products and, thereby, helped limit the window of expression that was compatible with obtaining viable transgenic mice. The accuracy of the assays of minigene expression relative to endogenous, however, made it possible

Table III: Gene Copy Number, Level of Expression of Exogenous Gene, and Phenotype of Transgenic Mice

transgenic line	copy number	expression relative to endogenous gene: mRNAs[a]	expression relative to endogenous gene: proα1(I) chains[b]	phenotype
73	>50	0.64 ± 0.13 (35)	1.21 ± 0.45 (42)	6% lethal
75–1	2–4		1.66 ± 0.59 (24)	90% lethal[c]
85	1	0.47 ± 0.11 (25)	0.88 ± 0.43 (48)	none

[a] Values from Table I. The numbers of samples assayed are in parentheses. [b] Values from Table II. [c] The F_0 founder of line 71–1 was a female with a normal phenotype. One of the F_1 male transgenic mice also had a normal phenotype, but 30 of 32 transgene offspring from the F_1 male had a lethal phenotype.

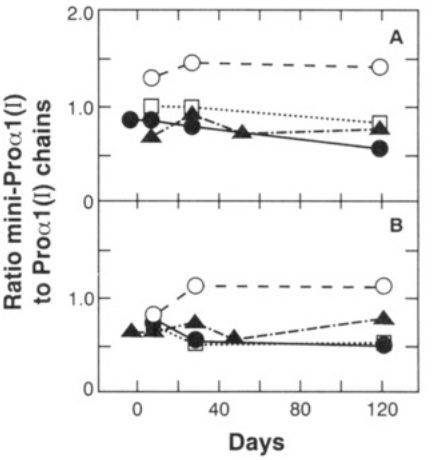

FIGURE 8: Age dependence of expression of the minigenes as proα1-(I) chains. (A) Line 73 expressing the intact minigene. (B) Line 85 expressing the minigene with the deletion in the first intron. Values are the means of at least two independent assays from the tissues from 1–3 transgenic mice of the line. Symbols: ●, skin; ○, bone; □, lung; ▲, tail.

Table IV: Expression of Human Mini-proα1(I) Chain in Skin and Cultured Fibroblasts from Homozygous and Heterozygous Transgenic Mice

transgenic line	tissue[b]	ratio of human mini-proα1(I) to mouse proα1(I) chains[a]: heterozygous	ratio of human mini-proα1(I) to mouse proα1(I) chains[a]: homozygous
line 73	skin	0.80 ± 0.16 (7)	1.79
	fibroblasts	0.72 ± 0.19 (3)	2.05 ± 0.60 (3)
line 85	skin	0.59 ± 0.28 (7)	1.11
	fibroblasts	0.61 ± 0.15 (4)	1.23

[a] Values are means and standard deviations. The numbers of independent assays are in parentheses. [b] Skin assayed directly or in cultures of skin fibroblasts.

to make meaningful comparisons with a limited number of transgenic lines.

The results given here demonstrate that the two minigene constructs contained all of the cis-regulatory elements needed

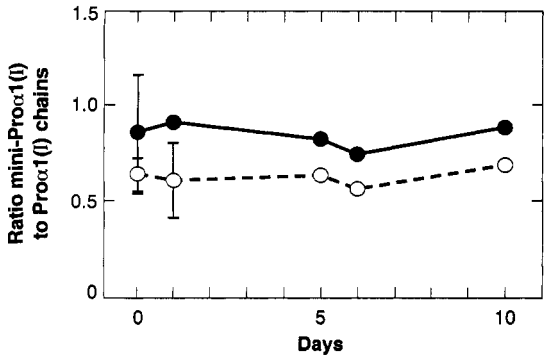

FIGURE 9: Effects of wound healing on expression of the minigenes. Values are from the Western blot assay of proα1(I) chains (see Figure 6). As discussed in the text, a wound was created by cutting a 1.5-cm section of the tail of each transgenic mouse. Immediately thereafter, a 1.0-mm adjacent section was cut as a zero-time control. An additional 1.0-mm section was cut from different mice on the days indicated and assayed as indicated in Figure 6. Values are the means for 1–4 mice. Symbols: ●, line 73 expressing the intact minigene; ○, line 85 expressing the minigene with the deletion in the first intron.

for correct expression of the genes when randomly inserted into the mouse genome. The level of expression in different lines was not related to the gene copy number and, therefore, probably was primarily dependent on the site of insertion. Tissue-specific regulation of expression, however, did not depend on the site of insertion. Assays of multiple tissues from a series of mice from the three independent lines demonstrated that the ratio of steady-state levels of mRNAs from the exogenous minigene and the endogenous gene were remarkably constant within a given line. Assays of expression of proα1(I) chains by Western blotting tissue extracts also showed little variation. The assays of proα1(I) chains reflected the steady-state levels of the unprocessed chains, and it may well be that the relative rates of processing or degradation of mini-proα1(I) and normal proα1(I) chains varied in different tissues. The observed values, however, were remarkably constant and were consistent with the mRNA assays. Most importantly, the ratio of expression of the minigene to the endogenous gene was the same in tissues such as skin and bone, in which more than one-half of the protein synthesized is type I collagen, and in tissues such as liver and brain, in which type I collagen accounts for only a small fraction of the protein synthesized. Also, the ratio of the mRNAs remained constant during the development from 15-day-old embryos to 120-day-old mice.

In addition, the ratio of mini-proα1(I) chains to endogenous proα1(I) chains remained constant during wound repair. The results indicated, therefore, that the two minigene constructs with about 2.3 kb of the promoter region and about 2 kb of the 3′-flanking region contain all of the sequences essential for correct expression of the genes in a tissue-specific and development-specific manner. It is important to note that, in similar studies in transgenic mice, Slack et al. (1991) found that growth hormone–reporter gene constructs driven by about 2.3 kb of the promoter of the human COL1A1 gene were expressed at anomalously high levels in lung tissue and low levels in muscle. More recently, it was found that CAT constructs driven by an even larger piece of COL1A1 gene promoter (about 3.6 kb) was expressed at anomalously low levels in aorta tissue (A. Bedalov, A. C. Lichter, I. Bedalov, S. H. Clark, C. O. Woody, B. E. Kream, and D. W. Rowe, personal communication). The discrepancies from previous observations may be explained by the difficulty of accurately assaying specific expression of collagen genes with reporter constructs. Alternatively, it is possible that expression of the COL1A1 gene in lung, muscle, and aorta tissue is mediated by sequences located within the body of the gene or in the 3′-flanking region. These sequences were absent in previously employed growth hormone and CAT constructs but present in the minigene constructs employed here. Interestingly, Slack et al. (1992) recently presented data suggesting that intragenic or far 3′-flanking sequences of the COL1A1 gene were critical in mediating the effects of ras on expression of the COL1A1 gene.

The results here also demonstrate that the putative regulatory elements within the relatively large first intron of the COL1A1 gene had no discernible effect on tissue-specific expression, development-specific expression, or injury-stimulated expression of the gene in transgenic mice. In the line of mice expressing the minigene with the deletion of most of the intron, the ratio of mRNAs was the same in all tissues examined and remained constant through development from 15-day-old embryos to 120-day-old mice. Also, the ratio of proα chains was essentially the same in all tissues and remained constant. The results, therefore, were similar to the results obtained by Slack et al. (1991) with a growth hormone–reporter gene construct containing 2300 bp of the human COL1A1 gene in transgenic mice, in that deletion of the first intron had no apparent effect on the expression of the gene. The results differ from previous reports indicating that important regulatory elements are present in the first intron of the COL1A1 gene (Barsh et al., 1984; Harbers et al., 1984; Bornstein et al., 1987; Rossouw et al., 1987; Bornstein & McKay, 1988; Boast et al., 1990; Sherwood & Bornstein, 1990), as well as in the first intron of several genes for other fibrillar collagens (Rossi & de Crombrugghe, 1987; Horton et al., 1987; Killen et al., 1988; Wang et al., 1991; Greenspan et al., 1991). It is of interest to note, however, that except for the experiments in transgenic mice by Slack et al. (1991), most of the data suggesting the presence of regulatory elements in the first intron of collagen genes are based on experiments with reporter gene constructs in transiently transfected cells.

ACKNOWLEDGMENT

The authors are grateful to Lev Yurgenev, Dan Bertolette, and Machiko Arita for expert technical assistance. We thank Dr. Larry Fisher of the National Institutes of Health for the antibodies to the proα1(I) chain of type I procollagen.

REFERENCES

Barnes, M. J., Morton, L. F., Bennett, R. C., & Bailey, A. J. (1975) *Biochem. Soc. Trans. 3*, 917–920.

Barsh, G. S., Rousch, C. L., & Gelinas, R. E. (1984) *J. Biol. Chem. 259*, 14906–14913.

Bernard, M. P., Chu, M.-L., Myers, J. C., Ramirez, F., Eikenberry, E. F., & Prockop, D. J. (1983) *Biochemistry 22*, 5213–5223.

Boast, S., Su, M.-W., Ramirez, F., Sanchez, M., & Avvedimento, E. V. (1990) *J. Biol. Chem. 265*, 13351–13356.

Bornstein, P., & McKay, J. (1988) *J. Biol. Chem. 263*, 1603–1606.

Bornstein, P., & Sage, H. (1989) *Prog. Nucleic Acid Res. Mol. Biol. 37*, 67–106.

Bornstein, P., McKay, J., Morishima, J. K., Devarayalu, S., & Gelinas, R. E. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8869–8873.

Choe, I., Aycock, R. S., Raghow, R., Myers, J. C., Seyer, J. M., & Kang, A. H. (1987) *J. Biol. Chem. 262*, 5408–5413.

Chomczynski, P., & Sacchi, N. (1987) *Anal. Biochem. 162*, 156–159.

Clark, R. A. F. (1988) In *Molecular and Cellular Biology of Wound Repair* (Clark, R. A. F., & Henson, P. M., Eds.) Chapter 1, pp 3–33, Plenum Press, New York.
Diegelmann, R. F., Rothkopt, L. C., & Cohen, I. K. (1975) *J. Surg. Res. 19*, 239–243.
Fine, A., & Goldstein, R. H. (1987) *J. Biol. Chem. 262*, 3897–3902.
Fine, A., Poliks, C. F., Donahue, L. P., Smith, B. D., & Goldstein, R. H. (1989) *J. Biol. Chem. 264*, 16988–16991.
Garofalo, S., Vuorio, E., Metsäranta, M., Rosati, R., Toman, D., Vaughan, J., Lozano, G., Mayne, R., Ellard, J., Horton, W., & deCrombrugghe, B. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9648–9652.
Greenspan, D. S., Lee, S.-T., Lee, B.-S., & Hoffman, G. G. (1991) *Gene Expression 1*, 29–39.
Hämäläinen, L., Oikarinen, J., & Kivirikko, K. I. (1985) *J. Biol. Chem. 260*, 720–725.
Harbers, K., Kühn, M., Delius, M., & Jaenisch, R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1504–1508.
Horton, W., Miyashita, T., Kohno, K., Hassel, J. R., & Yamada, Y. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8864–8868.
Ignotz, R. A., Endo, T., & Massague, J. (1987) *J. Biol. Chem. 262*, 6443–6446.
Khillan, J. S., Olsen, A. S., Kontussari, S., Sokolov, B., & Prockop, D. J. (1991) *J. Biol. Chem. 266*, 23373–23379.
Killen, P. D., Burbelo, P. D., Martin, G. R., & Yamada, Y. (1988) *J. Biol. Chem. 263*, 12310–12314.
Kratochwill, K., von der Mark, K., Kollar, E. J., Jaenisch, R., Mooslehner, K., Schwarz, M., Haase, K., Gmache, I., & Harber, K. (1989) *Cell 57*, 807–816.
Liska, D. J., Slack, J. L., & Bornstein, P. (1990) *Cell Regul. 1*, 487–498.
Martin, G. R., Timpl, R., Müller, P. K., & Kühn, K. (1985) *Trends Biochem. Sci. 10*, 285–287.
Mayne, R., & Burgeson, R. E., Eds. (1987) *Structure and Function of Collagen Types*, Academic Press, New York.
McPherson, J. M., & Piez, K. A. (1988) in *Molecular and Cellular Biology of Wound Repair* (Clark, R. A. F., & Henson, P. M., Eds.) Chapter 20, pp 471–495, Plenum Press, New York.
Metsäranta, M., Garofalo, S., Decker, G., Rintala, M., deCrombrugghe, B., & Vuorio, E. (1992) *J. Cell Biol. 118*, 203–212.
Mooslehner, K., & Harbers, K. (1988) *Nucleic Acids Res. 16*, 773.
Olsen, A. S., & Prockop, D. J. (1989) *Matrix 9*, 73–81.
Olsen, A. S., Geddis, A. E., & Prockop, D. J. (1991) *J. Biol. Chem. 266*, 1117–1121.
Pavlin, D., Lichtler, A. C., Bedalov, A., Kream, B. E., Harrison, J. R., Thomas, H. F., Gronowicz, G. A., Clark, S. H., Woody, C. O., & Rowe, D. W. (1992) *J. Cell Biol. 116*, 227–236.
Penttinen, R. P., Kobayashi, S., & Bornstein, P. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1105–1108.
Pereira, R., Khillan, J. S., Helminen, H. J., Hume, E. L., & Prockop, D. J. (1993) *J. Clin. Invest.* (in press).
Pierce, R. A., Glang, M. G., Greco, R. S., Mackenzie, J. W., Boyd, C. D., & Deak, S. B. (1987) *J. Biol. Chem. 262*, 1652–1658.
Prockop, D. J., & Sjoerdsma, A. (1961) *J. Clin. Invest. 40*, 843–849.
Prockop, D. J., & Kivirikko, K. (1984) *New Engl. J. Med. 311*, 376–386.
Raghow, R., & Thompson, J. P. (1989) *Mol. Cell. Biochem. 86*, 5–18.
Raghow, R., Kang, A. H., & Pidikiti, D. (1987a) *J. Biol. Chem. 262*, 8409–8415.
Raghow, R., Postlewaite, A. A., Keshi-Oja, J., Moses, H. L., & Kang, A. H. (1987b) *J. Clin. Invest. 79*, 1285–1288.
Rippe, R. A., Lorenzen, S.-I., Brenner, D. A., & Breindl, M. (1989) *Mol. Cell. Biol. 9*, 2224–2227.
Ritzenthaler, J., Goldstein, R. H., Fine, A., Lichtler, A., Rowe, D. W., & Smith, B. D. (1991) *Biochem. J. 280*, 157–262.
Rosenbloom, J., Feldman, G., Freundlich, B., & Jimenez, S. A. (1984) *Biochem. Biophys. Res. Commun. 123*, 365–372.
Rossi, P., Karensty, G., Roberts, A. B., Roche, N. S., Sporn, M. B., & deCrombrugghe, B. (1988) *Cell 52*, 405–414.
Rossouw, C. M. S., Vergeer, W. P., DuPlooy, S. J., Bernard, M. P., Ramirez, F., & de Wet, W. J. (1987) *J. Biol. Chem. 262*, 15151–15157.
Sandell, L. J., & Boyd, C. D. (1990) In *Extracellular Matrix Genes*, pp 1–56, Academic Press, New York.
Schwarz, M., Harbers, K., & Kratochwill, K. (1990) *Development 108*, 717–726.
Sherwood, A. L., & Bornstein, P. (1990) *Biochem. J. 265*, 895–897.
Slack, J. L., Liska, D. J., & Bornstein, P. (1991) *Mol. Cell. Biol. 11*, 2066–2074.
Slack, J. L., Parker, M. I., Robinson, V. R., & Bornstein, P. (1992) *Mol. Cell. Biol. 12*, 4714–4723.
Solis-Herruzo, J. A., Brenner, D. A., & Chojkier, M. (1988) *J. Biol. Chem. 263*, 5841–5845.
Stacey, A., Bateman, J., Choi, T., Mascara, T., Cole, W., & Jaenisch, R. (1988) *Nature 332*, 131–136.
Vandenberg, P., Khillan, J. S., Prockop, D. J., Helminen, H., Kontusaari, S., & Ala-Kokko, L. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7640–7644.
Walsh, M. J., LeLeiko, N. S., & Sterling, K. M., Jr. (1987) *J. Biol. Chem. 262*, 10814–10818.
Wang, L., Balakir, R., & Horton, W. E., Jr. (1991) *J. Biol. Chem. 266*, 19878–19881.
Westerhausen, A., Constantinou, C. D., Pack, M., Peng, M., Hanning, C., Olsen, A. S., & Prockop, D. J. (1991) *Matrix 11*, 375–379.