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Research Article

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Transgenic Mice Expressing a Partially Deleted Gene for Type I Procollagen (COL1A1)

A Breeding Line with a Phenotype of Spontaneous Fractures and Decreased Bone Collagen and Mineral

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Abstract

A line of transgenic mice was prepared that expressed moderate levels of an internally deleted human gene for the proα1(I) chain of type I procollagen. The gene construct was modeled after a sporadic in-frame deletion of the human gene that produced a lethal variant of osteogenesis imperfecta by causing biosynthesis of shortened proα1(I) chains. 89 transgenic mice from the line were examined. About 6% had a lethal phenotype with extensive fractures at birth, and 33% had fractures but were viable. The remaining 61% of the transgenic mice had no apparent fractures as assessed by x ray examination on the day of birth. Brother-sister matings produced eight litters in which ~ 40% of the mice had the lethal phenotype, an observation indicating that expression of the exogenous gene was more lethal in putative homozygous mice from the line. Examination of femurs from the transgenic mice indicated that the bones were significantly shorter in length and had a decrease in wet weight, mineral content, and collagen content. However, there was no statistically significant change in the mineral to collagen ratio. Biomechanical measurements on femurs from the mice at 6 wk indicated a decrease in force and energy to failure. There was also a decrease in strain to failure and an increase in Young's modulus of elasticity, observations indicating increased brittleness of bone matrix. The results suggested that the transgenic mice may be an appropriate model for testing potential therapies for osteogenesis imperfecta. They may also be a useful model for studying osteoporosis. (*J. Clin. Invest.* 1993. 91:709–716.) Key words: osteoporosis • osteogenesis imperfecta • brittle bones • biomechanical defect of bone • animal models

Introduction

Mutations in the genes for the proα1(I) and proα2(I) chains of type I procollagen (COL1A1 and COL1A2) cause osteogenesis imperfecta (OI),[1] a heritable disorder characterized by osteopenia and fragile bones in children (1–4). Over 90% of probands with severe OI have been shown to have mutations in one of the two genes for type I procollagen. With a few exceptions, the mutations in the type I procollagen genes produce their devastating effects because they cause synthesis of structurally abnormal but partially functional proα1(I) or proα2(I) chains. Most of the mutations change the primary structure of the triple-helical domain of proα chains without altering the COOH-terminal propeptides that direct the association of the proα chains. As a result, the structurally abnormal proα chains associate with and become disulfide-linked to normal proα chains synthesized by the same fibroblasts. The presence of a mutated proα chain in the molecule can have one of two effects (4). The first is that the mutation can prevent folding of the three proα chains into the triple-helical conformation that is characteristic of collagens and that is required for the biological functions of the protein. As a result, both normal and mutated proα chains are degraded as they are secreted in a process referred to as "procollagen suicide." The second possible effect is that the mutations permit folding of three proα chains, but they introduce subtle conformational changes in the triple helix, such as a flexible kink. The conformational change in the molecule does not prevent processing of procollagen to collagen, but the resulting abnormal collagen copolymerizes with normal collagen and thereby disrupts the assembly of fibrils.

Because most of the mutations causing OI produce their effects through the synthesis of structurally abnormal proα chains, it has seemed apparent for some time that expression of mutated genes for type I procollagen in transgenic mice would produce the same phenotypes as seen in patients with OI. Stacey et al. (5) reported preparation of transgenic mice expressing a mutated proα1(I) gene in which a codon for cysteine was substituted for a codon for glycine. Transgenic mice expressing the gene died shortly after birth or after cesarean section just before term. Several of the mice had unusually pliable limbs and unusually soft cranial bones. The mice, therefore, had some phenotypic changes seen in probands with OI. However, no breeding lines were developed. More recently, we prepared transgenic mice expressing an internally deleted human gene for the proα1(I) chain of type I procollagen (6). The gene construct was modeled after a sporadic in-frame deletion of the human gene that produced a lethal variant of OI by causing biosynthesis of shortened proα1(I) chains and procollagen suicide (1–4). Mice expressing relatively high levels of the transgene developed a lethal phenotype with extensive fractures of ribs and long bones similar to the fractures seen in lethal variants of OI (6).

Here, we have examined a line of mice expressing moderate levels of the internally deleted gene for the proα1(I) chain of human type I procollagen. We demonstrated that the mice can be developed into a breeding line that is characterized by a

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1. *Abbreviation used in this paper:* OI, osteogenesis imperfecta.

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phenotype of fractures, a decrease in the content of mineral and collagen in bone, and a mechanically brittle bone matrix.

Methods

Analysis of DNA from transgenic mice. Transgenic mice were prepared as described previously (6) in an inbred strain of mice (FVB/N). A line expressing moderate levels of the internally deleted COL1A1 gene (line V in reference 6) was selected for further study here. Transgenic mice in litters from the line were identified either by polymerase chain reaction (PCR) analysis (7) or by Southern hybridization (8). For PCR analysis, one toe was cut from each newborn pup and the tissue was incubated at 55°C for 3 h in 100 μl of 10 mM Tris buffer (pH 8.3) containing 50 mM KCl, 5 mM $MgCl_2$, 0.1 mg/ml gelatin, 1% polyoxylene laurel ester (Brij 35) and 40 mg proteinase K. The sample was boiled for 5 min, after which a 5-μl aliquot was taken for the PCR reaction. Oligomers A7 (5'-CCTCTCCATTCCAACTCCCA) and A8 (5'-CGCGATATAGAGTATCCTTGC) used for the PCR were located at −147 bp and +266 bp with respect to the start of transcription of the COL1A1 gene (9). For Southern analysis, genomic DNA was extracted from cut sections of tail (6). The DNA was digested with the restriction enzyme SacI, fractionated on a 1% agarose gel, and blotted onto a nylon filter. Filters were probed with the ^{32}P-radiolabeled transgene (6).

Protein assay. To assay the expression of the transgene as protein, ~ 50 mg of tissue was homogenized in 0.5 ml of 4 M guanidine thiocyanate, 0.25 M EDTA, 10 mM *N*-ethylmaleimide, and 1 mM *p*-aminobenzamidine (6). Samples were electrophoresed on 4 to 15% polyacrylamide gradient gels (Bio-Rad Laboratories, Richmond, CA) and the protein was electroeluted onto a filter (Immobilon P; Millipore Corp., Bedford, MA). The filter was incubated with a polyclonal antibody that reacted with the COOH propeptide of the proα1(I) chain from both mice and humans (6, 9). The antibody was kindly provided by Dr. Larry Fisher, National Institute of Dental Research, National Institutes of Health. The secondary antibody was anti-rabbit IgG coupled to horse radish peroxidase (Promega Corp., Madison, WI). Antibody binding was visualized on x ray film by the chemiluminescence produced by oxidation of a cyclic diacylhydrazine (Luminol; Amersham Corp., Arlington Heights, IL) by horseradish peroxidase in the presence of hydrogen peroxide.

Collagen assay of bone specimens. Freshly dissected femurs were first crushed in a mechanical grinder (Tissuemizer; Tekmar Co., Cincinnati, OH) and then homogenized with a motor-driven sintered glass homogenizer. The homogenate was dialyzed for 4 d against 0.5 M EDTA (pH 8.0) followed by a 1-d dialysis against water. The sample was hydrolyzed in 6 N HCl at 100°C overnight and hydroxyproline was assayed (10). Collagen content was calculated from the hydroxyproline values by assuming a content of 10.11% (11).

Assay of bone mineral. The wet weight of freshly dissected bones was assayed. To determine mineral content, the bones were wrapped in aluminum foil and reduced to mineral by heating at 600°C for 18 h after which they were weighed again. Preliminary experiments indicated there was no further decrease in weight if samples were incubated at 600°C for more than 18 h.

Biomechanical testing. A three-point bending test was performed on a commercial instrument (Universal Testing Machine; Instron Corp., Canton, MA) as described by Kiebzak et al. (12). The femurs were dissected free of soft tissues under a dissecting microscope, and the ends of the bone were supported on two fulcra separated by 5 mm. The load was applied to the anterior midshaft at a constant speed of 10 mm/min until failure.

A chart recorder was used to generate a force-deformation curve (12). Ultimate force, ultimate deformation, and ultimate energy were determined directly from the curve. The stiffness was assessed as the slope of the force-deformation curve. Bone endosteal and periosteal diameters, parallel and perpendicular to the line of force, were measured (Fig. 1). The diameters were used to calculate the area moment of inertia using the formula for an ellipse (12). The area moment of inertia (I) describes the distribution of the bone mass around the central axis, i.e., the bone geometry at the site at which the force is applied. It was determined by the equation

$$I(m^4) = \frac{\pi}{64}(BD^3 - bd^3)$$

in which B, D, b, and d are defined as in Fig. 1. The whole bone three-point load behavior is a function both of bone material and bone material distribution. The calculated area moment of inertia describes the bone distribution and allows the properties to be reduced to material properties. From the measured mechanical whole bone properties and from the area moment of inertia calculated from the bone diameters, the material properties of ultimate stress, ultimate strain, and Young's modulus can be calculated. These material properties are directly related to the matrix properties and have no relationship to the size of the bone or the size of the animal. The calculations then allow direct comparison of the matrix properties between different experimental groups.

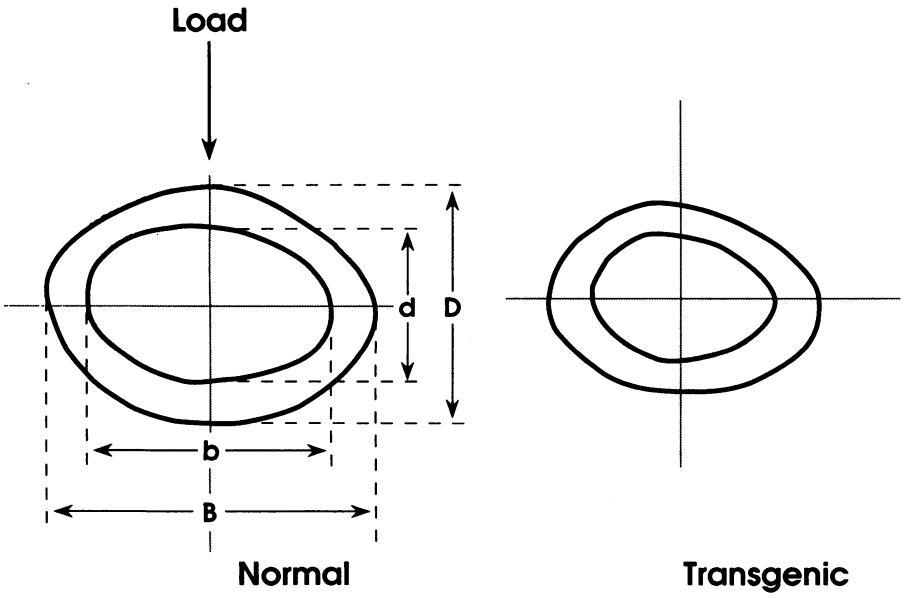

Figure 1. Diagrams of cross-sections of femurs from control and transgenic mice. After the bones were broken, the cross-sections were drawn with a camera lucida. The distances B, D, b, and d were indicated as defined to calculate the second moment of area (12). Femurs from the transgenic mice were smaller than from controls.

The ultimate stress (σu) is calculated as (12)

$$\sigma u\,(N/m^2) = \frac{Fu \times L \times C}{4 \times I}$$

where Fu is the ultimate force, L is the distance between the fulcra (5×10^{-3} m), and C is one-half of the diameter (D in Fig. 1).

The ultimate strain (ϵu) is calculated as (12)

$$\epsilon u = \frac{12 \times Du \times C}{L^2}$$

where Du is the ultimate deformation.

Young's modulus of elasticity (E) is calculated as (12)

$$E(N/m^2) = \frac{K \times L^3}{48 \times I}$$

where K is the stiffness.

Skeletal staining. Viscera and skin were removed from mouse carcasses before fixation in 95% ethanol. Length of fixation time varied from 3 to 7 d depending on the size of the animal. The carcasses were then dehydrated in 100% ethanol for 2–5 d. They were immersed in 1% KOH for 1 d to dissolve the soft tissues (13). The skeletons were stained in 1% KOH containing 0.001% alizarin red S for 1–2 d, after which they were taken through 25, 50, and 80% glycerol for 1 d each and finally stored in 100% glycerol.

Results

Phenotype of the mice. An internally deleted construct of the human gene for the proα1(I) chain of type I procollagen was used to prepare transgenic mice in an in-bred line (6, 9). The gene contained 2.5 kb of the promoter and 2 kb of the 3′-flanking sequence. Exons 6–46 and the adjacent introns were deleted by joining intron 5 to intron 45. Therefore, the gene caused synthesis of shortened proα1(I) chains that were unable to fold into a stable triple helix but associated with normal proα1(I) chains and caused depletion of normal type I procollagen through procollagen suicide (4, 6, 9). Progeny from a series of microinjected fertilized ova were examined for the presence of the exogenous gene and for phenotypic changes. Transgenic mice from one line (line V) were found to develop fractures but surviving mice bred effectively. Therefore, the line was selected for further study here.

Southern blot assay indicated that transgenic mice had ~ 20 copies of the exogenous gene (not shown). Expression of the exogenous gene as protein was assayed with a polyclonal antibody that cross-reacted with mouse and human proα1(I) chains. Assays of the founder mouse (not shown) and of transgenic progeny (Fig. 2) indicated that proα1(I) chains from the exogenous gene were synthesized together with proα1(I) chains from the endogenous mouse gene (Fig. 2). Densitometry of x ray films of the Western blots indicated that the ratio of shortened proα1(I) chains to normal proα1(I) chains was ~ 0.50. Values of 0.40±0.12 SD were reported previously (6).

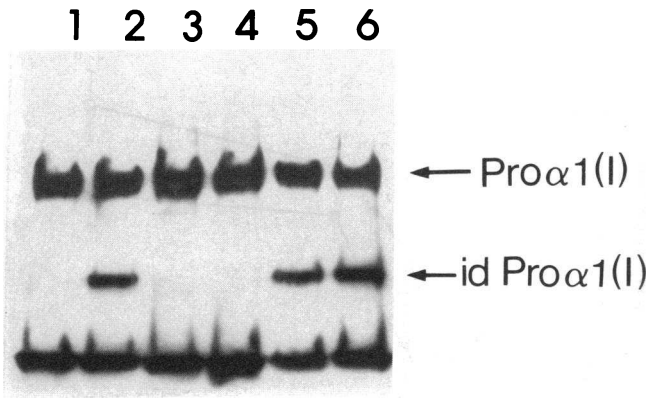

Figure 2. Western blot assay of expression of the exogenous and endogenous COL1A1 genes in transgenic mice. Skin from control and transgenic mice was homogenized and Western blotted as indicated in text. Lanes *1*, *3*, and *4*: controls. Lanes *2*, *5*, and *6*: transgenic mice.

Transgenic mice from the line were bred and 25 litters were examined. As indicated in Table I, 5 of 89 or 6% of the transgenic mice died shortly after birth with extensive fractures. X ray examination of the surviving transgenic mice from five litters indicated that ~ 33% had detectable fractures at the day of birth. No fractures were seen in the remaining transgenic mice. There was no apparent difference in the severity of the phenotype in male and female transgenic mice. The male/female ratio was about the same in transgenic mice and normal littermates.

In further experiments, brother-sister matings were carried out between transgenic siblings. Eight litters from such breedings were examined. 19 of 48 or 40% of the transgenic mice died shortly after birth with extensive fractures (Table I). Homozygous and heterozygous mice could not be distinguished because of the high copy number of the exogenous gene in the line. The results indicated, however, that expression of the exogenous gene was more lethal in putative homozygous mice from the line. Also, the results indicated that the phenotype was transmitted by both male and female transgenic mice.

The transgenic mice were the same size and weight as normal littermates at birth (Fig. 3). However, the transgenic mice grew more slowly than littermates and the difference in body weight persisted for up to 90 d (Fig. 3). Also, the transgenic mice had thinner skin at birth (not shown). The skin continued to appear more delicate thereafter, but the difference in

Table I. Summary of Mouse Phenotypes at Birth

	Mice		Phenotype of transgenic mice*		
	Total	Transgenic	Lethal with fractures	Nonlethal with fractures	No fractures
idCOL1 × Wt‡	195	89	6%	33%§	61%§
idCOL1 × idCOL1	70	48	40%	ND	ND

* Fractures detected by x ray. ‡ Matings of transgenic males with wild-type females from the same line of mice (FBV/N). Ratio of males to females was 1.20 in 55 transgenic mice versus 1.04 in 57 normal littermates. § Values based on x ray examination on day of birth of 15 viable transgenic mice from five litters.

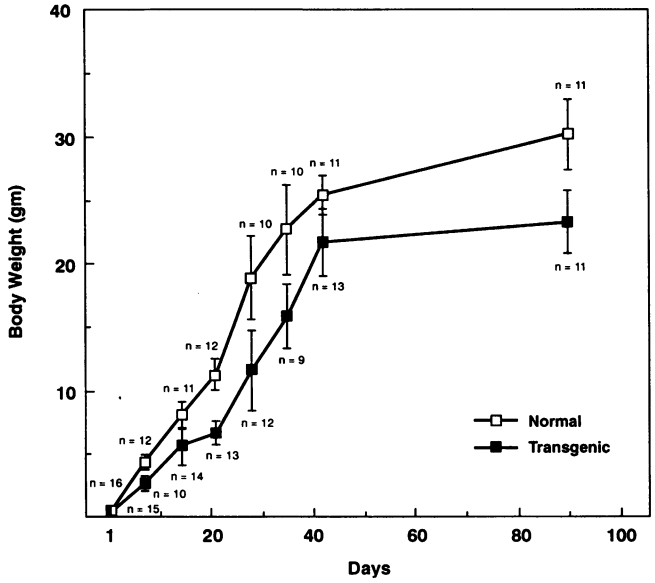

Figure 3. Growth curves for normal and transgenic mice. Bars indicate standard deviation. Numbers on bars indicate number of mice weighed.

skin between the transgenic mice and control littermates was not defined quantitatively. At three months, most of the transgenic mice in the first few litters had loose and broken incisors (Fig. 4). Subsequent litters were offered both a pelleted diet and a powdered diet. Thereafter, fewer broken teeth were seen, but the teeth grew abnormally thin and long (not shown).

Staining of skeletons from the newborn mice demonstrated that about one-third of the transgenic mice had readily apparent fractures of long bones and ribs (Fig. 5). In older mice healing fractures and callus formation were seen (Fig. 6).

Chemical composition of bone from the transgenic mice. Femurs from the normal male littermates and transgenic male mice were assayed for mineral content by ashing the bones. Also, they were assayed for collagen content by assay of nondialyzable hydroxyproline. As indicated in Table II, the length and the wet weight of the femurs were less than in normal littermates. Also, both the mineral and collagen content were less than the normals. The standard deviations were larger in the transgenic mice for the mean values of length, weight, and mineral content, apparently because of the variation in phenotype. However, the ratio of mineral to collagen did not show any significant difference. The results indicated, therefore, there was a proportional decrease in the mineral and collagen content of bone.

Biomechanical measurements of bone. Femurs from the transgenic male mice and normal male littermates at 6 wk of age were assayed biomechanically with a three-point bending test. The stiffness of femurs from the transgenic mice was not significantly different from controls (Table III). However, the force to failure was decreased ($P < 0.01$) and the energy to

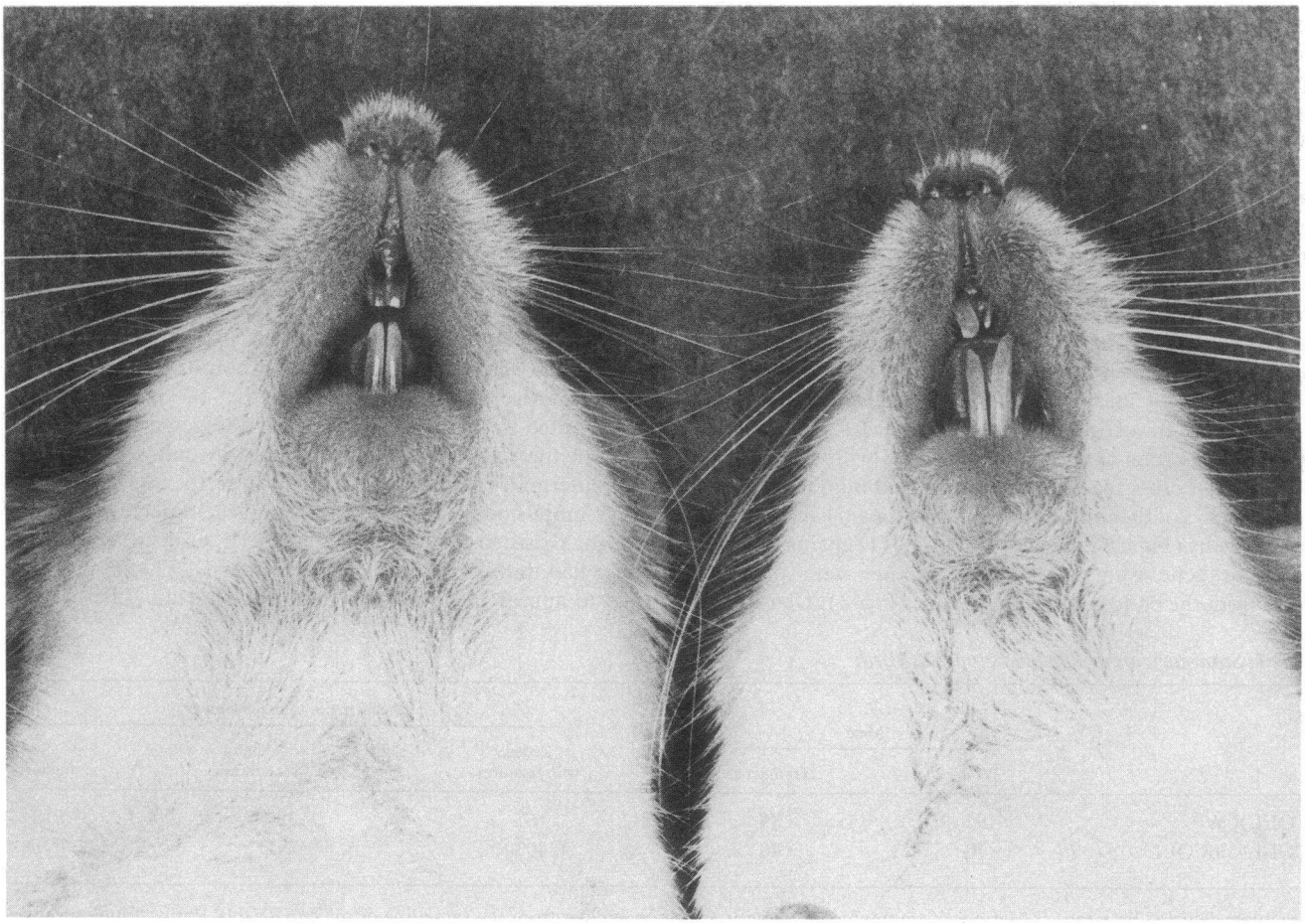

Figure 4. Photographs of normal (*left*) and transgenic (*right*) mice at 3 mo of age. The incisors in the transgenic mouse were longer, looser, and irregular.

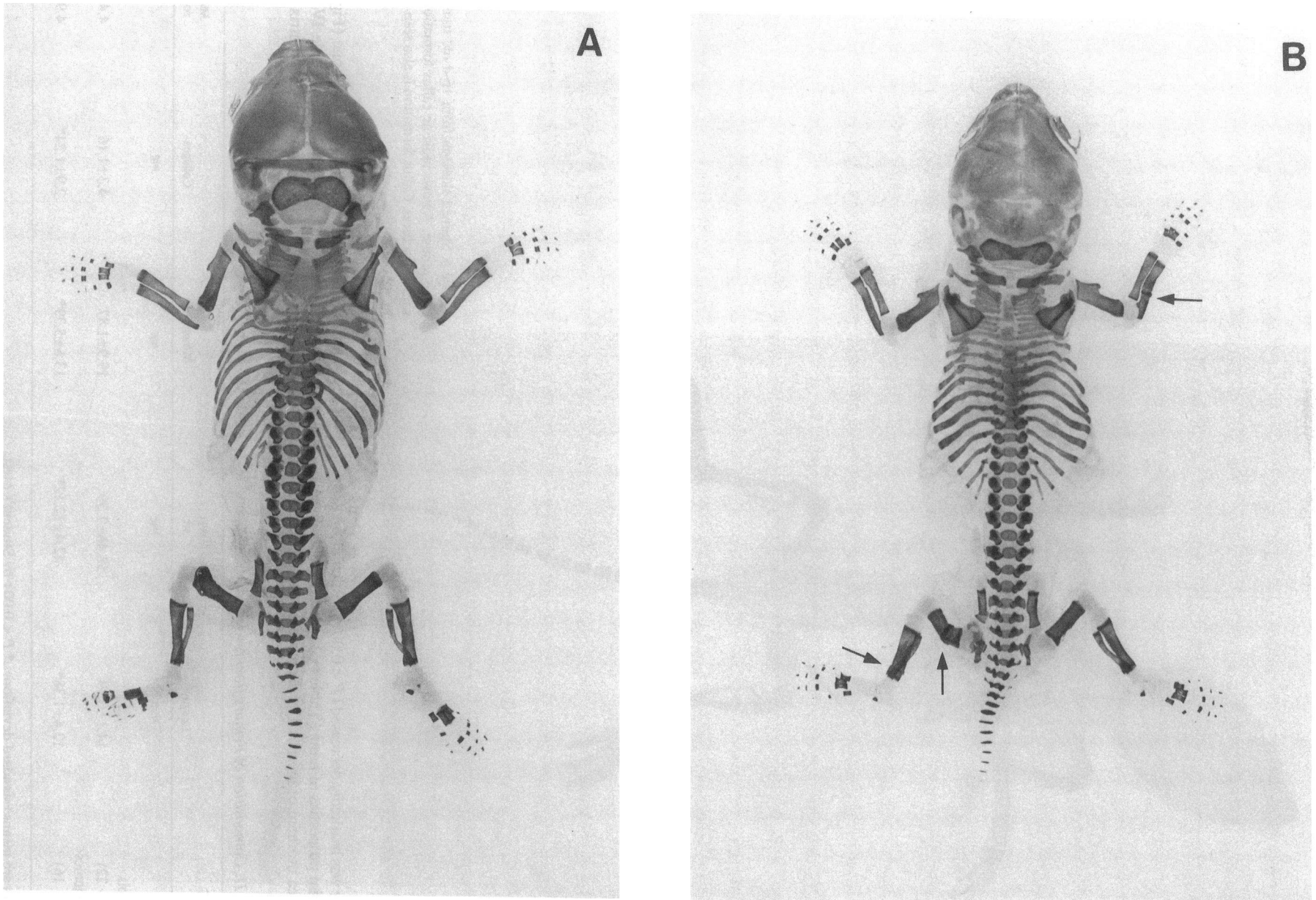

Figure 5. Stained skeletons from newborn mice. Control littermate (*A*) and transgenic mouse (*B*). Arrows indicate sites of fractures.

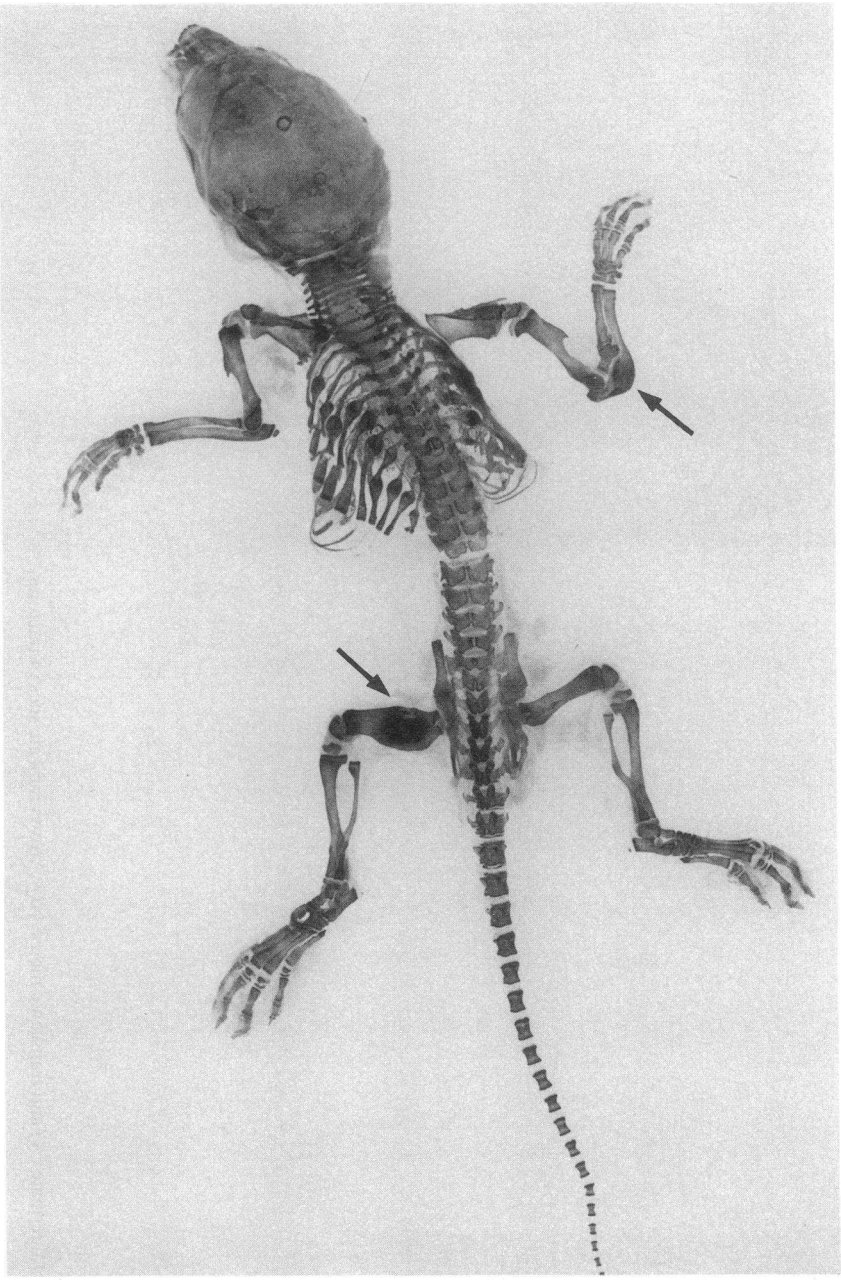

Figure 6. Stained skeleton from 16-d old transgenic mouse. Arrows indicate callus formation at sites of fractures. Callus formation is also seen on many of the ribs.

failure was markedly decreased ($P < 0.001$). Calculation of the material properties of bone (Table IV) indicated a decrease in the area moment of inertia. The decrease in area of bone mass was also apparent by visual inspection of the bone (Fig. 1). There was a decrease in the ultimate strain (Table IV), an observation indicating an increased brittleness of matrix. The

Table II. Composition of Femurs from 6-wk Old Male Mice

	Length	Wet wt	Mineral	Collagen	Mineral/ collagen
	mm	*mg*	*mg*	*mg*	
Controls (n = 22)	14.1±0.39	58.46±7.39	19.9±1.87	4.3±1.16	4.4±1.26
Transgenics (n = 16)	12.4±1.38*	42.4±12.12*	13.6±5.39*	2.9±1.24*	4.9±1.18

Values are means±standard deviation. * $P < 0.001$ by Student's t test.

Table III. Biomechanical Measurements on Femurs from 6-wk Old Male Mice

	K (Stiffness)	Fu (Force to failure)	Eu (Energy to failure)
	$N/m \times 10^4$	N	$N.m \times 10^{-3}$
Controls ($n = 22$)	3.9±0.74	11.6±1.46	6.8±2.61
Transgenic ($n = 16$)	3.3±1.37	9.3±3.35*	3.7±1.07‡

Values are means±standard deviation. * $P < 0.01$ by Student's t test. ‡ $P < 0.001$.

Young's modulus of elasticity was increased apparently because there was a minimal decrease in stiffness (Table III) but a larger decrease in the area moment of inertia (Table IV). As shown in Tables III and IV, the standard deviations for the mean values of most of the biomechanical parameters were larger in the transgenic mice, apparently because of phenotypic variation.

Discussion

The results here demonstrate that a gene construct causing synthesis of mutated proα1(I) chains of type I procollagen can be used to develop breeding lines of transgenic mice with defects of bone. The line examined expressed lower levels of the same internally deleted gene for the proα1(I) chain of type I procollagen than mice that developed a consistently lethal phenotype similar to lethal variants of OI (6). Expression of the internally deleted transgene paralleled expression of the endogenous gene in a tissue-specific and age-specific manner (14). About 6% of the mice in the line died shortly after birth with extensive fractures, but the remaining transgenic mice were viable. About one-third of the surviving transgenic mice had extensive fractures as assessed by x ray, but all the surviving mice matured sexually and were capable of breeding. The defects in bone were directly demonstrated by decreases in the collagen content and the mineral content. The defects in bone were also demonstrable by assaying the biomechanical properties. As expected, it was possible to increase the severity of the phenotype by brother-sister breeding of the line (Table I).

Previous attempts to develop transgenic mice with defects in bones did not produce breeding lines with spontaneous fractures. In the case of transgenic mice prepared with a mutation in a glycine codon in the proα1(I) chain of type I procollagen, all the mice died at birth or shortly before and none had demonstrable fractures (5). Some of the phenotypic features of OI, including more fragile bones, were seen in mice in which synthesis of type I procollagen was decreased because of a fortuitous insertion of a retrovirus into the first intron of the COL1A1 gene (15, 16). However, no spontaneous fractures (15, 16) were reported in the mice. More recently, spontaneous fractures (17) were observed in a line of mice with a naturally occurring mutation in the gene for the proα2(I) chains of type I procollagen (COL1A2). The fractures were seen in homozygous mice and were associated with progressive skeletal dysplasia and scoliosis.

Table IV. Calculated Material Properties of Femurs from 6-wk Old Male Mice

	I (Area moment of inertia)	σu (Ultimate stress)	εu (Ultimate strain)	E (Young's modulus of elasticity)
	$m^4 \times 10^{-13}$	$N/m^2 \times 10^7$		$N/m^2 \times 10^8$
Controls ($n = 22$)	1.4±0.32	6.8±1.24	0.13±0.02	7.5±1.97
Transgenics ($n = 16$)	0.8±0.33*	8.1±2.4	0.09±0.02‡	10.9±3.31*

Values are means±standard deviation. * $P < 0.001$; ‡ $P < 0.05$.

Biomechanical assays on femurs from the transgenic mice demonstrated that there was a decrease in the force and energy to failure. There was also a decrease in the area moment of inertia and ultimate strain. The results indicated that the bone matrix was brittle and that there was a decrease in the distribution of bone mass around the central axis. Therefore, the femurs from the transgenic mice demonstrated both abnormal matrix mechanical properties and abnormal modeling that together produced an increased risk of fractures.

Of special interest was the observation that not all the transgenic mice from the line developed spontaneous fractures even though the transgene was expressed in an inbred strain of mice. Breeding the mice through several generations repeatedly produced litters in which about one-third of the transgenic mice had fractures and ~ 6% had a lethal phenotype (Table I). Similar phenotypic variations are observed among affected members of families with mutations in type I procollagen genes that cause OI (1–3). The phenotypic variations in the families are frequently ascribed to variations in the genetic background of individual members of a family. This explanation is difficult, however, to apply to the phenotypic variations observed here. We are currently exploring the possibility that the phenotypic variation may be explained by variable levels of expression of the transgene.

Over 100 different mutations in the two genes for type I procollagen (COL1A1 and COL1A2) have been found in probands with OI (1–3). The most common mutations are single-base substitutions that introduce a codon for a bulkier amino acid in place of a codon for one of the glycine residues that is present as every third amino acid in the triple helical domain of the protein. Other mutations causing OI include RNA processing mutations, partial deletions, and insertions into the genes. Although gene deletions are relatively rare, internal in-frame deletions of the kind made in the transgene used here have more predictable consequences than other mutations in that they cause synthesis of shortened proα1(I) chains that produce procollagen suicide (1–4).

The fractures and decrease in bone collagen and mineral seen in the line of transgenic mice indicate that they have a phenotype similar to the phenotypes seen in many probands with OI expressing spontaneously mutated genes for type I procollagen (1–3). As seen in some variants of OI, the mice appeared to improve after birth in that there were fewer fractures and the difference in their appearance from control litter mates became less apparent with age (not shown). The mice should be appropriate for studying the pathoetiology of OI in terms of specific changes that occur in embryonic development in bone growth and morphogenesis. Also, the mice should be appro-

priate models for testing treatments for OI. In particular, the mice should provide a model for studying antigene or antisense strategies to specifically prevent expression of mutated alleles for collagen (18). Recent observations indicate that mutations in the genes for type I procollagen may also cause some forms of osteoporosis (19). Therefore the same transgenic mice may also be useful models for studying osteoporosis.

Acknowledgments

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