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Genetics

# Expression of a partially deleted gene of human type II procollagen (*COL2A1*) in transgenic mice produces a chondrodysplasia

(type II collagen/deletion mutations/collagen depletion)

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ABSTRACT **A minigene version of the human gene for type II procollagen (*COL2A1*) was prepared that lacked a large central region containing 12 of the 52 exons and therefore 291 of the 1523 codons of the gene. The construct was modeled after sporadic in-frame deletions of collagen genes that cause synthesis of shortened proα chains that associate with normal proα chains and thereby cause degradation of the shortened and normal proα chains through a process called procollagen suicide. The gene construct was used to prepare five lines of transgenic mice expressing the minigene. A large proportion of the mice expressing the minigene developed a phenotype of a chondrodysplasia with dwarfism, short and thick limbs, a short snout, a cranial bulge, a cleft palate, and delayed mineralization of bone. A number of mice died shortly after birth. Microscopic examination of cartilage revealed decreased density and organization of collagen fibrils. In cultured chondrocytes from the transgenic mice, the minigene was expressed as shortened proα1(II) chains that were disulfide-linked to normal mouse proα1(II) chains. Therefore, the phenotype is probably explained by depletion of the endogenous mouse type II procollagen through the phenomenon of procollagen suicide.**

Skeletal dysplasias in man are a heterogeneous group of >80 heritable disorders that are characterized by abnormalities in the size and shape of limbs and trunk and that usually produce short stature (see ref. 1). Many appear to involve defects in cartilage and, therefore, are referred to as chondrodysplasias. Several recent reports (2–8) indicate that some variants of the subgroup known as spondyloepiphyseal dysplasias are caused by mutations in the *COL2A1* gene for type II procollagen, the precursor of the major collagen of cartilage.

The data available to date on mutations in type II procollagen suggest a parallel to the mutations in the two genes for type I procollagen (*COL1A1* and *COL2A1*) that cause osteogenesis imperfecta (OI), a group of heritable disorders characterized by fragility of bone and other tissues rich in type I collagen (9–11). It is reasonable to assume, therefore, that the mutations in the type II procollagen gene recently found in probands with chondrodysplasias cause the disease phenotypes. However, linkage studies with the *COL2A1* gene have been performed on only a few families with mild chondrodysplasias. Also, cartilage is only rarely available from probands, and human cartilage cells do not grow well in culture (12). Therefore, it has been difficult to establish the causal link between mutations in the *COL2A1* gene and any effects that the mutations may have on either the biosynthesis of type II procollagen or the functional properties of the protein.

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Here we have prepared transgenic mice expressing a minigene version of the human *COL2A1* gene. The design of the gene construct was based on several mutations in the genes for type I procollagen that produced in-frame deletions in codons for the repeating -Gly-Xaa-Yaa- amino acid sequence of the collagen triple helix and caused lethal variants of OI (for reviews, see refs. 9 and 10). Because the collagen triple helix is formed by a series of hydrogen bonds and water bridges that link the -Gly-Xaa-Yaa- sequences in one proα chain to equivalent -Gly-Xaa-Yaa- sequences in the two other proα chains (see ref. 13), the presence of one shortened proα chain in a procollagen molecule can prevent folding into a stable triple helix and degradation of all three proα chains in a process referred to as "procollagen suicide" (11, 14, 15).

## MATERIALS AND METHODS

**Gene Construct.** The gene construct was prepared from a cosmid clone containing the human *COL2A1* gene (ref. 16; kindly provided by Francesco Ramirez, Brookdale Center for Molecular Biology, Mt. Sinai Medical Center, New York). The insert in the cosmid was cleaved with *Xba* I, *Sph* I, and *Cla* I to generate four fragments ranging in size from 5 to 12 kilobases (kb) (Fig. 1). Three of the fragments were then assembled into a modified cosmid vector (17, 18) by four-way ligation. The insert was digested with *Sal* I, electrophoresed in an agarose gel, electroeluted, extracted with phenol/chloroform/isoamyl alcohol (24:24:1), ethanol precipitated, and dissolved in 1 mM EDTA in 10 mM Tris·HCl buffer (pH 7.4) for microinjection.

**Preparation of Transgenic Mice.** One-cell stage mouse embryos (19) were obtained by mating of inbred FVB/N males and females. The partially deleted *COL2A1* gene was microinjected into embryos at a concentration of 2 μg/ml with about 600 copies per embryo. Inbred CD1 females were used as the pseudopregnant recipients.

**Assay of Protein and DNA from Transgenic Mice.** For the experiments with chondrocytes, joint cartilage was dissected and digested for 2 hr with 1:1 (wt/wt) collagenase (Boehringer Mannheim) in Dulbecco's modified Eagle's medium (DMEM) (20). Matrix-free chondrocytes obtained (typically $5 \times 10^5$ cells) were cultured in suspension for 4 hr at 37°C in 100 μl of the above DMEM containing 10 μCi of [$^{14}$C]proline (1 Ci = 37 GBq; NEN), 10% fetal calf serum (GIBCO), and 5 mM EDTA. Samples of cells and medium (15) were electrophoresed in SDS/polyacrylamide gels with a 3.5%

Abbreviations: *COL2A1*, gene for type II procollagen; *COL1A1*, gene for proα1(I) chain of type I procollagen; OI, osteogenesis imperfecta; hproα1(II)$^S$, shortened chain of human type II procollagen; hα1(II)$^S$, shortened chain of human type II collagen; mproα1(II), proα1(II) chain of mouse type II procollagen; m/hproα1(II), disulfide-linked hybrid molecules of hproα1(II)$^S$ and mproα1(II) chains.

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stacking and 6% separating gel using a minielectrophoresis unit (Protean II, Bio-Rad). $^{14}$C-labeled proteins were assayed with storage phosphor screens (PhosphorImager; Molecular Dynamics, Sunnyvale, CA). Proteins from the gels were then electroblotted onto nitrocellulose and allowed to react with polyclonal antibodies specific for the COOH-terminal telopeptide of the human α1(II) chain of type II collagen (S. Jimenez, C. Merryman, L.A.-K., R. Jankowski, and D.J.P., unpublished data). The secondary antibodies were anti-rabbit IgG coupled to alkaline phosphatase (Promega).

For assay of DNA, a terminal piece of tail was minced in 0.7 ml of 100 mM NaCl/100 mM EDTA/1% SDS in 50 mM Tris·HCl buffer (pH 8.0) and digested overnight at 55°C with 0.5 μg of proteinase K per ml. The digested sample was extracted with phenol/chloroform/isoamyl alcohol (24:24:1) and then extracted with chloroform/isoamyl alcohol (24:24:1). The DNA was precipitated with ethanol and dissolved in 1 mM EDTA in 10 mM Tris·HCl buffer (pH 7.4). For copy number, filters were probed with a 10-kb *Eco*RI–*Eco*RI fragment from the human gene and a 15-kb *Eco*RI–*Eco*RI fragment of the mouse gene for type II procollagen (S.-W. Li, J.S.K., and D.J.P., unpublished data).

**Skeletal Morphology and Histology.** Mineralized tissue of selected newborn mice was stained with alizarin red S (21). For microscopy of tissues, whole embryos were perfused with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) shortly after birth. Tissues were decalcified by incubation in 10% EDTA (pH 7.4) for 2–6 days. Samples were postfixed in 1% osmium tetroxide in 0.15 M sodium cacodylate buffer (pH 7.4) for 2–4 hr at 20°C, washed three times with the cacodylate buffer, and then placed in 1% tannic acid in 0.15 M sodium cacodylate buffer (pH 7.4). For polarized light microscopy, specimens embedded in paraffin were stained with sirius red (picrosirius staining) after incubation in xylol at 37°C overnight and with 2 mg of hyaluronidase per ml (Sigma) in 0.1 M phosphate buffer (pH 6.9) at 37°C overnight. The sections were analyzed with an Ortholux 2 Pol-Bk microscope (Leitz) operated in monochromatic light [λ = 543 nm; filter IL 543 (Schott, Mainz, FRG)] and using the de Senarmont compensation technique (22). For electron microscopy, samples embedded in Epon were cut into 50- to 60-nm sections, stained with uranyl acetate for 30 min and lead citrate for 2–4 min, and examined with a JEM-1200 EX electron microscope (JEOL).

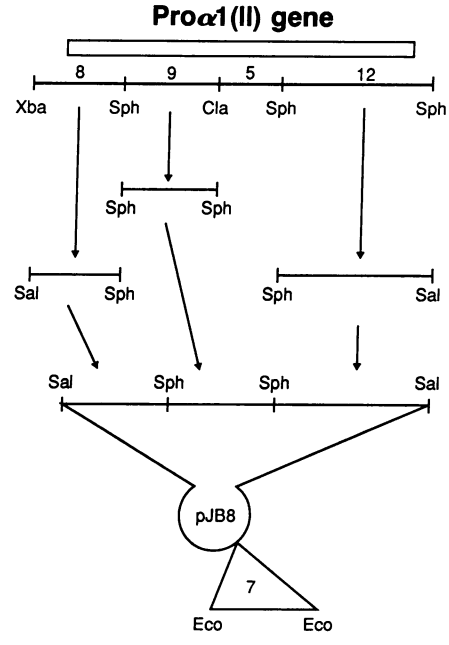


FIG. 1. Minigene construct of the human gene for type II procollagen (see text).

## RESULTS

**Transgenic Mice Expressing the Minigene Construct.** The minigene construct (Fig. 1) was designed so as to create a deletion that extended from intron 15 to intron 27 and eliminated 12 exons of the 52 exons of the gene. The deleted exons contained 291 of the 1523 codons. The partially deleted gene was in-frame in terms of the coding sequences and the requirement for glycine as every third amino acid in the collagen triple helix (see ref. 23).

Five lines expressing the gene construct were prepared (Table 1). Four of the five $F_0$ founders had slightly shortened limbs but no definitive phenotype. The $F_0$ founder of the fifth line (line 5 in Table 1) was phenotypically normal, and several observations suggested that he was mosaic for the transgene: (*i*) the copy number in tail tissue from the $F_0$ mouse was lower than in tail tissue from $F_1$ transgenic mice from the line, (*ii*)

Table 1. Phenotypes of transgenic mice

| Line | Generation | Transgenic mice | Gene copy number | Transgenic mice: Dwarfism* | Delayed mineralization† | Cleft palate | Dead at birth |
|---|---|---|---|---|---|---|---|
| 5 | $F_0$‡ | | 4 | 0 | 0 | 0 | |
| | $F_1$ | 25/135 | 12 | 25/25 | 25/25 | 25/25 | 25/25 |
| 7 | $F_0$ | | 2 | ± | ± | 0 | |
| | $F_1$ | 60/156 | 2 | 60/60 | 60/60 | 10/60§ | 20/60 |
| | $F_2$¶ | 80/118 | 2, 4 | 80/80 | 80/80 | 28/80§ | 39/80 |
| 42 | $F_0$‡ | | | ± | ± | 0 | |
| | $F_1$ | 1/30 | | 1/1 | ND‖ | 0/1 | 0/1 |
| 44 | $F_0$ | | | ± | ± | 0 | |
| | $F_1$ | 9/14 | | 9/9 | 9/9 | 2/9§ | 2/9 |
| 47 | $F_0$ | | | ± | ± | 0 | |
| | $F_1$ | 1/2 | | 1/1 | 1/1 | 0/1 | 0/1 |

*Short and thick limbs, short snout, and cranial bulge (see Figs. 2 and 3).
†Less than 10 mineralized caudal vertebrae visible in newborn mice in roentgenograms (not shown) or in alizarin red S-stained skeletons (Fig. 3). In control newborn mice, 11 caudal vertebrae were mineralized.
‡Probable mosaic founder (see text).
§All mice with cleft palate died at birth or shortly thereafter.
¶Mice from $F_1 \times F_1$ matings. $F_1$ transgenic mice from line 7 had a gene copy number of 2. Among $F_2$ transgenic mice, all mice with a copy number of 4 had the severe and lethal phenotype, but some with a copy number of 2 also had the severe and lethal phenotype.
‖Not determined.

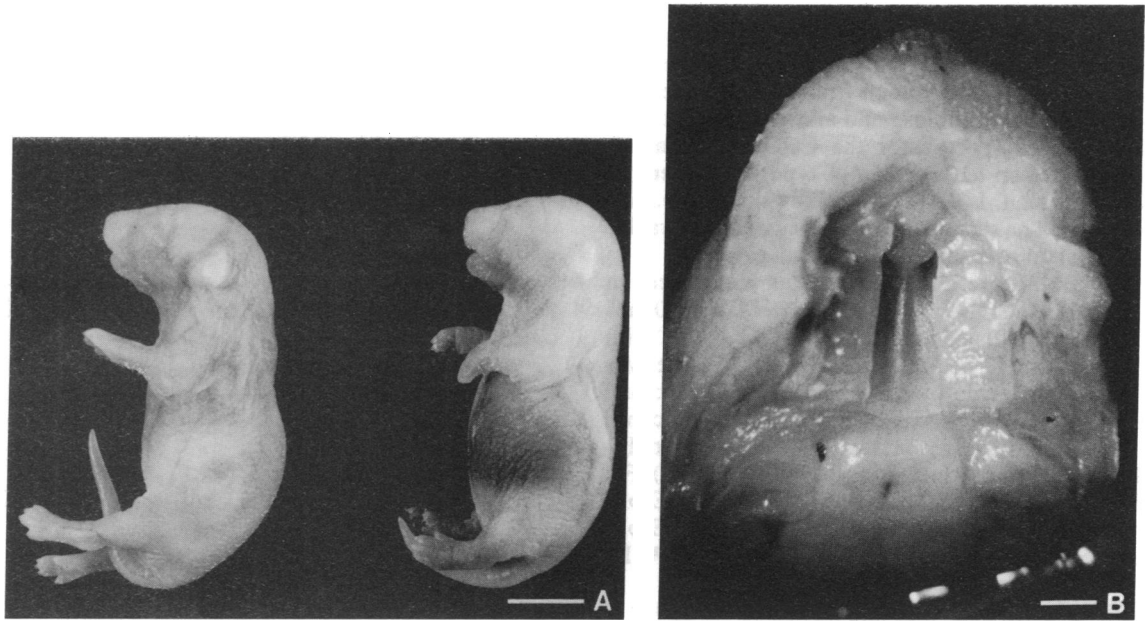


FIG. 2. (*A*) Photograph of normal (left) and transgenic (right) newborn mice. The transgenic mouse shows short limbs, short tail, short snout, and a cranial bulge. (Bar = 5 mm.) (*B*) Caudocranial view of a cleft palate from a gene-positive $F_2$ mouse of line 7 (Table 1). For better visualization, the mandible was removed. (Bar = 1 mm.)

all $F_1$ transgenic mice had a severe phenotype (see below) and died shortly after birth, and (*iii*) only 19% of the progeny of the $F_0$ founders inherited the transgene (Table 1). The $F_0$ founder of line 42 (Table 1) may also have been a mosaic, since only 1 of 30 $F_1$ mice inherited the gene. In the other lines, the absence of a distinctive phenotype in the $F_0$ founders may have been explained by its mildness, or a failure to examine the mice thoroughly before maturity when the skeletal changes became less apparent.

Some of the transgenic $F_1$ mice from three separate lines (line 5, line 7, and line 44 in Table 1) had a severe phenotype. The severe phenotype in each of the three lines was indistinguishable. The newborn mice were smaller than most other littermates and many died shortly after birth. X-ray films (not shown) showed no air in the lungs and, therefore, death was probably caused by respiratory failure. The mice had short limbs, a short tail, a short snout, and a cranial bulge (Figs. 2*A* and 3). Most of the pups had a wide cleft palate (Fig. 2*B*). Staining of the skeleton with alizarin red S demonstrated that the bones were short and thick compared to controls (Fig. 3). In addition, there was delayed mineralization of bone (Fig. 3 and Table 1).

Although some mice from each of three lines had the same severe phenotype, there was some variability in phenotype among transgenic littermates in two of the lines. With line 7, all $F_1$ transgenic mice were dwarfs and showed delayed mineralization of bone. However, only one-fifth of the transgenic mice had a cleft palate (10/60) and only one-third died at birth or shortly thereafter (20/60). Several of the surviving $F_1$ transgenic mice from line 7 had increased cervical lordosis on x-ray examination (not shown) and two developed a spastic lower leg paresis, apparently secondary to the increased cervical lordosis. As expected, the incidence of the lethal phenotype increased in line 7 when $F_2$ mice were prepared by in-breeding of $F_1$ animals (Table 1). However, some of the $F_2$ apparent heterozygotes had no obvious cleft palate and were viable. A similar variability in phenotype was seen in line 44 (Table 1) in that only 2 of 9 $F_1$ transgenic mice had a cleft palate and died at birth or shortly thereafter.

**Expression of the Minigene in Mouse Tissues.** Western blots of tissue extracts revealed that the minigene was expressed

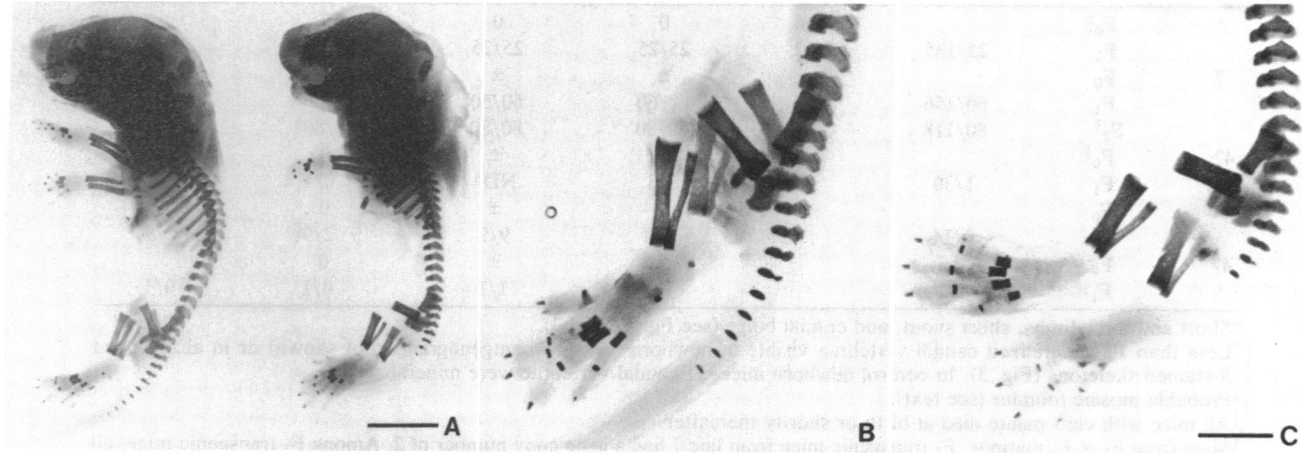


FIG. 3. Skeleton of normal (left) and transgenic (right) mouse. Mineralized tissues were stained with alizarin red S. (*A*) Photograph of whole skeletons. (Bar = 5 mm.) (*B*) Enlarged photograph of control mouse. (*C*) Enlarged photograph of transgenic mouse. (Bar = 2 mm.)

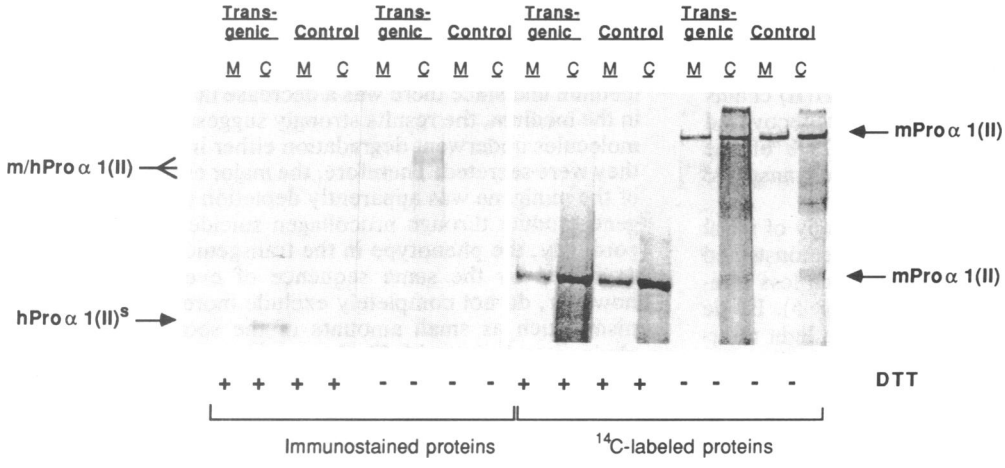


FIG. 4. Synthesis of type II procollagen by matrix-free chondrocytes from a control littermate and a transgenic mouse. $^{14}$C-labeled proteins and immunostained proteins are of the same gel. C, cell fraction; M, medium fraction; mPro$\alpha$1(II), mouse pro$\alpha$1(II) chains (lower band) and disulfide-linked trimers of mouse pro$\alpha$1(II) chains (upper band); DTT, with or without reduction with dithiothreitol; m/hPro$\alpha$1(II), disulfide-linked hybrid molecules of hpro$\alpha$1(II)$^S$ and mpro$\alpha$1(II) chains. $^{14}$C-labeled hpro$\alpha$1(II)$^S$ chains are not visible, apparently because of low levels of expression.

as shortened human pro$\alpha$1(II) chains (not shown). To examine expression of the transgene further, samples of cartilage were dissected from the joints of transgenic mice, and the tissues were digested with collagenase to obtain matrix-free chondrocytes (20). The chondrocytes were then incubated in suspension in DMEM for 4 hr with [$^{14}$C]proline. Western blot analyses demonstrated the hpro$\alpha$1(II)$^S$ chains (shortened chains of human type II procollagen) synthesized from the exogenous gene were found in cells from the transgenic mice (Fig. 4). The hpro$\alpha$1(II)$^S$ chains were not detected in the medium. In unreduced samples of the cells, the hpro$\alpha$1(II)$^S$ chains appeared in cell extracts as at least three bands migrating more rapidly than disulfide-linked trimers of endogenous mpro$\alpha$1(II) chains [pro$\alpha$1(II) chains of mouse type II procollagen]. Therefore, the hpro$\alpha$1(II)$^S$ chains in unreduced samples of cells were probably present as homotrimers of hpro$\alpha$1(II)$^S$ chains and heterotrimers containing one or two

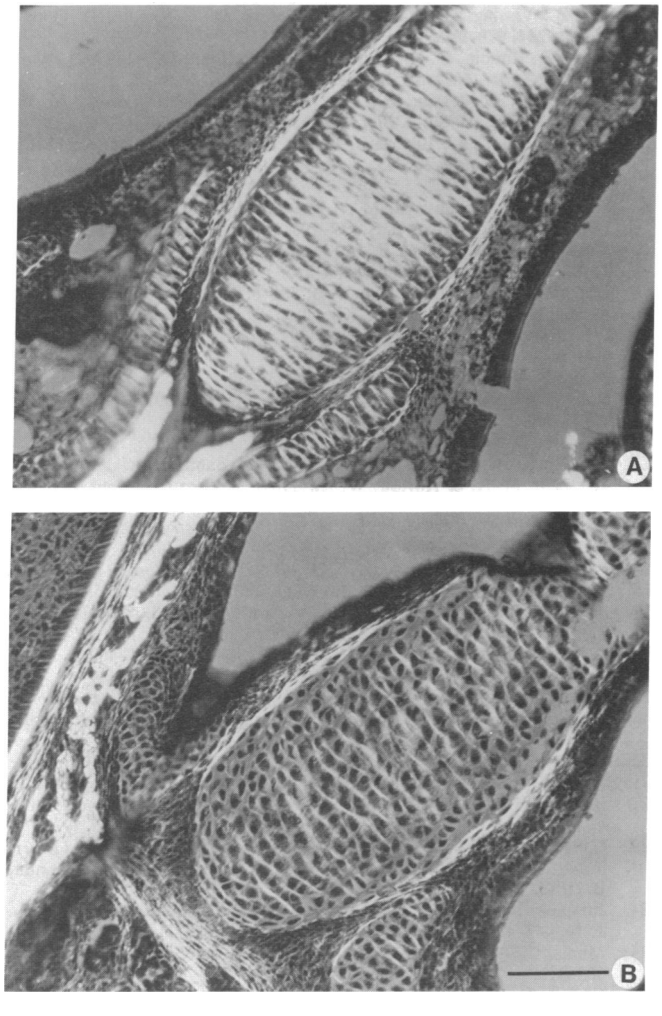


FIG. 5. Polarized light microscopy of a section of nasal septal cartilage stained with sirius red. Polarizer and analyzer vibration directions (crossed) run parallel to the picture margins. (*A*) Sample from normal mouse. (*B*) Sample from transgenic mouse. (Bar = 100 $\mu$m.)

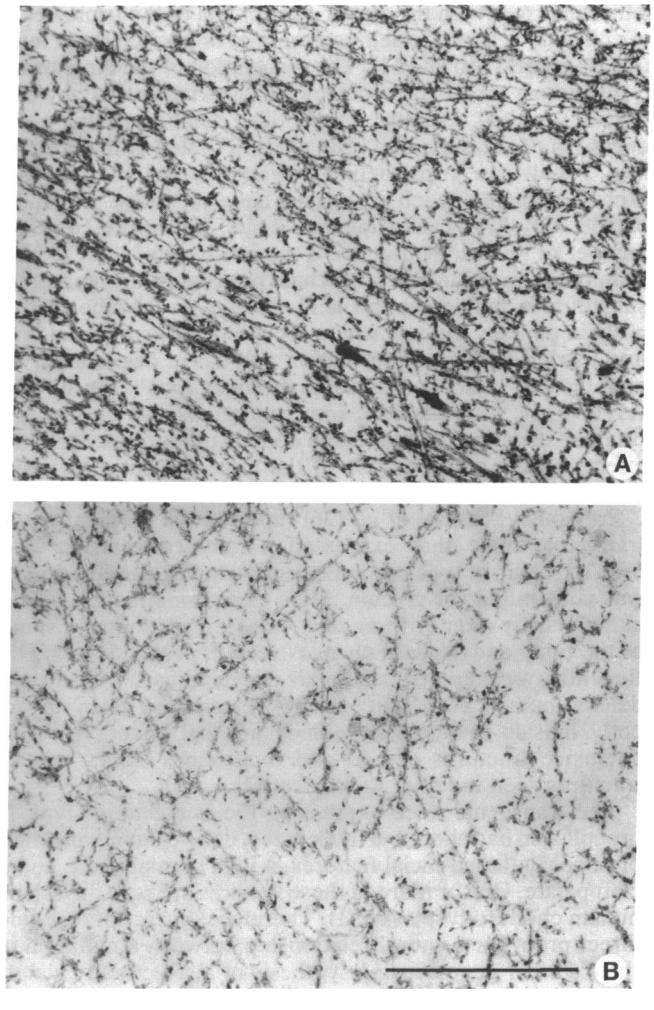


FIG. 6. Transmission electron microscopy of sections of hyaline cartilage from elbow joint. (*A*) Cartilage from control mouse. (*B*) Transgenic mouse. (Bar = 1 $\mu$m.)

endogenous mproα1(II) chains. Analysis of $^{14}$C-labeled proteins in the same filters demonstrated $^{14}$C-labeled mouse proα1(II) chains in cells and medium from the transgenic mice. In four experiments, 33–40% of the mproα1(II) chains synthesized in 4 hr by cells from normal mice were recovered in the medium. In contrast, only 14% and 27% of the mproα1(II) chains synthesized by cells from two transgenic mice were recovered in the medium.

**Microscopic Changes.** Polarized light microscopy of nasal septum after staining with sirius red (24, 25) demonstrated that cartilage from the transgenic mice was much less birefringent than cartilage from control animals (Fig. 5). By de Senarmont compensation analysis, the polarized light retardation in the samples from two transgenic mice was 4–15 nm. The value was 20–33 nm in a control littermate (5-μm sections). Electron microscopy of sections of the cartilage of spine (not shown) and elbow joint (Fig. 6) demonstrated that the collagen fibrils were less dense and more poorly organized in the transgenic mice than in controls. Also, individual collagen fibrils in the cartilage from the transgenic mice were thinner than in cartilage from controls. The amount and distribution of proteoglycan granules appeared to be about the same in the transgenic and control cartilage (Fig. 6).

## DISCUSSION

Mutations in the two structural genes for type I procollagen produce trans-dominant effects, because they cause synthesis of structurally abnormal proα1(I) and proα2(I) chains that either interfere with the zipper-like folding of the triple-helix of collagen or alter the self-assembly of normal collagen into fibrils (see refs. 10 and 11). The association of the three proα chains into a procollagen molecule is directed entirely by the COOH-terminal globular propeptides that represent only one-sixth of the mass of a proα chain. Folding of the protein into a collagen triple-helix, however, requires that the α-chain domain of each proα chain has a repetitive sequence of 338 -Gly-Xaa-Yaa- tripeptide units. Therefore, a large number of mutations that spare the structure of the COOH-terminal propeptide of a proα chain but change the length, or substitute a bulkier amino acid residue for glycine in the α-chain domain, allow chain association but prevent folding into a triple-helical conformation and cause degradation of mutated and normal proα chains through procollagen suicide (11, 14, 15). Alternatively, some mutations that change the primary structure of the α-chain domain produce more subtle effects on the conformation of the molecule but drastically alter self-assembly of collagen into fibrils.

Previously, Stacey *et al.* (26) reported the preparation of transgenic mice that expressed a mutated gene for the proα1(I) chain of type I procollagen in which a cysteine codon was substituted for glycine. Mice expressing the gene died either shortly after birth or after cesarean section with some of the bony and skeletal changes seen in probands with OI. Recently, we prepared transgenic mice expressing a partially deleted human proα1(I) gene (27). $F_1$ progeny from apparently mosaic founders developed a lethal phenotype with extensive fractures of ribs and long bones similar to the fractures seen in lethal variants of OI.

The transgenic mice expressing the minigene of type II procollagen had short and thick limbs, delayed mineralization of bone, flattened facial features, a high cranial vault, and a cleft palate. One or more of these features are seen in various human chondrodysplasias (1). Therefore, the results provide direct proof for a causal relationship between a mutation in the *COL2A1* gene causing synthesis of a structurally abnormal proα1(II) chain and a chondrodysplasia.

The experiments with chondrocytes from the transgenic mice demonstrated the hproα1(II)$^S$ chains synthesized from the minigene formed intracellular hybrid molecules with mproα1(II) chains synthesized from the endogenous mouse genes. Since the hproα1(II)$^S$ chains were not found in the medium and since there was a decrease in mproα1(II) chains in the medium, the results strongly suggested that the hybrid molecules underwent degradation either intracellularly or as they were secreted. Therefore, the major effect of expression of the minigene was apparently depletion of the endogenous gene product through procollagen suicide (11, 14, 15). Accordingly, the phenotype in the transgenic mice is probably explained by the same sequence of events. The results, however, do not completely exclude more complex mechanisms such as small amounts of the secreted hproα1(II)$^S$ chains interfering with fibril assembly (11).

The transgenic mice with a chondrodysplasia should be of considerable interest in studying the role of type II collagen in embryonic development and in exploring possible treatments for human disorders of cartilage (2–8, 28).

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