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Genetics

Assembly of cartilage collagen fibrils is disrupted by overexpression of normal type II collagen in transgenic mice

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ABSTRACT **Cartilage collagen fibrils, which are characterized by their thin, uniform diameters, are formed of a multicomponent system of three collagen types (II, IX, and XI) and interacting proteoglycans. We have used a genetic approach to test whether the proper assembly of this multiprotein structure was altered by overexpression of one of its normal components. Here we show that in transgenic mice in which the normal mouse α1(II) collagen is overexpressed, thick abnormal collagen fibrils are generated. Mice that showed the highest expression of the transgene also displayed a larger proportion of abnormal fibrils and died at birth. We propose that an imbalance among the constituents of the cartilage collagen fibrils disrupts the mechanism that controls their assembly. The results show the applicability of the transgenic mice system to studies of complex multicomponent protein assemblies in intact animals.**

Collagens form a variety of supramolecular structures that have different functions in extracellular matrices (1, 2). In cartilages, collagen fibrils have specific properties characterized by their nearly uniform, thin diameter of ≈10–40 nm (3, 4) and their multicomponent nature consisting of three different collagens (types II, IX, and XI). Type II collagen, the major structural component of these fibrils, is a homotrimer of three α1(II) chains. Heterotrimeric type XI collagen, which is a minor component, is thought to reside in the central portion of the fibril (4), whereas type IX collagen, another heterotrimeric minor component, associates laterally on the fibril surface (5). Given the complexity of most collagen fibrils, the role of each component in fibril assembly is difficult to evaluate by using *in vitro* reconstitution experiments. We have therefore chosen a genetic approach to examine the role of specific components in fibrillogenesis. Specifically, we hypothesized that the increased expression of one of the normal components of the fibrils would alter the proper assembly of this multiprotein structure. To test this hypothesis we generated transgenic mice that harbored a normal polymorphic variant of the mouse α1(II) procollagen gene (*Col2a-1*), and we show that increased expression of normal type II collagen disrupts the mechanism that controls the growth of cartilage fibrils to generate giant collagen fibrils.

MATERIALS AND METHODS

Introduction of a Silent Mutation in *Col2a-1*. The oligonucleotide used for mutagenesis (5′-TGCAGGGCCCTATGGGACCCCGTGG) corresponds to part of the sequence of exon 7 and contains an *Apa* I recognition site (underlined) and a single base mutation (in boldface type) that changes the *Nco* I site CCATGG to CTATGG. The PCR product was digested with *Apa* I, and the 150-bp *Apa* I–*Apa* I fragment was first cloned into a *Pst* I–*Eco*RI subclone, which was subsequently cloned into a 2.2-kb *Kpn* I–*Cla* I genomic subclone, from which a 1.9-kb *Xho* I–*Xho* I fragment was derived and inserted into a 13-kb clone p8045. The reconstruction of the gene was completed by addition of the 28-kb *Cla* I fragment (p8023) containing the 3′ end of the gene, as described (6, 7).

Generation of Transgenic Mice. The 42-kb insert was released from the cosmid vector pWE15 by *Not* I digestion. Purified DNA was microinjected into pronuclei of one-cell mouse embryos, obtained from C57BL/6 × DBA/2J F_1 (hereafter called B6D2F_1) females mated with B6D2F_1 males and were implanted into BALB/c × DBA1 (hereafter called CD1) pseudopregnant foster mothers (8). Founder animals were identified by Southern blot after digestion of tail genomic DNA with *Nco* I (6).

Histology and EM. For light microscopy, limbs were removed from newborn mice, fixed in 4% (vol/vol) buffered formalin, dehydrated, and embedded in Spurr's resin. Sections were stained with 1% toluidine blue. For EM, growth plates of long bones were fixed in 1.5% glutaraldehyde/1.5% paraformaldehyde/0.1 M cacodylate buffer. Ruthenium hexamine trichloride (0.05%) was added to the fixative. The tissue was postfixed in 1% osmium tetroxide/0.1 M cacodylate buffer, dehydrated, and embedded in Spurr's resin. Sections were stained with 4% uranyl acetate and Reynolds' lead citrate.

Measurements of the Relative Levels of Transgene mRNA by PCR. Total RNAs were extracted from mouse limbs. Synthesis of cDNA and its amplification by PCR using mouse α1(II) procollagen-specific primers was done as described (6, 7). The digestion products were fractionated on a nondenaturing 15% polyacrylamide gel. For quantitation of the transgene mRNA [^{32}P]dNTPs were incorporated during the PCR amplification of the cDNAs. The radioactivities in the 94-bp fragment (wild-type cDNA) and 103-bp fragment (transgene and heteroduplex cDNAs) were determined by a betascanner, and the ratio of the transgene mRNA/wild-type mRNA was calculated. To avoid the bias caused by formation of *Nco* I-indigestible heteroduplexes, the amplified cDNA fragments were heat-denatured and slowly reannealed at room temperature before *Nco* I digestion. Formation of double-stranded molecules should follow a random distribution according to equation $wt^2 + 2\ wt{\cdot}tg + tg^2 = 1$, where *wt* denotes the concentration of wild-type strands and *tg* indicates the concentration of transgene-derived strands. The fraction of *wt* strands was calculated as the square root of the proportion of radioactivity in the 94-bp band, and the fraction of the *tg* strands was calculated from equation $tg = 1 - wt$. Because the ratio between transgene mRNA and wild-type mRNA is believed to remain constant during the cDNA synthesis and the logarithmic phase of PCR amplification, the amount of

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transgene mRNA was measured as percentage of the wild-type mRNA.

Expression of Type II Collagen in Mov13 Cells. The SIL construct was cloned in a modified pWE15 cosmid vector that carried one copy of the simian virus 40 enhancer derived from plasmid pAZ1009 (9) and was designated SVenh-SIL. In this cosmid that also contained the SV2neo gene, the simian virus 40 enhancer was located ≈3 kb 5′ of the *Col2a-1* ATG codon. The cosmid DNA was transfected into Mov13 cells that do not express any type I collagen because of a retroviral integration in the α1(I) procollagen gene (10). Clones of Mov13 cells were selected with G418 and screened for the expression level of Col2a-1 mRNA by Northern analysis (data not shown). The clone with the highest level of Col2a-1 mRNA was labeled with [^{3}H]proline for 48 hr in DMEM/10% fetal calf serum/4-aminopropionitrile fumarate at 100 μg/ml and ascorbic acid at 100 μg/ml. The collagen types in the culture medium were determined after pepsin digestion (0.2 mg/ml in 0.5 M acetic acid for 3 hr at 15°C, followed by neutralization with 100 mM Tris·HCl, pH 8.0, and extensive dialysis against water. Collagens were separated by SDS/6% PAGE under nonreducing conditions. Fluorographs were exposed for 7 days at −80°C.

RESULTS

Construction of SIL Transgene. A polymorphic variant of the mouse α1(II) procollagen gene, in which the amino acid sequence of the polypeptide is unchanged, was generated by the introduction of a silent mutation (SIL) in exon 7. The single-base substitution that produces a CCC (Pro) → CCU (Pro) change destroys a *Nco* I site. CCU is the most frequently used codon for proline throughout the *Col2a-1* gene (11).

Generation of SIL Transgenic Mice. Transgenic mice were generated using the SIL α1(II) procollagen gene containing 3 kb of 5′ and 7 kb of 3′ flanking sequences (6); as was shown previously, these sequences are sufficient to secure the same expression pattern as that of the endogenous *Col2a-1* gene (7). Mice carrying the construct were identified by Southern hybridization (Fig. 1) and were designated SIL2 for those that contained ≈16 copies of the transgene and SIL3 for those harboring ≈4 copies. From these lines we also generated SIL3/SIL3 homozygotes and SIL2-SIL3 double heterozygotes. In line SIL2 the transgenes are integrated in the Y chromosome because only male-to-male transmission was observed. The genotypes of the four different lines and their transgene copy numbers are presented in Table 1.

Expression of SIL Transgenes. The level of transgene mRNA was estimated in limb cartilages of the different lines using a reverse transcription-PCR method (Fig. 2 *A* and *B*). Digestion of the [^{32}P]dNTP-labeled PCR products with *Nco* I resulted in a DNA fragment of 103 bp diagnostic of the transgene and a fragment of 94 bp characteristic of the wild-type gene. The levels of SIL transgene mRNA were in direct proportion to the transgene copy number in the four different lines (Fig. 2*B*; Table 1).

Because the α1(II) collagen produced by the SIL transgene was identical to the wild-type protein, it was impossible to distinguish it from the endogenous protein in cartilages of SIL mice. To demonstrate that the reconstituted SIL transgene directed the synthesis of α1(II) collagen, it was placed under the control of the simian virus 40 enhancer and stably transfected into Mov13 cells (10), as these cells do not produce α1(I) collagen chains that would comigrate with α1(II) collagen chains. Electrophoretic analysis of collagen secreted into the medium showed an α1(II) chain with normal mobility (Fig. 2*C*).

Phenotypic Abnormalities. No overt phenotypic abnormality was detected in newborn mice of lines SIL3/+, SIL3/

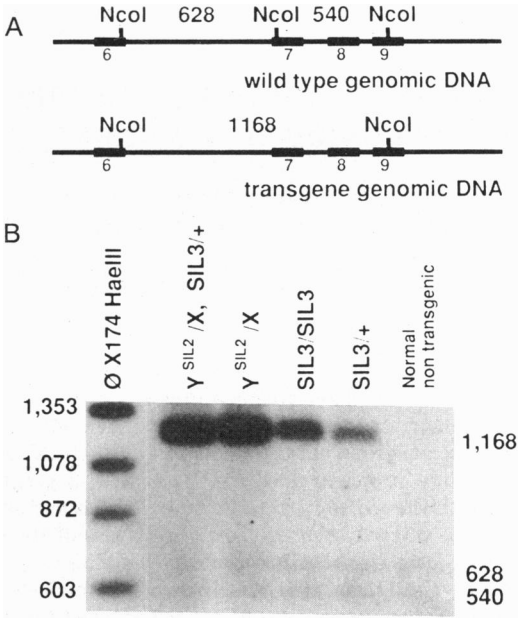

FIG. 1. Southern analysis of the DNA of founder animals carrying SIL transgenes. (*A*) Schematic representation of the structure of mouse α1(II) procollagen gene flanking the silent mutation, which removes a *Nco* I site in exon 7. (*B*) Ten micrograms of *Nco* I-digested tail genomic DNAs of the different transgenic lines was hybridized with a genomic DNA probe covering a segment from intron 6 to intron 8. This probe hybridizes to 540- and 628-bp fragments in the wild-type gene and to a 1168-bp fragment in the transgene carrying the silent mutation (8). DNA size standards are shown at left, sizes of the gene fragments are at right, and the genotypes are on top of each lane.

SIL3, and Y^{SIL2}/X. However, the double heterozygous, Y^{SIL2}/X, SIL3/+ mice did not survive the perinatal period and died from respiratory distress; histological examination indicated that their lung alveoli were mostly collapsed. Unlike mice carrying mutant Col2a-1 transgenes, which also die at birth in acute respiratory failure (6, 7), these mice did not exhibit a cleft palate, abnormal craniofacial features, or other skeletal deformities. There was, however, a small reduction in the length of the long bones in the limbs of Y^{SIL2}/X, SIL3/+ double heterozygous 19-day embryos compared with SIL3/+ littermates (9.7 ± 1.2% for the forelimbs, 19.7 ± 5.7% for the hindlimbs).

Structural and Ultrastructural Abnormalities of Cartilages. The epiphyseal and growth plate cartilages of long bones are

Table 1. Genotypes of the transgenic mouse lines generated by microinjection of the α1(II) procollagen transgene carrying the silent mutation

Genotype	Transgene copy number	Transgene mRNA, % of wild-type mRNA	Abnormal fibrils
SIL3/+	4	22	−
SIL3/SIL3	8	43	+
Y^{SIL2}/X	16	82	++
Y^{SIL2}/X, SIL3/+	20	144	+++

Transgene copy numbers were estimated from Southern hybridization (Fig. 1), and the relative expression levels were calculated according to the equation discussed in *Materials and Methods* from the reverse transcriptase-PCR experiment of Fig. 2. It should be noted that in a previous report (6) from this laboratory the levels of transgene RNA of the SIL3/+ mice, which served as control for mice harboring a mutant *Col2a-1* transgene, were overestimated. The amounts of abnormally thick collagen fibrils were assessed visually by light and electron microscopy (see Figs. 3–5).

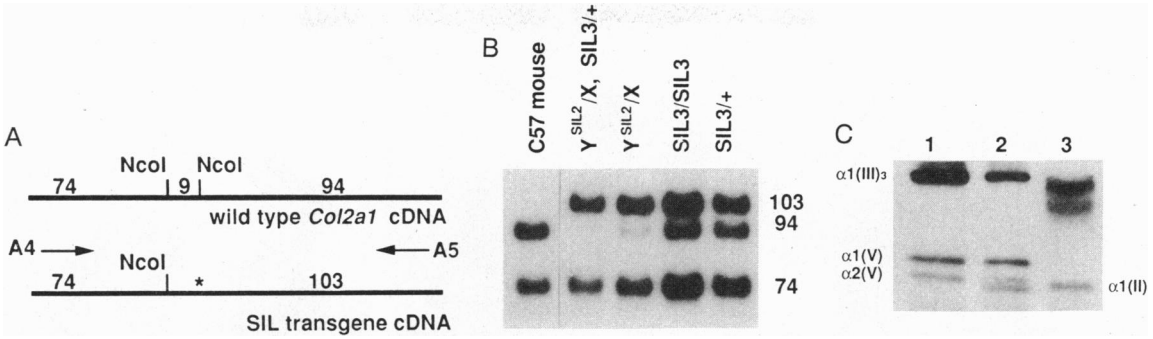

FIG. 2. Expression of the SIL transgene. (*A*) Schematic representation of the α1(II) procollagen cDNA flanking the silent mutation (marked with *) in exon 7. Numbers denote the sizes (in bp) of *Nco* I fragments of wild-type and transgene-derived cDNAs. A4 and A5 are the primers used for PCR amplification (6, 7). (*B*) Autoradiogram of electrophoretically fractionated, *Nco* I-digested, PCR-amplified ^{32}P-labeled cDNAs of normal C57 mouse and transgenic SIL mice limb RNAs. Genotypes are marked above each lane; DNA sizes are indicated in bp. The 94-bp species is the wild-type cDNA, whereas the 103-bp fragment corresponds to the transgene cDNA plus heteroduplex cDNAs. The level of transgene RNA was computed from the 103/94 bp ratio, according to the equation described in *Materials and Methods*. (*C*) Production of α1(II) collagen in Mov13 cells. Lanes: 1, Mov13 cells; 2, Mov13 cells stably transfected with the SVEnh-SIL construct; 3, rat chondrosarcoma cells that produce α1(II) procollagen. The different collagen polypeptides in Mov13 cells are identified as in ref. 10.

sites of very active *Col2a1* gene expression. We therefore examined their structure in SIL newborn mice by light microscopy. No abnormalities were found in SIL3/+ mice (Fig. 3 *A* and *D*); however, when homozygous (SIL3/SIL3) mice were produced, intensely staining fibrous material was found to be distributed randomly in the interterritorial matrix compartments of the cartilages (Fig. 3*B*). These fibrous structures were even more prominent in the cartilages of Y^{SIL2}/X mice and in double heterozygous Y^{SIL2}/X, SIL3/+ mice (Fig. 3 *C* and *F*). Transmission EM revealed the fibrous material was composed of large banded collagen fibrils in the cartilages of SIL3/SIL3 (Fig. 4 *B* and *E*), Y^{SIL2}/X, and

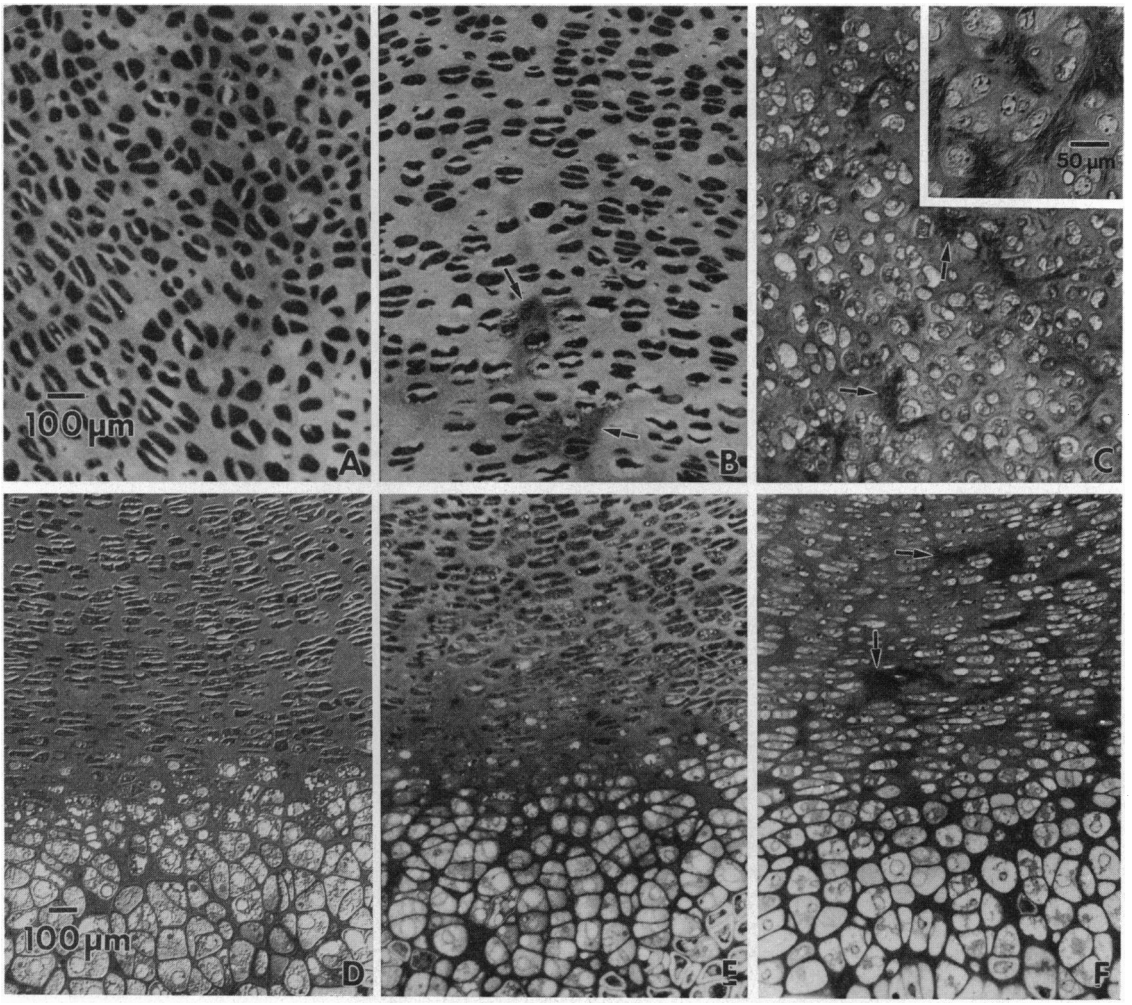

FIG. 3. Histological analysis of growth plate cartilages in SIL3/+(*A* and *D*), SIL3/SIL3 (*B* and *E*), and Y^{SIL2}/X, SIL3/+ mice (*C* and *F*). (*A–C*) Epiphyseal cartilage. (*D–E*) Growth cartilage. Arrows point to some of the intensely staining fibrous materials. (*Inset*) Higher magnification of fibrous materials of *C*. (Magnification is the same in *A–C*; magnification is the same in *D–F*.)

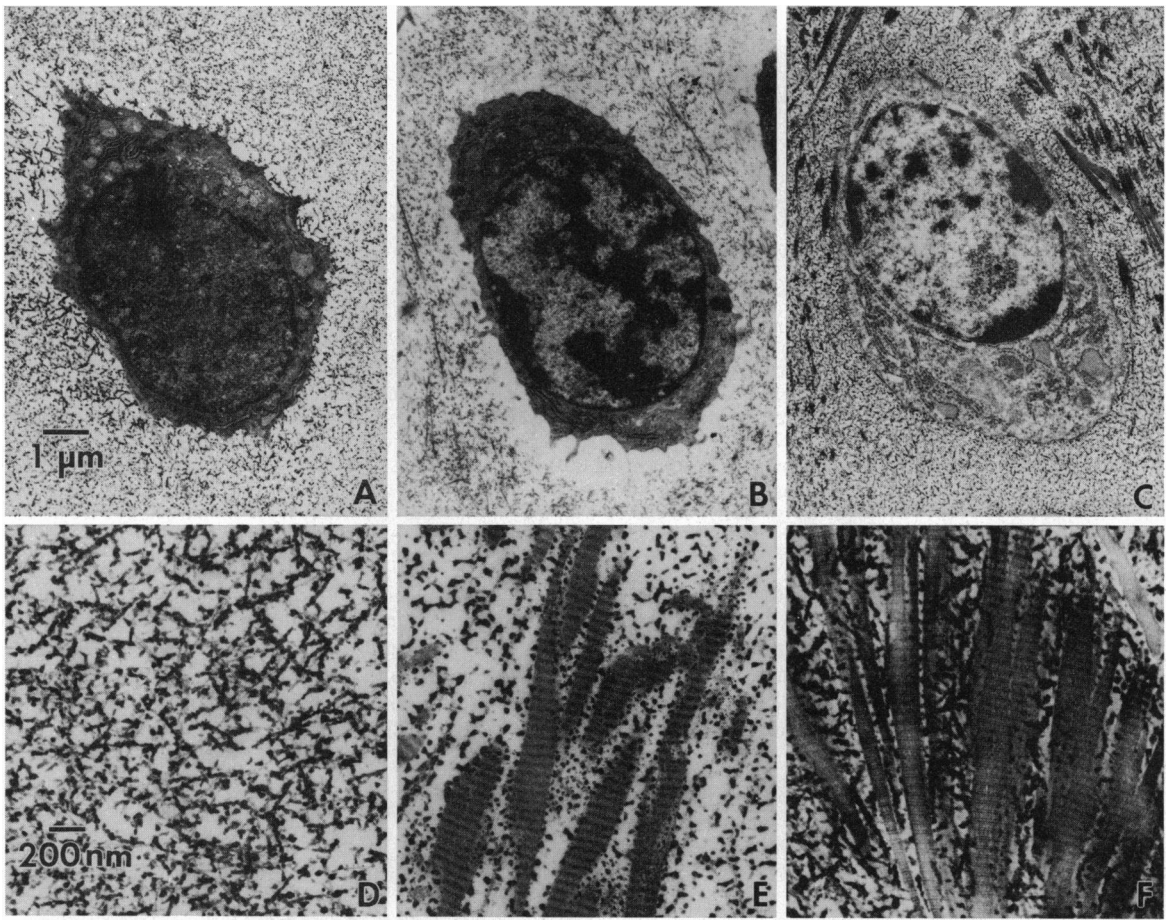

FIG. 4. EM of the growth plate cartilages in SIL3/+ (*A* and *D*), SIL3/SIL3 (*B* and *E*), and Y^{SIL2}/X, SIL3/+ mice (*C* and *F*). No abnormality is detectable in SIL3/+ mice, but the matrix of SIL3/SIL3 and Y^{SIL2}/X, SIL3/+ mice contains large aggregates of banded collagen fibrils dispersed among the regular thin 20-nm fibrils. (Magnification is the same in *A–C*; magnification is the same in *D–F*.)

double heterozygous Y^{SIL2}/X, SIL3/+ mice (Fig. 4 *C* and *F*). These abnormal fibrils were not detected in SIL3/+ or normal mice cartilages (Fig. 4 *A* and *D*). Another characteristic of the thick collagen fibrils was their extensive branching, visible as bush-like structures in cross-sections (Fig. 5*A*). Many of these fibrils reached a diameter of 150–250 nm, more than 10 times the uniform diameter of normal cartilage collagen fibrils, which were also abundant in all cartilage matrices analyzed (Fig. 5*B*).

DISCUSSION

One advantage of the genetic approach described here to study fibrillogenesis over *in vitro* reconstitution experiments is that the changes in expression of the *Col2a-1* gene occurred in intact animals in an otherwise normal environment. The simple increase in expression of a normal mouse *Col2a-1* gene in transgenic mice resulted in a dominantly inherited phenotype of large-banded collagen fibrils in cartilages. The abundance of these fibrils appeared proportional to the level

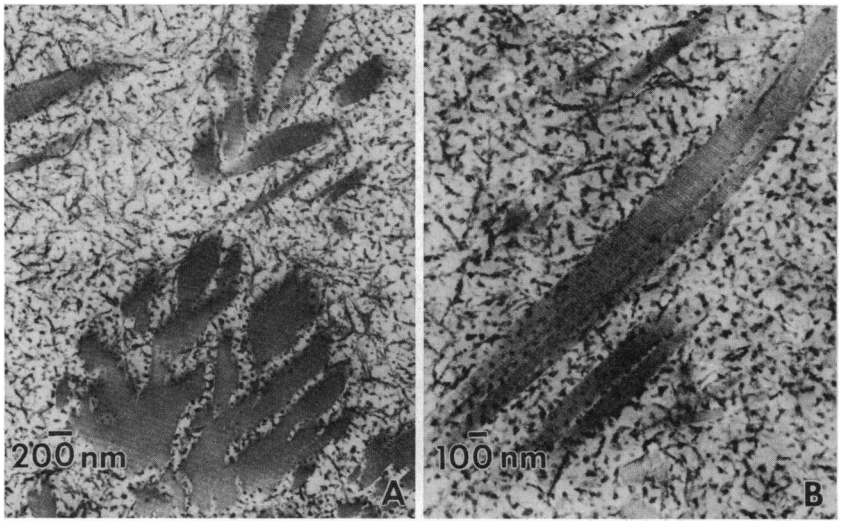

FIG. 5. EM of the abnormal collagen fibrils in Y^{SIL2}/X mouse cartilages. Cross-section (*A*) and longitudinal section (*B*) show the abnormal branching of thick fibrils, their irregular diameters, and typical collagenous banding pattern. In both *A* and *B* normal thin cartilage collagen fibrils are also present.

of expression of the transgene, although their precise quantitation was not possible. Abnormal fibrils were detected when the total (endogenous plus transgene) level of type II collagen expression exceeded the level of expression of the endogenous genes by ≈40%.

The simplest interpretation of our results is that the overexpression of normal α1(II) procollagen caused an imbalance among the constituents of the multicomponent cartilage fibrils and that this imbalance disrupted the mechanism that controls the diameter of the fibrils. In yeast, overexpression of wild-type components of other multicomponent structures, such as the cytoskeleton and the nucleosome (12, 13), also resulted in mutant phenotypes caused by an imbalance in the concentration of one of the constituents. We hypothesize that an excess of type II collagen molecules competes with other molecules whose role it is to arrest fibril growth. Once the mechanism responsible for this arrest has been overridden, excess molecules are free to assemble into large fibrillar structures that branch randomly (Fig. 5). Consistent with this idea, *in vitro* reconstitution experiments with purified type II, IX, and XI collagens have indicated that excess type II collagen results in thick tactoidal fibrils similar to those seen in SIL mice and different from the ≈20-nm-diameter fibrils observed when the three collagens were present in proper concentrations (14, 15).

Although *in vitro* reconstitution experiments using only type I procollagen molecules retaining the aminopropeptide have revealed sheet- and cauliflower-like structures that bear some resemblance to the abnormal fibrils in SIL mice, it is unlikely that insufficient extracellular processing of the procollagen aminopropeptide would account for the observed phenotype in our transgenic mice (16). Human (17, 18), bovine (19), and ovine (20) dermatosparaxis are recessive diseases affecting the aminopeptidase. No abnormal fibrils have been described in heterozygous carriers that exhibit 50% enzyme activity, suggesting that this enzyme activity is not a rate-limiting factor in fibrillogenesis (17, 18).

Large abnormal collagen fibrils were also observed in the cartilages of mice carrying an α1(II) procollagen transgene with a Gly-85 → Cys mutation (6); these large fibrils were shown to contain type II collagen by immunofluorescence (6). Similarly we believe that the large abnormal cartilage collagen fibrils reported here contain mainly type II collagen, but it is possible that other collagen types are also present in these large fibrils. In the mice harboring a Gly-85 → Cys mutation, pronounced alterations in the typical columnar arrangement of chondrocytes in the growth plate were also found when the mutant transgene was expressed at levels comparable to those found in Y^{SIL2}/X and SIL3/SIL3 mice. This severe phenotype was probably due to the much reduced amount of cartilage fibrils in the matrix, but it is likely that the presence of abnormal, large fibrils also contributed to the severity. Thick-banded cartilage collagen fibrils have previously also been detected in other conditions affecting both mouse and human. Four mouse mutants, chondrodysplasia (*cho*), cartilage-matrix deficiency (*cmd*), spondylometaphyseal chondrodysplasia (*smc*), and brachypodism (*bp*), show abnormally large cartilage collagen fibrils (21, 22); however, it is only in *cho* mice that these fibrils reach an average diameter of 200 nm and demonstrate branching (23). Among human chondrodysplasias, broad abnormal collagen fibrils are a characteristic finding in cartilages of patients with diastrophic dysplasia and have also been observed in some cases of Kniest dysplasia (24, 25). However, in none of these genetic diseases has the identification of the mutant gene been reported. One could speculate that in some of these diseases a similar type of imbalance occurred among the components of cartilage collagen fibrils. Given the multicomponent nature of cartilage and other collagen fibrils, one may also expect that several different genetic changes could disrupt their correct assembly.

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