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A Fibrillar Collagen Gene, *Col11a1*, Is Essential for Skeletal Morphogenesis

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Summary

Mice that are homozygous for the autosomal recessive chondrodysplasia (*cho*) mutation die at birth with abnormalities in cartilage of limbs, ribs, mandible, and trachea. Limb bones of newborn *cho/cho* mice are wider at the metaphyses than normal bones and only about half the normal length. By linkage analysis, the *cho* gene and the gene encoding the α1(XI) chain of cartilage collagen XI were mapped to the same region of chromosome 3. Deletion of a cytidine residue about 570 nt downstream of the translation initiation codon in *cho* α1(XI) mRNA causes a reading frame shift and introduces a premature stop codon. The data demonstrate that collagen XI is essential for normal formation of cartilage collagen fibrils and the cohesive properties of cartilage. The results also suggest that the normal differentiation and spatial organization of growth plate chondrocytes is critially dependent on the presence of type XI collagen in cartilage extracellular matrix.

Introduction

Autosomal recessive chondrodysplasia (*cho*) in mice is characterized by skeletal defects caused by abnormalities in the cartilage of limbs, ribs, mandible, and trachea (Seegmiller et al., 1971). Homozygous (*cho/cho*) mice are born with shortened snouts, cleft palate, protruding tongue, and short limbs. The airway is reduced, and tracheal cartilage rings are soft (Seegmiller et al., 1972). The thoracic cage is small, causing lung hypoplasia (Seegmiller et al., 1986; Hepworth et al., 1990), and the spine is shortened (Figure 1A). The homozygotes die at birth, probably as a result of tracheal collapse, lung hypoplasia, or both. Previous studies of *cho/cho* cartilage have shown that collagen fibrils in the matrix are unusually thick and that proliferating epiphyseal chondrocytes do not show the normal columnar arrangement (Seegmiller et al., 1971; Monson and Seegmiller, 1981; Figure 1B). It has also been found that proteoglycans are more easily extracted from *cho/cho* than from normal cartilage and that mutant cartilage lacks cohesiveness (Stephens and Seegmiller, 1976; Seegmiller et al., 1981).

Identification of the *cho* mutation should lead to an understanding of the molecular basis for the cohesive properties of normal cartilage. Also, since the features of *cho* resemble those of certain human chondrodysplasias, identification of the *cho* mutation is likely to provide insights into the molecular basis for human disorders. In the present paper, we report the mapping of the *cho* locus to the *Amy1–Amy2* region of mouse chromosome 3. This region corresponds to the p21 region of human chromosome 1 (Seldin et al., 1993). The human gene encoding α1(XI) collagen, a component of cartilage collagen fibrils, had previously been localized by in situ hybridization to this region (Henry et al., 1988), making α1(XI) collagen a candidate for the *cho* mutation. We demonstrate that *Col11a1* indeed is linked to the *cho* locus of mouse chromosome 3. We also show that α1(XI) collagen chains are undetectable in *cho/cho* cartilage and that the *cho* mutation is deletion of a cytidyl residue, causing a premature translational stop about 570 nt downstream of the initiation codon of α1(XI) collagen mRNA. Finally, analysis of α1(XI) collagen transcripts in *cho/+* heterozygous cartilage revealed lower levels of the *cho* allele than the wild-type allele transcripts. We conclude, therefore, that the *cho* mutation affects the synthesis, stability, or both of α1(XI) collagen mRNA and suggest that *cho/cho* mice are functionally equivalent to homozygous knockouts for *Col11a1*.

Results

Mapping of the *cho* Locus to Mouse Chromosome 3

To map the *cho* locus, C57BL/6FrSe (*cho/+*) mice were crossed with the MEV retroviral linkage testing strain (Taylor and Rowe, 1989), F1 animals were intercrossed, and 51 F2 (*cho/cho*) homozygotes were collected. An additional 54 first generation backcross homozygotes were obtained by backcrossing (C57BL/6FrSe × MEV) *cho/+* heterozygotes with the *cho/+* parents. Analysis of Southern blots of DNA from the 105 *cho/cho* mice, the DNA of which had been hybridized with a probe for ecotropic MuLV proviruses, showed absence of the 9.4 kb *Emv27*-derived PvuII band in all samples except one, thus placing the *cho* locus about 0.6 cM from the *Emv27* provirus on

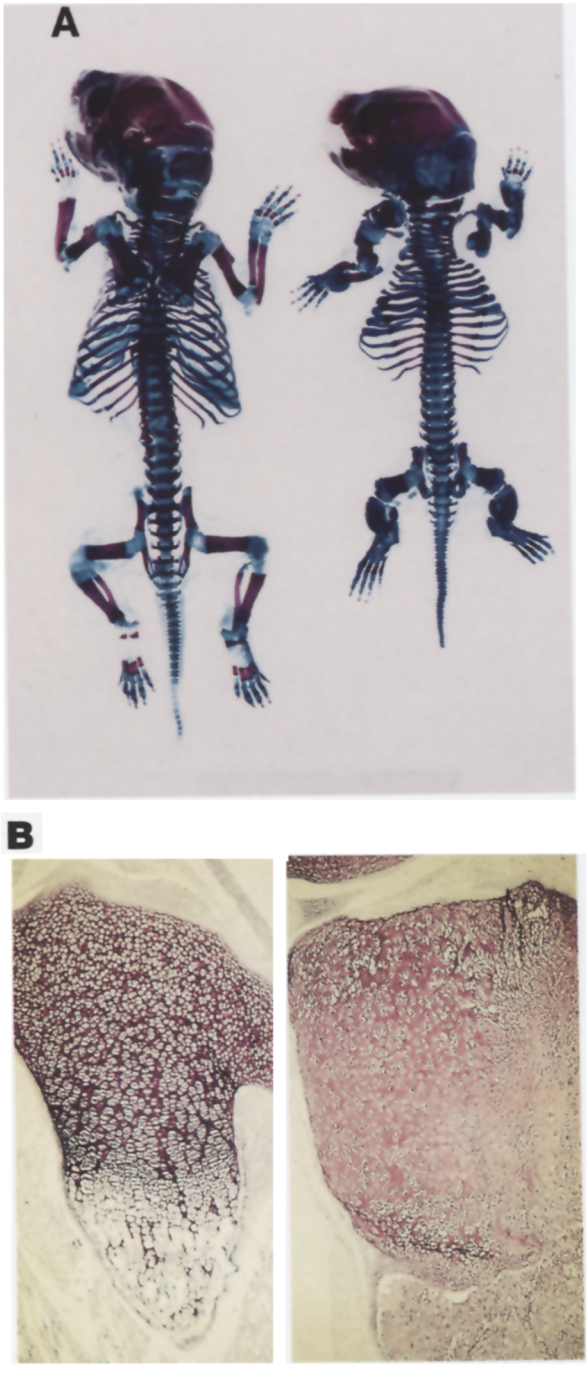

Figure 1. Comparison of Normal and Mutant Skeletal Tissues

(A) Comparison of normal (left) and *cho/cho* (right) specimens reveals the shortened spine and markedly shortened bones with flared metaphyses of the appendicular and thoracic skeleton. Calcified bone and cartilage are differentially stained with Alizarin red and Alcian blue, respectively.

(B) Sections of tibial epiphyses from 19-day-old *cho/cho* mutant (right) and wild-type (left) embryos stained with toluidine blue. The growth plate region, while showing an ordered arrangement of chondrocytes in different stages of differentiation in the wild-type tissue, is completely disorganized in *cho/cho* mutant tissue.

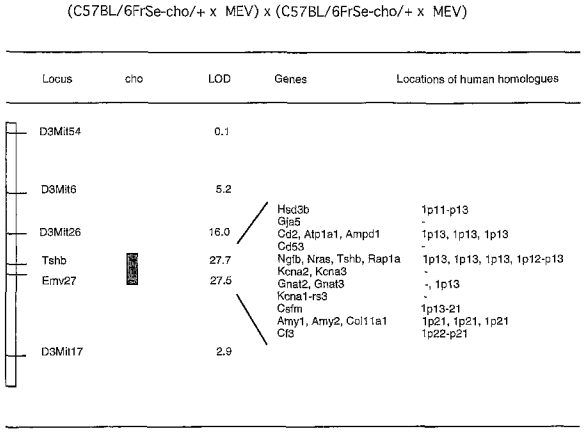

Figure 2. Partial Linkage Map of Mouse Chromosome 3

The maximum likelihood position estimates for the location of the *cho* locus on chromosome 3 are given based on analysis of the F2 intercross population.

chromosome 3 (95% confidence interval: 0.02–3.5 cM). To confirm this location of the *cho* gene, we also screened the F2 and first generation backcross DNAs using five microsatellite markers that distinguished between C57BL/6FrSe and MEV mice. These analyses placed the *cho* locus in the interval between *Tshb* and *Emv27* on chromosome 3 (Figure 2).

Mapping of *Col11a1* to Mouse Chromosome 3

Linkage mapping of *Col11a1* was accomplished using a sequence length polymorphism between different inbred strains in the 3′ untranslated region of α1(XI) collagen mRNA. Flanking primers were used to amplify this polymorphic region with a panel of DNAs from the BXD recombinant inbred series as template for polymerase chain reaction (PCR). Strains BXD-5, BXD-6, BXD-9, BXD-14, BXD-16, BXD-18, BXD-19, BXD-20, BXD-23, BXD-24, BXD-27, BXD-29, BXD-31, and BXD-32 exhibited the larger fragment of C57BL/6J, while strains BXD-2, BXD-8, BXD-11, BXD-15, BXD-21, BXD-22, BXD-25, BXD-28, and BXD-30 exhibited the smaller DBA/2J fragment. The strain distribution pattern was concordant in all 23 strains with the *Amy1* locus on chromosome 3, suggesting a genetic distance between *Col11a1* and *Amy1* of less than 3.7 cM (95% upper confidence limit).

Abnormal Expression of α1(XI) Collagen in *cho/cho* Mice

The expression of the *cho* abnormality in cartilage and colocalization of the *cho* gene with *Col11a1* made *Col11a1* a candidate for the *cho* mutation. To test this possibility, we used polyclonal antibodies against a peptide from within the carboxyl telopeptide of α1(XI) collagen chains (Figure 3A) to examine extracts of normal and *cho/cho* cartilage by Western blotting. The antibodies were made against rat α1(XI) sequences but cross-react with mouse α1(XI) chains. As shown in Figure 3B, extracts of normal costal cartilage contained a positive band with the mobility expected for α1(XI) collagen chains, while extracts of *cho/*

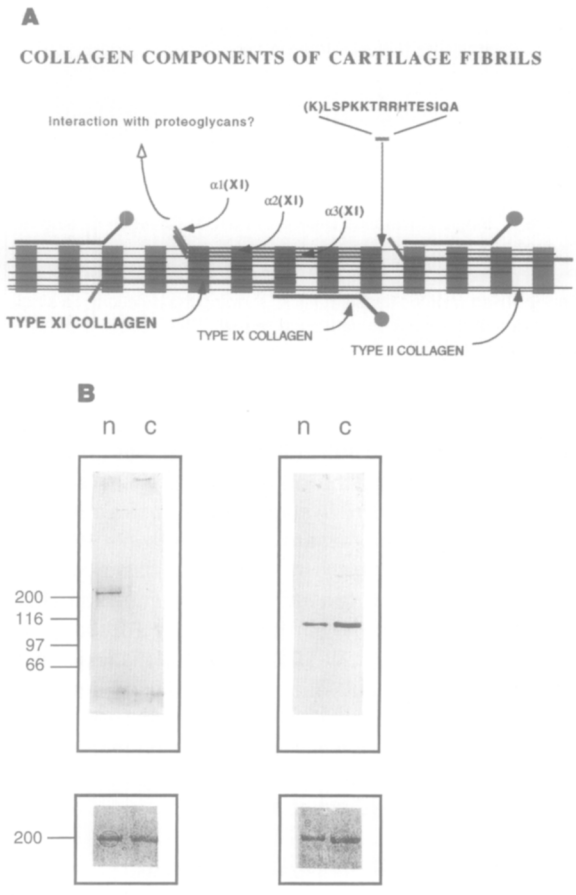

Figure 3. Diagram of Collagen XI Molecules in Cartilage Fibrils and Detection of α1(XI) Chains in Extracts

(A) Diagram of a cartilage collagen fibril, composed of the fibrillar collagens II and XI and the FACIT component type IX (Shaw and Olsen, 1990). Type IX molecules are localized on the surface of the fibrils, while type XI collagen molecules may be partially embedded within the fibrillar structure. The peptide sequence (K)LSPKKTRRHTESIQA from within the carboxyl region of α1(XI) collagen chains, used to generate polyclonal antibodies, is shown above the diagram.

(B) Western blot showing the reactivity of cartilage extracts with polyclonal anti-α1(XI) antibodies (left panel) and monoclonal antibodies against α1(II) collagen (right panel). Before incubation with the primary antibodies, the filters were stained for protein with Ponceau red. As shown in the insets below the Western blots, the amount of protein loaded in each lane was similar since a band with the mobility of myosin heavy chain appeared with about the same intensity in all lanes. The positions of molecular mass markers (in kilodaltons) are indicated to the left of the figure. n, extract of wild-type C57BL/6FrSe rib plates; c, extract of *cho/cho* rib plates.

cho cartilage did not. In contrast, when immunoblots were stained with an antibody specific for the major collagen of cartilage, α1(II), both normal and *cho/cho* extracts contained positive bands of equal intensity. This observation suggests that α1(XI) collagen is absent from *cho/cho* cartilage. The conclusion was supported by immunohistochemical analyses with the α1(XI) collagen antibodies. The antibodies stained normal tracheal, costal, and epiphyseal cartilage intensely, but did not stain *cho/cho* cartilage (Figure 4 shows the results for epiphyseal cartilage). Both types of cartilage showed positive staining, however, with antibodies against type II collagen.

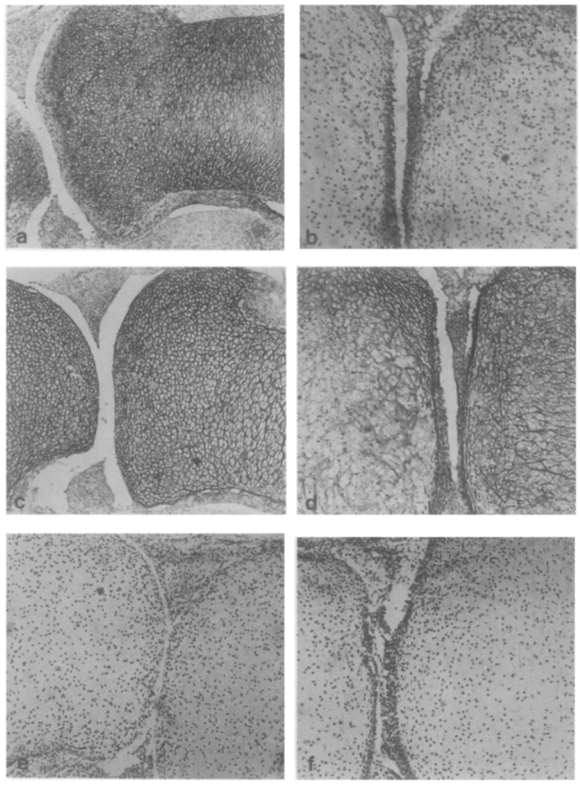

Figure 4. Immunohistochemistry of Normal and Mutant Cartilage

Sections of knee joints from *cho/cho* mutants (right) and normal littermates (left) stained with antibodies against α1(XI) collagen (a and b) and α1(II) collagen (c and d). For sections shown in (e) and (f), only secondary antibody was used.

Identification of the *cho* Mutation

To identify the *cho* mutation, we used reverse transcription (RT)–PCR with mRNA isolated from homozygous *cho/cho* embryos to generate a set of cDNAs spanning about 5700 nt of α1(XI) collagen mRNA (Figure 5A). These cDNAs, labeled with [^{32}P]dCTP, were digested with restriction endonucleases into smaller fragments and were compared with similar fragments obtained from wild-type α1(XI) collagen cDNAs by single strand conformational polymorphism (SSCP) analysis. Only one cDNA (*YL10-2*) showed a difference between wild-type and *cho* (Figure 5B); a difference was observed even when the fragments of *YL10-2* were analyzed on a polyacrylamide sequencing gel under denaturing conditions (Figure 5C). Direct nucleotide sequencing of *YL10-2* demonstrated that this difference was due to a single nucleotide deletion in the *cho* RT–PCR product (Figure 6). To confirm the presence of this deletion in genomic DNA from *cho* mice, we also performed PCR analysis of genomic DNA, using oligonucleotide primers that flank the deletion. As shown in Figure 7, this analysis showed amplification of the expected 96 nt and 95 nt bands with wild-type and *cho/cho* DNA, respectively, and a double band with heterozygous *cho/+* DNA.

Analysis of *cho* mRNA Levels

To examine the relative levels of *cho* and wild-type mRNAs, we used RT–PCR to amplify a portion of the 3′

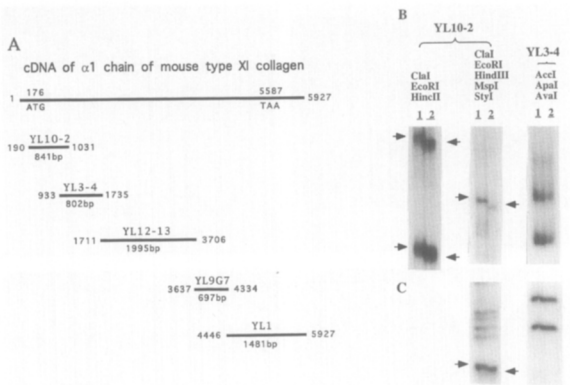

Figure 5. Electrophoretic Analysis of α1(XI) Collagen cDNA Fragments

(A) Diagram showing the 5927 nt long mouse α1(XI) collagen mRNA (Yoshioka et al., submitted) (top) and five DNA fragments generated by RT–PCR. The length of each PCR product (in base pairs) obtained with mRNA from wild-type mice is indicated below each fragment. For each fragment, the first and last nucleotides (as counted from the 5′ end of the mRNA sequence) are indicated at the beginning and the end of each fragment. Within the mRNA sequence, the initiation codon starts with nucleotide 176; the TAA stop codon starts with nucleotide 5587.

(B) The results of SSCP analysis of fragments *YL10-2* and *YL3-4* (see [A]) obtained with wild-type mRNA (lanes 1) and *cho*/*cho* mRNA (lanes 2). For the analysis, the ^{32}P-labeled cDNAs were digested with restriction endonucleases, as indicated above the lanes. All cDNAs shown in (A) (except *YL1*; this was completely sequenced) were analyzed, but only *YL10-2* showed differences between the wild-type and *cho*/*cho* products. Therefore, only the results with *YL3-4* are shown as a control. As indicated by the arrows fragments of *YL10-2* generated by two different sets of restriction endonucleases showed different mobilities between wild-type and *cho*/*cho* lanes.

(C) When fragments obtained from *YL10-2* were analyzed on a 6% polyacrylamide sequencing gel, a difference in mobility between wild-type and *cho*/*cho* DNA was observed (arrows), indicating a single nucleotide deletion in the *cho*/*cho* product. In contrast, fragments derived from *YL3-4* showed no difference.

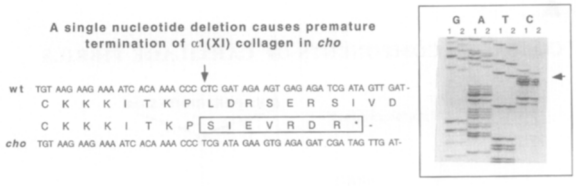

Figure 6. Single Nucleotide Deletion in α1(XI) Collagen Coding Sequence

Partial nucleotide sequence of *YL10-2* (see Figure 5A) from wild-type (lanes 1) and *cho*/*cho* (lanes 2) mice, showing the deletion of a single C residue in the *cho*/*cho* sequence (arrow). This causes premature termination of α1(XI) collagen in *cho*.

untranslated region of α1(XI) mRNA. Since this region gave rise to products of different size with RNA from DBA/2J and C57BL/6FrSe mice, RNA was extracted from animals derived from a cross between DBA/2J (+/+) and C57BL/6FrSe (*cho*/+) mice and used as template for PCR. As shown in Figure 8, RT–PCR of (C57BL/6FrSe × DBA/2J) *cho*/+ RNA produced mostly the wild-type product, while RT–PCR of (C57BL/6FrSe × DBA/2J) +/+ RNA produced bands of equal intensity. In contrast, when genomic DNA was used as template for PCR, no difference was observed between products obtained with DNA from (C57BL/6FrSe × DBA/2J) *cho*/+ and +/+ animals.

Discussion

The *cho* Phenotype Is Caused by an Absence of α1(XI) Collagen

Based on the colocalization of the *cho* gene with *Col11a1*, the absence of immunodetectable α1(XI) collagen in *cho*/*cho* cartilage, and the presence of a single nucleotide deletion in the coding region of *cho* α1(XI) collagen mRNA, we conclude that the *cho* phenotype is caused by the absence of α1(XI) collagen in extracellular matrices. The mutation would allow the synthesis of a truncated α1(XI) collagen chain, containing 195 amino acid residues (including the signal peptide), but the low level of *cho* mRNA as compared with wild-type mRNA suggests that such a truncated polypeptide would be present at very low levels. We conclude, therefore, that *cho* represents a loss-of-function phenotype and is not caused by the accumulation of an abnormal protein. The reason for the low level of *cho* α1(XI) transcripts is not clear, but frameshift mutations in many other proteins frequently lead to a decreased synthesis or reduced stability of the mRNA. For example, fibrillin mutations in Marfan patients leading to a premature stop codon have been shown to reduce the expression level of the mutant allele (Dietz et al., 1993).

The Role of Type XI Collagen in Cartilage

Type XI collagen molecules are heterotrimers composed of α1(XI), α2(XI), and α3(XI) chains (Morris and Bachinger, 1987). Their genes, including *Col11a1*, belong to the fibrillar class of collagen genes (Jacenko et al., 1991) and encode precursor chains, procollagens, that are processed to mature collagen XI chains after secretion from cells. Type XI molecules copolymerize with molecules of types II and IX collagen to form the collagen fibrils of cartilage (Figure 3A) (Mendler et al., 1989). In the vitreous of the mammalian eye in which type II collagen is also the major fibrillar collagen, a unique hybrid form of collagen XI is found, consisting of α1(XI), α2(V), and α3(XI) chains (Mayne et al., 1993).

It has been hypothesized that collagen XI plays a role in regulating the diameter of collagen fibrils, similar to the manner in which type V collagen is thought to regulate the diameter of type I–containing fibrils (Eyre and Wu, 1987; Linsenmayer et al., 1993). The molecular basis for such a role is thought to be the incomplete processing of the amino propeptide domains of these two types of procollagens (Linsenmayer et al., 1993; Sheren et al., 1986; Broek et al., 1985). The presence of amino-terminal extensions on the processed molecules would sterically restrict the addition of type II collagen molecules to the surface of fibrils in which type XI molecules are incorporated. Thus, the diameter of cartilage collagen fibrils would be determined by the ratio between type XI and type II molecules in the fibrils. The hypothesis predicts that an increased ratio leads to thinner fibrils, while a decreased ratio results in thicker fibrils. The data reported here on the

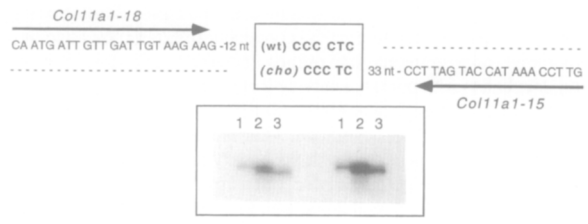

Figure 7. Mutation Detection in Genomic DNA

Gel electrophoresis of PCR products generated with primers *Col11a1-18* and *Col11a1-15* and genomic DNA from wild-type mice (lanes 1), *cho*/+ heterozygotes (lanes 2), and *cho*/*cho* homozygotes (lanes 3). For clarity, we show results obtained with two different amounts of sample loaded on the gel. The wild-type product is 96 nt in length; the *cho*/*cho* product is 95 nt.

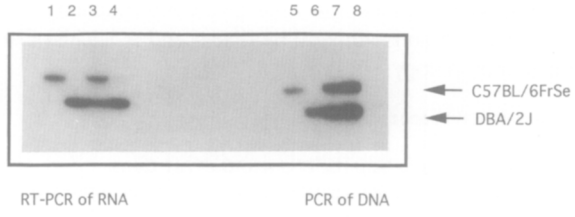

Figure 8. Reduced Levels of *cho* α1(XI) Transcripts

Gel electrophoresis of PCR products with primers flanking a portion of the 3′ untranslated region of the α1(XI) collagen gene. As described in the text, this PCR product is larger with template cDNA and genomic DNA from C57BL/6FrSe than from DBA/2J mice (lanes 1, 2, 5, and 6). As expected, with DNA from C57BL/6FrSe × DBA/2J mice, both products were generated (lane 7). RT–PCR also generated two bands with RNA from (C57BL/6FrSe × DBA/2J) +/+ mice (lane 3). With RNA from (C57BL/6FrSe × DBA/2J) *cho*/+ mice (lane 4) mostly the smaller product was generated, while the use of DNA from (C57BL/6FrSe × DBA/2J) *cho*/+ mice (lane 8) as template produced two bands of almost equal intensity.

absence of α1(XI) collagen in *cho*/*cho* cartilage, combined with the previous electron microscope demonstration of abnormally thick collagen fibrils in mutant cartilage (Seegmiller et al., 1971), provide strong and direct support for this hypothesis. Consistent with the hypothesis is also the finding of thicker than normal fibrils in the cartilage of transgenic mice overexpressing type II collagen (Garofalo et al., 1993).

Smith et al. (1985) reported that type XI collagen binds with high affinity to proteoglycans and speculated that such binding may be important in vivo for anchoring cartilage proteoglycans to the collagen fibrillar network. The unusual extractability of proteoglycans from *cho*/*cho* cartilage (Stephens and Seegmiller, 1976) clearly indicates that type XI collagen is essential for the interaction between the large protoglycan aggregates and the collagen, but this interaction may not involve a direct molecular association of proteoglycan components and collagen XI. An alternative explanation for the increased extractability and the lack of cohesiveness is that the presence of fewer, albeit thicker, fibrils in *cho*/*cho* cartilage provides less of a physical entrapment of large proteoglycan aggregates.

Potential Effect of *Col11a1* Haploinsufficiency

Heterozygous carriers for the *cho* mutation do not manifest an obvious skeletal phenotype. This is somewhat surprising, since haploinsufficiency for structural matrix components, such as collagens I, II, and X, have been associated with clinical phenotypes in humans. Several explanations for the lack of obvious abnormalities in heterozygous *cho*/+ mice can be postulated. First, the phenotypic effects in heterozygotes may be quite subtle. For example, high frequency hearing loss or myopia may not be detected in the absence of specialized testing. Second, humans and mice differ substantially with respect to growth, development, and lifestyle. It is reasonable to assume, therefore, that stresses upon bones and joints effecting cartilage might differ between the two species. Consequently, provocative testing may be required to elicit a phenotype in mice. Third, the regulatory pathways responsible for control of collagen XI synthesis may be able to compensate for haploinsufficiency states. Collagen XI is a quantitatively minor component of collagen fibrils, accounting for less than 10% of total cartilage collagen content. Therefore, up-regulation of the wild-type allele could negate the effect of haploinsufficiency.

Murine Chondrodysplasia as a Model for Human Chondrodysplasias

The *cho* mice provide an animal model for human disorders due to mutations in type XI collagen chains. Several human chondrodysplasias share inheritance patterns and phenotypic similarities, such as abnormally wide collagen fibrils, with *cho* (Spranger and Maroteaux, 1990; Burton et al., 1986). The collagen XI genes can be considered candidates for these conditions. Also of interest is the identification of abnormal collagen fibrils in several types of Ehlers–Danlos syndrome, potentially implicating collagen V, which is closely related to collagen XI, in the pathogenesis of these disorders (Hausser and Anton-Lamprecht, 1994).

As has previously been noted for other structural matrix components, a spectrum of phenotypes may occur depending upon the effect of the gene mutation on the protein product. For example, haploinsufficiency of collagen II causes a form of Stickler syndrome, whereas dominant negative mutations cause a spectrum of phenotypes ranging from mild chondrodysplasia with arthritis to lethal achondrogenesis (Kivirikko, 1993). We anticipate a similar spectrum of phenotypes for collagen XI mutations. This conclusion is supported by the results reported in the accompanying paper (Vikkula et al., 1995 [this issue of *Cell*]). In a family with autosomal dominant Stickler syndrome linked to the *COL11A2* gene (Brunner et al., 1994), we demonstrate a splice junction mutation causing an in-frame deletion of 18 amino acid residues in α2(XI) collagen. Members of this family do not show the ophthalmological abnormalities usually associated with chondrodysplasia in the Stickler syndrome, consistent with the absence of the α2(XI) chain in the mammalian vitreous form of type XI collagen (Mayne et al., 1993). In the accompanying paper (Vikkula et al., 1995), we further demonstrate linkage to *COL11A2* in a

kindred with an autosomal recessive bone dysplasia, associated with sensorineural deafness and a Stickler-like facial morphology.

Experimental Procedures

Linkage Analysis to Map the *cho* and *Col11a1* Genes

C57BL/6FrSe mice heterozygous for the *cho* gene (*cho*/+) were crossed with a linkage testing stock, MEV, containing multiple ecotropic proviruses (Taylor and Rowe, 1989). F1 animals were intercrossed, and 51 dwarfed, nonviable F2 homozygotes (*cho*/*cho*) were collected. An additional 54 homozygotes were obtained from a backcross of C57BL/6FrSe/MEV (*cho*/+) heterozygotes with *cho*/+ mice of the parental stock. Genomic DNA was isolated from livers or body tissue according to Bell et al. (1981). For Southern blotting, DNA digested with PvuII was electrophoresed through 0.7% Seakem ME agarose (FMC) gels and blotted onto Biotrace HP membranes (Gelman Biosciences). Hybridization was with a ^{32}P-labeled MuLV probe (pEcB4) at 42°C.

For linkage analysis using simple sequence length polymorphisms, primers for PCR were synthesized based on published data (Dietrich et al., 1992) and from sequences available in the Whitehead Institute/Massachusetts Institute of Technology Genome Center Genetic Database. The PCR products were electrophoresed through 4% agarose gels. The maximum likelihood genetic map and positional estimate for the *cho* locus were generated using the MAPMAKER program (Lander et al., 1987).

For linkage mapping of *Col11a1*, we utilized a sequence length polymorphism between different inbred strains in the 3′ untranslated region of α1(XI) collagen mRNA. A panel of DNA from the BXD (C57BL/6J × DBA/2J) recombinant inbred series was used as template for PCR with primers flanking this polymorphic region, as described (Beier, 1993).

Preparation of Antibody Specific for α1(XI) Collagen

The synthetic peptide, KLSPKKTRRHTESIQA, corresponding to the distal 15 amino acids of the rat α1(XI) carboxyl telopeptide domain (Bernard et al., 1988) was provided by J. Gambee of the Shriners' Hospital analytical core facility. The proximal six amino acids of this domain, IQPLPI, were not included because they are shared by α2(XI) (Kimura et al., 1989) and α1(V) (Greenspan et al., 1991). The amino-terminal lysine is noncoded and was used to enhance coupling to keyhole limpet hemocyanin carrier. Details of the methods of antibody production and characterization are presented elsewhere (Oxford et al., 1994).

Western Blotting and Immunohistochemistry

For Western blot analysis, rib plates of normal C57BL/6FrSe and *cho*/*cho* newborn mice were pulverized in liquid nitrogen. About 50 mg of tissue powder were transferred to 400 μl of hot sample buffer (2% SDS, 10% glycerol, 12.5 μM Tris–HCl [pH 6.8], 0.1% bromophenol blue) and immersed in boiling water for 5 min. After spinning in a microcentrifuge for 1 min, the supernatant was used for gel electrophoresis.

Samples were reduced by addition of 5% 2-merceptoethanol and electrophoresed through 6% polyacrylamide gels. For Western blots, the nitrocellulose strips were blocked for 2 hr in 3% nonfat dry milk in PBS and incubated overnight at 4°C with polyclonal α1(XI) collagen antibodies or with monoclonal antibodies (2B1) against α1(II) collagen. After washing in PBS, the blots were treated with secondary antibodies conjugated to alkaline phosphatase (Promega; anti-rabbit IgG or anti-mouse IgG). The membranes were washed in PBS and developed with the Bio-Rad alkaline phosphatase reagent kit.

From a C57BL/6FrSe *cho*/+ female in day 19 of pregnancy, *cho*/*cho* mutants and normal littermates were derived and processed for microscopy. Eviscerated embryos were fixed for 48 hr in 100% ethanol containing 3.2% (w/v) polyvinylpyrrolidone (MW 40,000) and 20% dimethyl sulfoxide, washed in 100% ethanol, cleared in Histosol, and infiltrated and embedded in paraffin wax (Paraplast, mp 56–57°C).

For immunohistochemistry, slides were deparaffinized and quenched for endogenous peroxidase activity by soaking in 1% hydrogen peroxide in 100% methanol for 30 min. After rinsing in PBS, sections were treated with hyaluronidase (1,000 U/ml of Sigma type 1-S from bovine testis) in PBS and incubated in PBS containing 10% fetal bovine serum for 2 hr at room temperature to reduce nonspecific background staining. Incubation with primary antibody (polyclonal anti-α1(XI) collagen, diluted 1:50, or monoclonal anti-α1(II) collagen antibodies (2B1), diluted 1:1000) was at 4°C overnight. After washing in PBS for 30 min, the sections were incubated with secondary antibodies (Sigma anti-rabbit or anti-mouse IgG conjugated with peroxidase) at room temperature for 2 hr. Following incubation with the secondary antibody, sections were washed in PBS and peroxidase activity was visualized by reacting with a diaminobenzidine solution containing hydrogen peroxide (kit from Sigma). Slides were washed, counterstained with Harris Modified Hematoxylin, dehydrated, cleared, and mounted with Permount.

Controls included sections that had the primary antibody omitted.

Identification of the *cho* Mutation

Total RNA was isolated from whole newborn C57BL/6FrSe +/+ and *cho*/*cho* mice, using the USB Rex Total RNA Extraction Kit. mRNA was purified using the Promega PolyATract Kit.

The mRNAs served as templates for first-strand cDNA synthesis using either oligo(dT)s or random primers and the SuperScript kit from Bethesda Research Laboratories. Following removal of the mRNA template with RNase H, the cDNAs were used for PCR amplification of α1(XI) collagen sequences, using the mouse α1(XI) collagen cDNA sequence as a basis for designing specific primers (Yoshioka et al., submitted). Several fragments were amplified. Their designation and associated sense and antisense primers are as follows: *YL10-2* (5′-GAGGTCCTTCAGCACCAGC-3′ and 5′-GCATCTCTGTTACACTCTCAG-3′); *YL3-4* (5′-GCCTCACATTGATGAGTATGC-3′ and 5′-CTGCTGAAGAATCGCCTGAGC-3′); *YL12-13* (5′-CAAGCTCAGGCGATTCTTCAG-3′ and 5′-AAACATTCCCTGCTGTCCTCG-3′); *YL9G7* (5′-CCTGTTGGTGCTCCTGGAATC-3′ and 5′-GAGGTCCTTCAGCACCAGC-3′); *YL1* (5′-AAAGGTGATGAGGGTGC-3′ and 5′-ACTATATCTTTGATGGACGGG-3′). Amplification reactions were in 25 μl of 1 × PCR buffer containing 1 μl of first strand cDNA, 1 μM of each primer, 0.2 mM of each dNTP, 0.5 μl of [^{32}P]α-dCTP (3000 Ci/mmole), and 1 U of Amplitaq Polymerase (Perkin Elmer Cetus). Amplification conditions were 95°C for 5 min, followed by 30 cycles at 94°C for 1 min, 56°C for 90 s, 72°C for 2 min, and a final 10 min extension at 72°C.

Before analysis of *YL10-2*, *YL3-4*, and *YL12-13* by SSCP, the three fragments were digested with several restriction endonucleases (*YL10-2* with ClaI, EcoRI, HincII, HindIII, MspI, and StyI; *YL3-4* with AccI, ApaI, and AvaI; *YL12-13* with BglI and Sau3AI). The digested PCR products were loaded on MDE gels (AT Biochem) and 6% denaturing polyacrylamide sequencing gels. The cDNAs *YL9G7* and *YL1* were analyzed by direct nucleotide sequencing.

The fragment *YL10-2* was subcloned into the TA cloning vector from Invitrogen and sequenced by dideoxy sequencing. Genomic DNA from wild-type (*cho*/+) and mutant (*cho*/*cho*) animals was used as template for PCR. The 5′ sense primer was 5′-CAATGATTGTTGATTGTAAGAAG-3′ (*Col11a1-18*) and the 3′ antisense primer was 5′-GTTCCAAATACCATGATTCC-3′ (*Col11a1-15*). Each reaction contained 10 μl of 1 × PCR reaction buffer, 50 ng genomic DNA, 1 μM of each primer, 0.2 mM of each dNTP, 0.2 μl of [^{32}P]α-dCTP (3000 Ci/mmole), and 0.5 U of Amplitaq Polymerase (Perkin Elmer Cetus). Amplification conditions were 30 cycles at 94°C for 90 s, 55°C for 1 min, 72°C for 40 s, and a final 10 min extension at 72°C. The PCR products were analyzed by electrophoresis through a 6% denaturing polyacrylamide sequencing gel.

Analysis of *cho* and Wild-Type mRNA

Total RNA was isolated from whole newborn C57BL/6FrSe +/+ and *cho*/*cho* mice and from costal cartilage of adult (C57BL/6FrSe × DBA/2J) *cho*/+ heterozygotes using the USB Rex Total RNA Extraction Kit.

Total RNA, treated with RNase-free DNase I, served as template for first-strand cDNA synthesis using oligo(dT) priming and the SuperScript kit from Bethesda Research Laboratories. Following removal of the RNA template by digestion with RNase H, the cDNA was used for PCR amplification of the 3′ untranslated region of mouse α1(XI) collagen mRNA. The 5′ sense primer (ST3) was 5′-ACCAACCATTTACGCACATGC-3′ and the 3′ antisense primer (*RU2*) had the sequence 5′-ACTATATCTTTGATGGACTGGG-3′. Amplification reactions were

in 50 μl of 1× PCR buffer containing 1 μM of each primer, 0.2 mM of each dNTP, 1 μl of [^{32}P]α-dCTP (3000 Ci/mmole), and 2 U of Amplitaq Polymerase (Perkin Elmer Cetus). Amplification conditions were 95°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 90 s, and a final 10 min extension at 72°C. PCR products (2 μl) were heat denatured at 95°C and electrophoresed through 6% acrylamide sequencing gels.

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