THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 8, Issue of February 20, pp. 4761–4768, 1998

Printed in U.S.A.

Structurally Abnormal Type II Collagen in a Severe Form of Kniest Dysplasia Caused by an Exon 24 Skipping Mutation*

(Received for publication, June 23, 1997, and in revised form, November 17, 1997)

Mary Ann Weis‡, Douglas J. Wilkin§¶, Hyon J. Kim§‖, William R. Wilcox§, Ralph S. Lachman§‡‡, David L. Rimoin§**, Daniel H. Cohn§**, and David R. Eyre‡§§**

*From the ‡Orthopaedic Research Laboratories, University of Washington, Seattle, Washington 98195-6500, §Ahmanson Department of Pediatrics, Steven Spielberg Pediatric Research Center, Burns and Allen Cedars-Sinai Research Institute, Cedars-Sinai Medical Center, Los Angeles, California 90048-1869, Departments of **Pediatrics and ‡‡Radiology, UCLA School of Medicine, Los Angeles, California 90048-1869, and ‖Department of Medical Genetics, Ajou University College of Medicine, Suwon, South Korea*

Type II collagen mutations have been identified in a phenotypic continuum of chondrodysplasias that range widely in clinical severity. They include achondrogenesis type II, hypochondrogenesis, spondyloepiphyseal dysplasia congenita, spondyloepimetaphyseal dysplasia, Kniest dysplasia, and Stickler syndrome. We report here results that define the underlying genetic defect and consequent altered structure of assembled type II collagen in a neonatal lethal form of Kniest dysplasia. Electrophoresis of a cyanogen bromide (CNBr) (CB) digest of sternal cartilage revealed an α1(II)CB11 peptide doublet and a slightly retarded mobility for all major CB peptides, which implied post-translational overmodification. Further peptide mapping and sequence analysis of CB11 revealed equal amounts of a normal α1(II) sequence and a chain lacking the 18 residues (361–378 of the triple helical domain) corresponding to exon 24. Sequence analysis of an amplified genomic DNA fragment identified a G to A transition in the +5 position of the splice donor consensus sequence of intron 24 in one allele. Cartilage matrix analysis showed that the short α1(II) chain was present in collagen molecules that had become cross-linked into fibrils. Trypsin digestion of the pepsin-extracted native type II collagen selectively cleaved the normal length α1(II) chains within the exon 24 domain. These findings support a hypothesis that normal and short α-chains had combined to form heterotrimeric molecules in which the chains were in register in both directions from the deletion site, accommodated effectively by a loop out of the normal chain exon 24 domain. Such an accommodation, with potential overall shortening of the helical domain and hence misalignment of intermolecular relationships within fibrils, offers a common molecular mechanism by which a group of different mutations might act to produce the Kniest phenotype.

The chondrodysplasias are a clinically and genetically heterogeneous group of skeletal disorders characterized by abnormal endochondral ossification (1, 2). Phenotypes within the group of chondrodysplasias caused by mutations in the gene for type II collagen, COL2A1, range in severity from the neonatal lethal dwarfing conditions achondrogenesis type II and hypochondrogenesis to mild phenotypes, such as Stickler syndrome, in which there is little observed skeletal growth abnormality (3). Together with the spondyloepiphyseal dysplasias and spondyloepimetaphyseal dysplasias, Kniest dysplasia is a moderately severe disorder within the clinical spectrum of type II collagenopathies (1–4). The phenotype is characterized by disproportionate dwarfism, a short trunk and small pelvis, kyphoscoliosis, and short limbs with prominent joints that can have restricted mobility. Craniofacial anomalies may include a flat face, myopia and retinal detachment, cleft palate, and hearing loss. Radiographic features include narrowed joint spaces, platyspondyly, vertical clefts of the vertebral bodies, short tubular bones, large epiphyses, and flared metaphyses.

Cartilage from Kniest dysplasia patients displays a distinct morphology, termed "Swiss cheese" cartilage (5), which is diagnostic for the phenotype. The matrix has a foamy appearance with sparse, thin collagen fibrils surrounding the chondrocytes and thickened fibrils in the periphery. Abnormal type II collagen can be isolated in high yield from Kniest dysplasia cartilage (6, 7), suggesting that the incorporation of mutant type II collagen into the matrix produces the characteristic histologic appearance.

Of about 10 distinct mutations known to cause Kniest dysplasia, most result in whole or partial exon deletions that cluster in the amino-terminal half of the triple-helical domain of the type II collagen molecule (8). Previously we suggested two models for the assembly of type II collagen trimers composed of normal chains and chains with a deletion: 1) maintenance of the triple-helix along the length of the molecule from the carboxyl terminus, resulting in a misregister of short chains relative to normal chains from the deletion site to the amino terminus and 2) "loop out" of the normal chain(s) with maintenance of chain registration and a normal amino-terminal structure (6). Here, we describe a new dominant mutation that results in the skipping of exon 24 and produces a severe form of Kniest dysplasia. Analysis of the abnormal type II collagen from the tissue provides evidence for the second model, that the normal chain sequences in effect loop out at the site of the deletion.

MATERIALS AND METHODS

Clinical Summary

The proband, the female product of a 37-week gestation, died of respiratory distress at 10 days of age. The infant had short limbs, club

* This work was supported in part by National Institutes of Health Grants HD22657, AR37318, and GM16219 (to D. W.). Additional support was from the Division of Intramural Research, National Human Genome Research Institute, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Current address: Medical Genetics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892-1267.

§§ To whom correspondence should be addressed: Dept. of Orthopaedics, University of Washington, P. O. Box 356500, Seattle, WA 98195-6500. Tel.: 206-543-4700; Fax: 206-685-4700.

feet, cleft palate, midface hypoplasia, and a narrow chest. X-rays revealed flattened vertebral bodies with coronal clefts, slight shortening of the ribs, and dumbbell-shaped femurs. These findings were compatible with a diagnosis of Kniest dysplasia (9). Cartilage and other tissues were obtained for analysis.

Protein Analysis

Pepsin Extraction of Collagen—Sternal cartilage was extracted in 4 M guanidine HCl, 50 mM Tris/HCl, pH 7.0, at 4 °C for 48 h. The washed residue was digested with pepsin (10). Cartilage from a 4-day-old infant was similarly treated as a control.

Collagen Peptide Analysis—Sternal cartilage was digested with CNBr in 70% (w/v) formic acid for 24 h at room temperature (11), and the resulting peptides were fractionated by sequential cation-exchange and reverse-phase HPLC[1] (12, 13). Fractions containing α1(II)CB11 (identified by SDS-PAGE) were pooled, dried, and digested further with either trypsin or endoproteinase Asp-N. Resulting peptides were fractionated by reverse-phase HPLC (13). Peptide yields were estimated by integration of peak areas of 220-nm absorbance and by the recoveries of phenylthiohydantoin-derivatives on subsequent sequence analysis.

Trypsin Susceptibility Assay—Pepsin-solubilized native collagens from patient and control tissues were dissolved in 0.1 M NH_4HCO_3, 0.01% (w/v) SDS, pH 7.8, at 24 °C. Trypsin was added at a 1:100 (w/w) enzyme:substrate ratio, and aliquots were incubated at various temperatures ranging from 24 to 37 °C for 18 h. The digests were then run on 6% gels (SDS-PAGE) and electroblotted to polyvinylidene difluoride membrane for sequence analysis.

SDS-PAGE—The method of Laemmli (14) was used with 6% and 12.5% gels, respectively, for whole α-chains and CNBr peptides.

Protein Microsequencing—Amino-terminal sequence analysis of individual peptides was carried out by Edman chemistry on a Porton 2090E machine equipped with on-line HPLC analysis of the phenylthiohydantoin-derivatives. The standard program was modified to resolve 4-hydroxyproline, hydroxylysine, and the two hydroxylysine glycosides.

Collagen Cross-linking Analysis—Cartilage was acid-hydrolyzed (6N HCl, 110 °C, 24 h) for analysis by reverse-phase HPLC (15) of the pyridinoline cross-links of collagen. Hydroxyproline was quantified in the same hydrolysate by colorimetric assay (16). Pyridinoline concentration was expressed in mol/mol of collagen for comparison of tissues.

The CNBr digests of pepsin-extracted type II collagen were also analyzed by molecular sieve HPLC (Toso-Haas G3000SW, 7.5 mm x 60 cm, two columns in series (6); eluent, 0.1 M sodium phosphate, pH 6.8, 30% (v/v) acetonitrile) monitoring for pyridinoline cross-link fluorescence in the resolved peptides.

Histology

Cartilage from the proband was prepared for light and electron microscopy as described previously (17).

COL2A1 Analysis

Total RNA was isolated from cartilage by the method of Chomczynski and Sacchi (18). RT-PCR was carried out essentially as described previously (19). Briefly, COL2A1 cDNA was synthesized by reverse transcription using 1 μg of total RNA and a gene-specific primer complementary to sequences in exon 25 (5′-CCAGGACGACCATCTTCACCA-3′). Amplification by PCR used the reverse transcription primer and a second gene-specific primer that annealed within exon 23 (5′-CAAGGGAGCCAACGGTGACC-3′). DNA was denatured at 94 °C for 2 min followed by 35 cycles of 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C in a DNA Thermal Cycler (Perkin-Elmer). The final 72 °C step was extended to 10 min. PCR products were analyzed by electrophoresis through 6% polyacrylamide gels.

Genomic DNA was isolated from fibroblasts or lymphoblastoid cell lines by standard methods. A genomic DNA fragment was amplified by PCR using a COL2A1 intron 23 forward primer (5′-CAGCCCTGCACTGCCAGGAT-3′) and the exon 25 reverse primer described above. Cycling conditions were as described above. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Inc.) and cloned using the TA cloning kit (Invitrogen Corp.). Colonies were grown and plasmid DNA was isolated by standard methods. Sequencing was with the fluorescent dideoxy terminator method of cycle sequencing on a Perkin-Elmer/ABI 373a automated DNA sequencer following ABI protocols.

[1] The abbreviations used are: HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; bp, base pair(s); RT, reverse transcription; CB, cyanogen bromide.

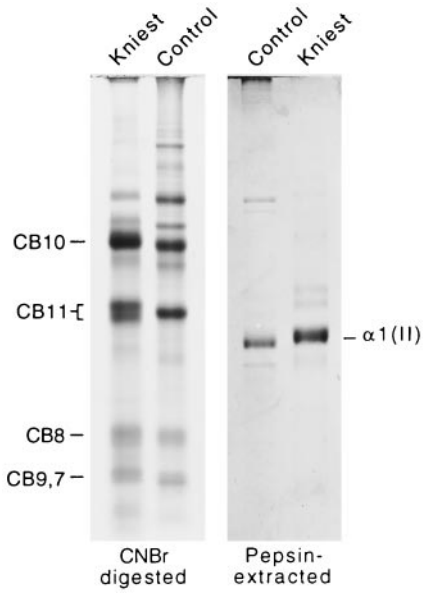

FIG. 1. **SDS-PAGE of cyanogen bromide-digested collagen peptides and pepsin-extracted collagen from human control and Kniest dysplasia cartilages.** The cyanogen bromide digests (12.5% gel; *left lanes*) show a doublet for peptide α1(II)CB11 from Kniest tissue, with the lower form running slightly faster and the upper form slightly slower than control CB11. Peptide CB10 also ran slower for Kniest compared with control. The pepsin-extracted collagen (6% gel, *right lanes*) shows a broad α1(II) band running slightly slower than control α1(II).

RESULTS

The collagen peptides from CNBr-digested Kniest cartilage migrated more slowly than their control counterparts on SDS-PAGE (Fig. 1). Peptide CB11 appeared to run as a doublet of bands, one migrating more slowly and the other faster than the control CB11 (Fig. 1, *left lane*). The pepsin-solubilized α1(II) chains from Kniest cartilage migrated as a broad band, slightly slower than control α1(II). These observations suggested a deletion in peptide CB11 in about half the α1(II) chains of the tissue. The faster CB11 band was the deletion candidate, and the slower CB11 band behaved as a post-translationally overmodified normal peptide. There was no obvious enrichment or abnormality of collagen α1(II) chains in the guanidine HCl extract of Kniest cartilage compared with control cartilage (not shown).

Enrichment from the cartilage CNBr digest by cation-exchange HPLC and analysis by SDS-PAGE confirmed that CB11 was the source of the doublet (Fig. 2). Pooled fractions of CB11 were purified by reverse-phase HPLC (not shown), and aliquots were digested with trypsin or endoproteinase Asp-N for peptide mapping in comparison with control CB11. Fig. 3 shows the resulting tryptic peptide profiles. (The endoproteinase Asp-N results are not shown.) Peptides that appeared to be unique to the patient were selected for amino-terminal sequence analysis. The observed peptide sequence shown *underlined* in Fig. 3*a* would result from trypsin cleavage if the exon 24 domain (residues 361–378 of the triple helix; Ref. 20) were deleted to create a new tryptic cleavage site between the terminal Arg-360 of exon 23 and the initial Gly-379 of exon 25. Deletion of the exon 24 sequence was confirmed by sequence analysis of an endoproteinase Asp-N peptide, which spanned the deletion site. It was estimated from densitometry of the two CB11 bands on SDS-PAGE and the yields of the various peptides on sequence analysis that the proportion of deletion-bearing α-chains in the Kniest tissue was 40–50% that of total α1(II).

The pepsin-solubilized molecule was probed with trypsin in an attempt to distinguish between two hypotheses concerning

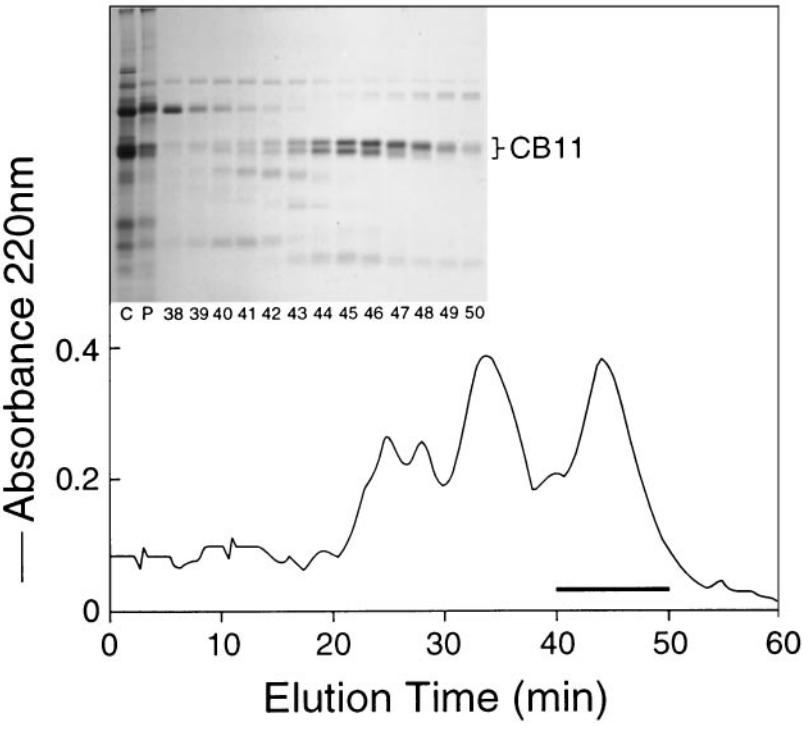

FIG. 2. **Chromatography of α1(II)CB peptides on cation-exchange HPLC.** The *inset* shows the results of SDS-12.5% PAGE on fractions across the peak, indicated by the *bar*. The column (Mono S HR 5/5, Pharmacia Biotech Inc.) was eluted with a linear gradient of 0.05–0.25 M NaCl in 0.02 M sodium formate, 10% (v/v) acetonitrile, pH 3.8, over 70 min at 1 ml/min. The remainder of the fractions indicated by the *bar* were pooled for reverse-phase HPLC. Peptide CB11 and the partial cleavage product CB11,8 ran as doublets. Samples in *lanes C* and *P* were from the whole cyanogen bromide digests of control and patient cartilages, respectively.

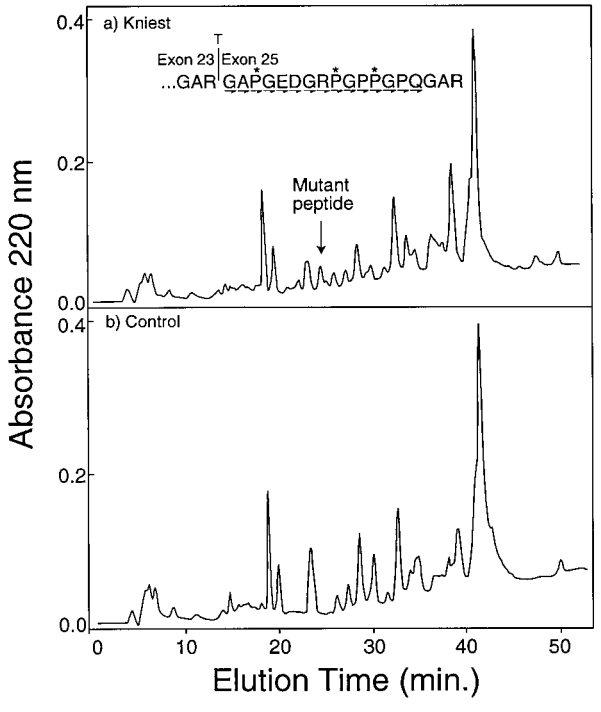

FIG. 3. **Reverse-phase HPLC of tryptic peptides from α1(II)CB11.** The column (C8, 25 cm x 4.6 mm, RP-300 Brownlee Laboratories) was eluted with a linear gradient (0–30% in 60 min) of acetonitrile:*n*-propanol (3:1, v/v) in 0.1% (v/v) trifluoroacetic acid at 1 ml/min.

the structural consequences of forming trimers from a mixture of normal and deleted chains (6). The first possibility was that the α-chains were in correct register with respect to their X- and Y-position amino acids, both amino-terminal and carboxyl-terminal to the disrupted region at the deletion site. This hypothesis requires that the normal chains in effect loop out to preserve the chain registration. The second possibility is that the molecules fold into a continuous triple helix without regard to chain registration. This means that the X- and Y-position residues would be out of register amino-terminal to the deletion and that the chain(s) with the deletion would stop short of the amino terminus of the molecule.

The pepsin-solubilized collagen used as the substrate contained a ratio of short to normal chains of about 2:3 based on the CB11 doublet on SDS-PAGE of a cyanogen bromide digest. On digestion with trypsin at 24 °C, this collagen yielded two cleavage products when analyzed by SDS-PAGE. Microsequence analysis of the larger product (2/3 fragment, Fig. 4*A*) gave two sequences; one beginning at residue 361 of the triple helical domain (GLTGRPG . . .) and the other at residue 375 (VGPSGAP . . .). Both originate in the exon 24-coded sequence (Fig. 4*B*) and so must have derived from normal α1(II) chains that presumably had been incorporated into heterotrimers that also included one or two deletion-bearing chains. Amino-terminal sequence analysis of the smaller product (*1/3* fragment, Fig. 4*A*) revealed the NH_2 terminus of the α1(II) chain beginning in the NH_2-telopeptide and running into the triple-helical domain (GVMQGPM . . .). Trypsin digestion of the collagen at 34 °C also yielded two cleavage products when analyzed by SDS-PAGE. The larger product (2/3 fragment, Fig. 4*A*) yielded the same two amino-terminal sequences found at 24 °C plus a third sequence starting at residue 379 (GAPGEDG . . .) in the domain encoded by exon 25. This trypsin cleavage product can only arise from the exon 24-deleted product of the mutant allele. At 37 °C, trypsin digestion produced little or no observable 2/3 and 1/3 fragments, although at least half of the starting collagen had been degraded, as seen by the decrease in α1(II). Presumably, this reflects rapid melting of the two triple helical fragments and further proteolysis of their denatured chains at the higher temperature. The faint band in the 37 °C Kniest lane (Fig. 4*A*) that appears to be the 1/3 fragment may in fact be a degradation product of the 2/3 fragment. (The minor band seen running midway between the 2/3 and 1/3 fragments in the 34 °C Kniest lane proved by sequence analysis to be a COOH-terminal piece of the 2/3 fragment.)

To explore the apparent melting properties of the collagen in more detail, the trypsin digestion experiment was repeated with brief digestion (2 min) and a higher enzyme:substrate

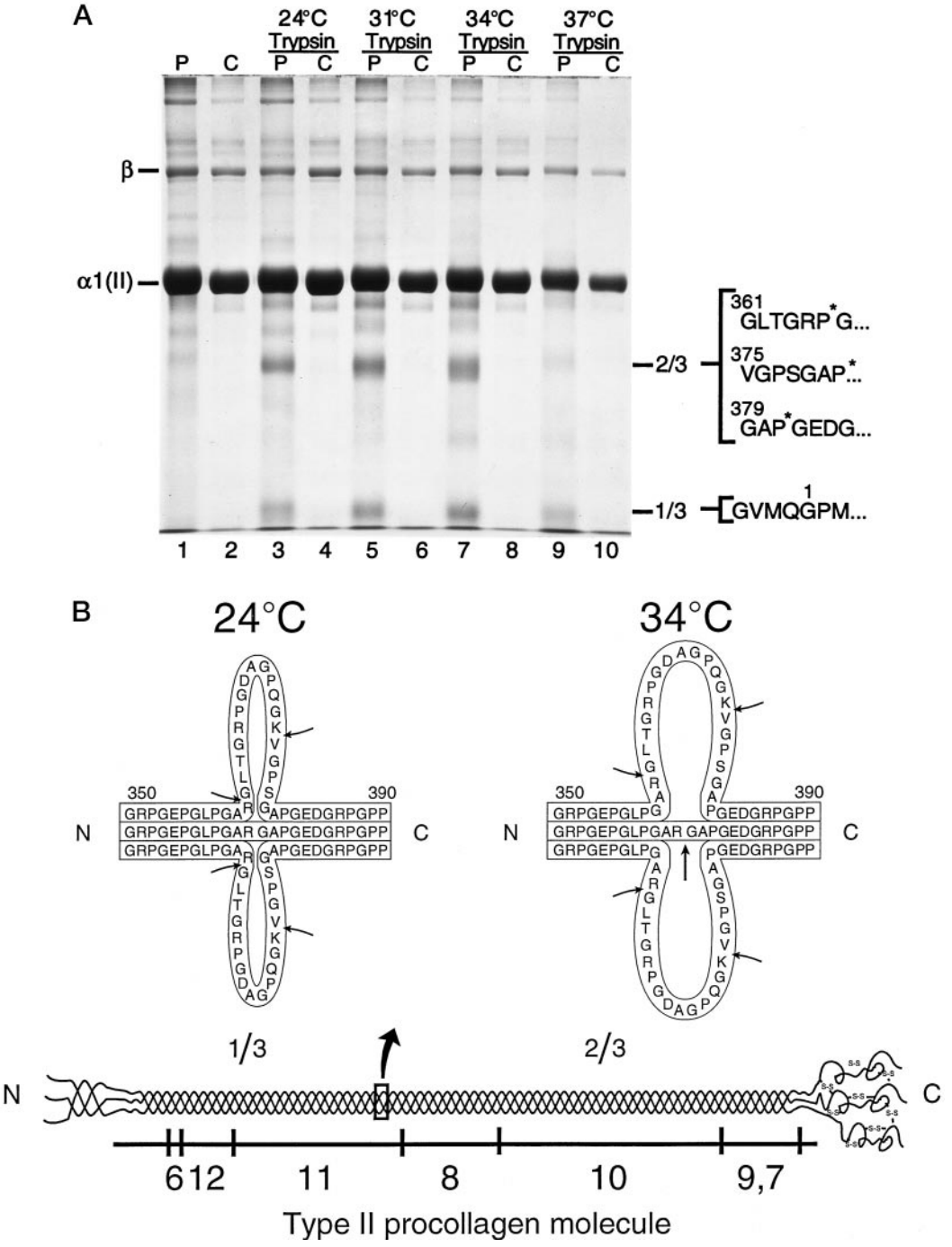

FIG. 4. **Trypsin susceptibility of native collagen type II molecules from the Kniest cartilage.** *A*, SDS-6% PAGE of trypsin digests (18 h) of pepsin-extracted collagen at 24, 31, 34, and 37 °C. Resulting cleavage products of the chains (*2/3*, *1/3* lengths) were subjected to NH_2-terminal sequence analysis after electroblotting to polyvinylidene difluoride membrane, giving the NH_2 termini shown. *B*, conceptual model showing trypsin cleavage sites within the exon 24 region of normal α1(II) chains and in an exon 24-deleted α1(II) chain that would yield the NH_2-terminal sequences and ⅔, ⅓ chain fragments identified in *A*.

ratio (1:4). Electrophoresis of the products formed at 31–43 °C in 2 °C increments revealed the same 2/3 and 1/3 fragments at 31–35 °C (results not shown). No other discrete cleavage products were seen, and the remaining full-length molecules melted and were degraded to small fragments with an apparent T_m above 39 °C for both Kniest and control collagens.

Analysis of the CNBr digest of type II collagen from the Kniest cartilage by molecular sieve HPLC gave a similar peptide profile to that of control cartilage (Fig. 5). The fluorescence profiles each had two peaks that contain the cross-linked peptides from the two sites of pyridinoline cross-linking, two NH_2-telopeptides linked to α1(II)CB9,7 and two COOH-telopeptides linked to α1(II)CB12. For both control and Kniest tissue, the ratio of fluorescence between these two peptide pools was similar. This indicates that for the total pool of collagen in the Kniest tissue there is no obvious imbalance in relative occupancy of the two cross-linking sites, which lie at opposite ends of the triple-helix (13). The concentration of hydroxylysyl pyridinoline cross-links in the Kniest cartilage collagen was 0.87 residues/collagen molecule, compared with a range of 0.5–1.5 residues/collagen molecule for control infant cartilage. The collagen content of the Kniest tissue, however, was 19% that of the dry weight, compared with 50–60% for neonatal control cartilage.

Light microscopy of the Kniest cartilage showed the typical

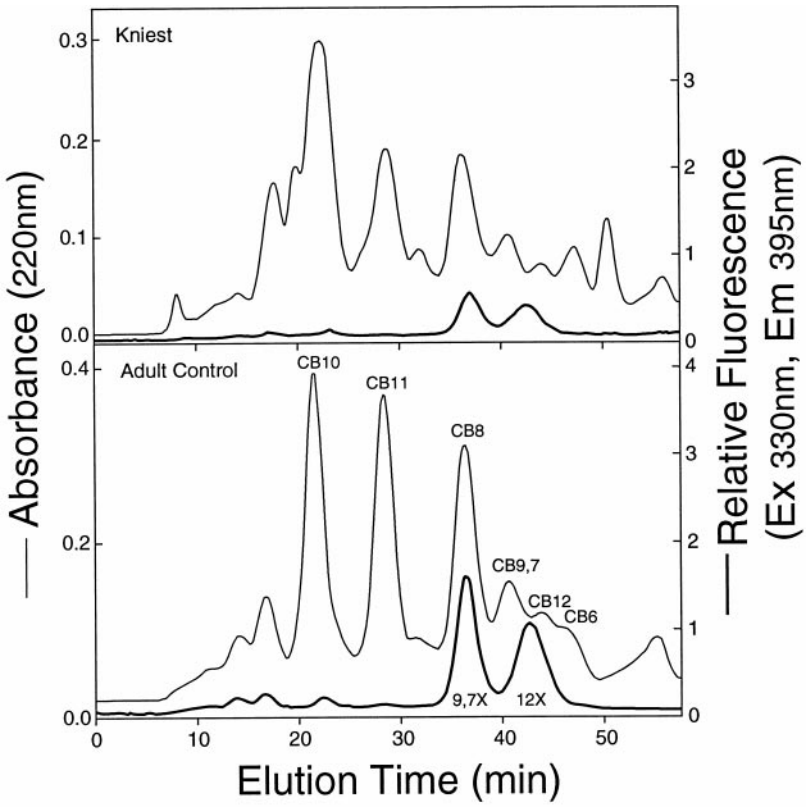

FIG. 5. **Molecular sieve HPLC of cyanogen bromide-digested collagen (pepsin-extracted) from control human and Kniest patient cartilages.** The heat-denatured samples (0.4 mg) were eluted from twin columns (Toso Haas G 3000 SW, 7.5 mm x 60 cm, × 2 in series) in 0.1 M sodium phosphate, pH 6.8, 30% (v/v) acetonitrile, monitoring for pyridinoline cross-link fluorescence (13) and peptide absorbance. Peptides *9,7X* and *12X* are derived from the two molecular sites of cross-linking in type II collagen, NH_2-telopeptide to helix, and COOH-telopeptide to helix, respectively.

Swiss cheese and a perilacunar foamy appearance characteristic of the phenotype (Fig. 6*A*; Refs. 1 and 5). Transmission electron microscopy showed sparse, thin collagen fibrils immediately surrounding the chondrocytes and thickened fibrils in the periphery (not shown). The chondrocytes contained large inclusion bodies of rough endoplasmic reticulum swollen with a granular material that is presumably abnormal type II collagen (Fig. 6*B*; Ref. 21).

To identify the underlying mutation, a cDNA fragment designed to contain the exon 24 coding domain was amplified from total RNA prepared from the Kniest cartilage. This analysis revealed the expected 189-bp fragment, also amplified from control cartilage RNA, plus a shorter fragment of approximately 135 bp (Fig. 7). This smaller fragment would result from mRNA in which exon 24 was deleted, compatible with an exon skipping mutation and consistent with the protein data. Direct sequence analysis of an amplified genomic DNA fragment containing the consensus splice sequences flanking exon 24 demonstrated that the patient was heterozygous for a G to A transition at position +5 of the intron 24 splice donor (Fig. 8).

DISCUSSION

The results define the effects at the protein level of a new mutation in the type II collagen gene that causes Kniest dysplasia. This extends the number of distinct COL2A1 mutations known to cause this phenotype to 10. They include splice-site mutations that cause or predict skipping of exons 12 (22), 15 (23), 18 (8), 20 (Kniest-like; Ref. 24), and now exon 24. Short deletions of seven amino acids in exon 12 (6), six amino acids in exon 21 (25), 34 (8), and 49 (26) also cause Kniest dysplasia. Two single amino acid substitutions, G103D (7) and G127D[2] have also been found in Kniest-like cases. It is notable that the exon 20 skipping mutation manifested as a spondyloepiphyseal dysplasia phenotype in the father and a Kniest-like radiographic phenotype in an affected fetus (24). Similarly, the G103D substitution resulted in a Kniest diagnosis from birth to 2 years (7), but the child went on to develop features of an spondyloepiphyseal dysplasia phenotype.[3]

The present molecular defect, a G to A transition at the +5 position of the donor splice site of intron 24, results in skipping of exon 24 in the spliced product of the mutant allele. The other allele and its spliced transcript are normal. This gene defect explains the recovery of both normal α1(II) protein sequences and exon 24-deleted α1(II) sequences from the structural collagen of the Kniest cartilage. Our detection of mRNA lacking exon 24 was nonquantitative and based on RT-PCR using primers for exons 23 and 25. It is possible that other mRNA splice forms had resulted from the mutation that were not detected by this strategy and also that some of the abnormal transcripts were degraded in the nucleus.

Yields of cyanogen bromide peptides on SDS-PAGE and of sequenced peptides indicate a ratio of short α1(II)/normal α1(II) of about two-thirds in the tissue. The inextractability of the short α1(II) chains in 4 M guanidine HCl and their recovery in triple-helical collagen molecules after pepsin digestion of the tissue indicate that they had become incorporated into the structural fabric of the extracellular matrix as components of cross-linked fibrils. The pyridinoline content/mol of tissue collagen and the distribution of these cross-links between the two intermolecular cross-linking sites in type II collagen (27) were in the normal range for neonatal cartilage. The content of collagen/tissue dry weight (19%) was low, however, consistent with the sparse distribution of collagen fibrils in Kniest cartilage seen by electron microscopy (28). Matrix deposition of collagen-containing mutant α1(II) chains appears to be a common feature in Kniest dysplasia. Including the present results, we and others have observed this for at least five different COL2A1 mutations that showed a Kniest-like phenotype and

[2] D. R. Eyre, M. A. Weis, R. S. Lachman, and D. L. Rimoin, unpublished results.

[3] D. L. Rimoin, unpublished observations.

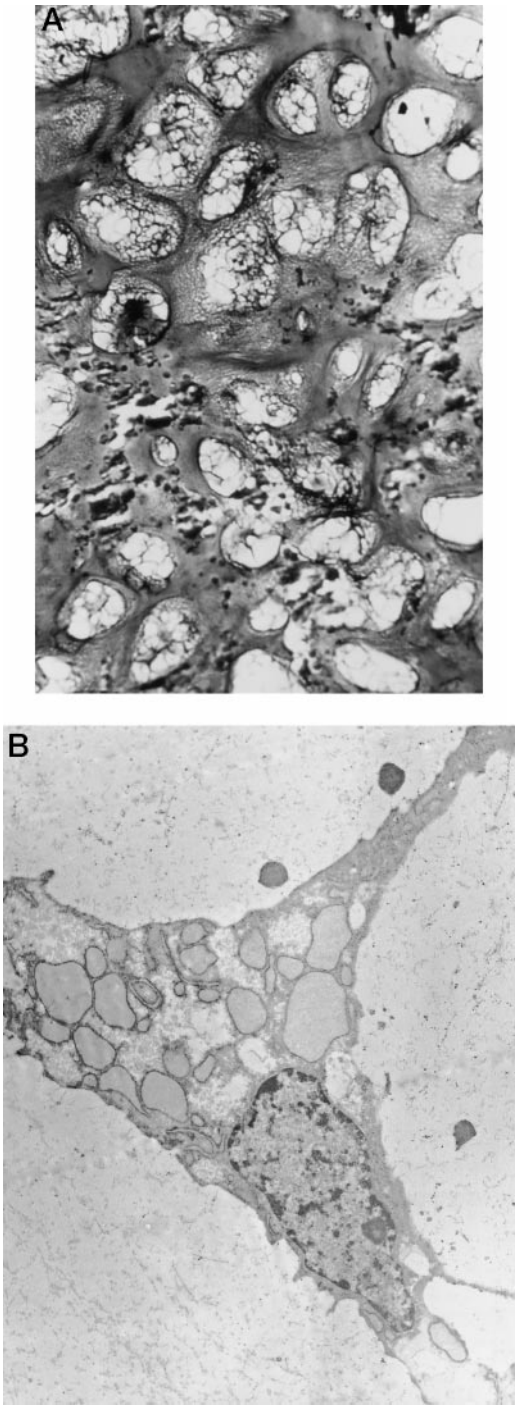

FIG. 6. **Light microscopy (*A*) and transmission electron microscopy (*B*) of hyaline cartilage from the Kniest patient.** An abnormal extracellular matrix with vacular degeneration (Swiss cheese and pericellular foamy appearance) is evident in *A*. Chondrocytes contain many large inclusion bodies filled with granular material (*B*).

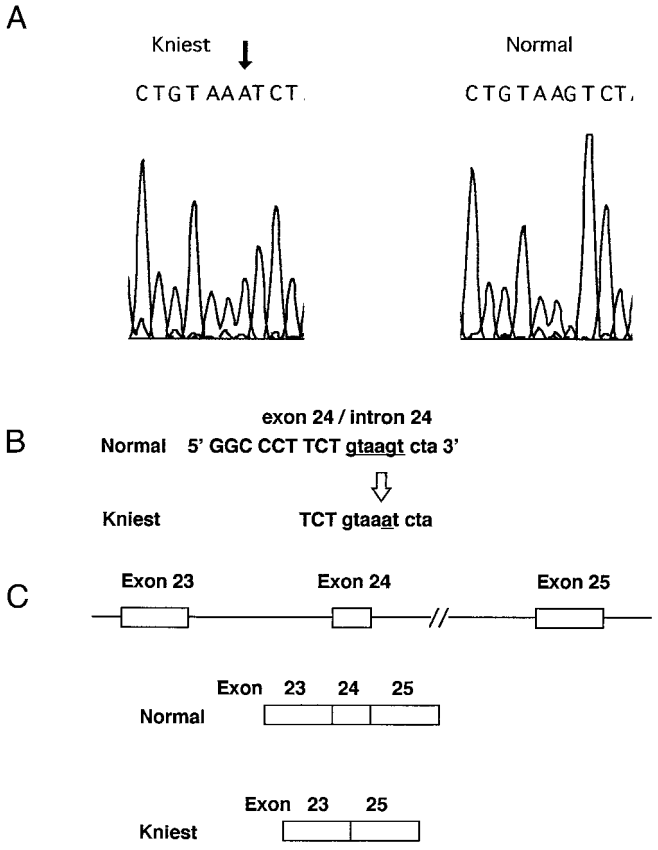

FIG. 7. **Genomic sequences.** *A*, direct sequencing of PCR product revealed a G to A at the +5 position of the donor splice site of COL2A1 intron 24. The Kniest proband is heterozygous for this mutation. *B*, the normal and Kniest sequences are shown with the splice site and mutation *underlined*. *C*, spliced products from the normal and mutant alleles.

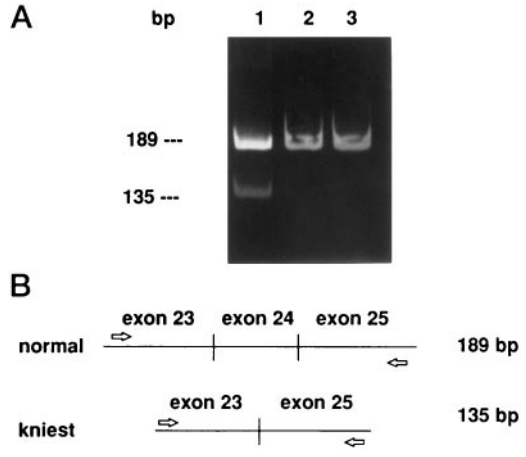

FIG. 8. **RT-PCR.** *A*, cartilage mRNA from the proband (*lane 1*) and two control individuals (*lanes 2* and *3*) were reverse-transcribed, and sequences surrounding COL2A1 exon 24 were amplified by PCR. RT-PCR from the proband revealed two bands of 189 and 135 bp, whereas RT-PCR from the control individuals only resulted in a 189-bp fragment. *B*, as depicted here, RT-PCR using mRNA that lacked sequences corresponding to COL2A1 exon 24, resulted in a 135-bp fragment (Kniest), whereas RT-PCR from normal alleles resulted in an 189-bp fragment. The position of the primers is shown (*arrows*).

from which cartilage was available for analysis (6, 7, 23–25, 29).[2] The phenotype may be owing, therefore, to the dominant negative effect of a particular form of mutant α1(II) chain being incorporated into molecules and fibrils of the extracellular matrix of cartilage. The deficiency of extracellular collagen and effects on chondrocytes of retaining abnormal protein presumably also must contribute greatly to the pathology. However, deficiency alone is unlikely to explain the unique phenotype since a matrix deficiency of type II collagen and chondrocyte accumulation of inclusion bodies is seen in the other type II collagenopathies (achondrogenesis II, hypochondrogenesis, spondyloepiphyseal dysplasia congenita, spondyloepimetaphyseal dysplasia), but their clinical presentation and cartilage histopathology are different from Kniest dysplasia.

An extensive body of published data on similar but not identical mutations that affect type I procollagen genes and cause

osteogenesis imperfecta provides the basis for interpreting the results (30, 31). These include glycine substitutions and several multi-exon (32) and single exon (33) deletions in COL1A1 and COL1A2 that cause in-frame shortening of the triple-helical domain of the expressed collagen chains. Cultured skin fibroblasts were routinely studied to define the consequences of such mutations on the molecular assembly of procollagen type I. There are relatively few data, however, on the structural collagen of osteogenesis imperfecta patient tissues, particularly from cases of single exon deletion. There is one report on a patient with osteogenesis imperfecta type IV caused by a mutation in COL1A2 that resulted in skipping of exon 12 (33). Both skin fibroblasts and osteoblasts in culture incorporated the product of the mutant allele into secreted procollagen (33). On pepsin treatment, some of the molecules were shortened at the amino terminus of the helix in both α1(I) and α2(I) chains, consistent with a helix disruption at the deletion site. The present study, therefore, is novel in providing data on structural collagen, in this case type II collagen, extracted from the extracellular matrix of a tissue assembled *in vivo*.

The trypsin digestion results reveal how exon 24-deleted α1(II) chains affect the structure of molecules into which they become incorporated. In theory, from equal pools of normal and exon 24-deleted chains, three kinds of molecule could assemble. One-eighth would be normal homotrimers, ⅛ would be short-chain homotrimers and ⅝ would be heterotrimers of normal and short chains (34). Whether all these forms assemble into stable molecules and get secreted from the chondrocytes is unknown. What is clear is that a significant fraction of molecules containing short chains do get out of the cell and become cross-linked into extracellular fibrils. The best explanation for the trypsin cleavage results is that heterotrimers present a locally disrupted domain at the deletion site (35) in which the normal chain exon 24 domains lack a triple-helical conformation and so can be cleaved after arginine and lysine (Fig. 4*B*). At 24–31 °C, the normal α1(II) chains were clipped but only in their exon 24 domain, indicating a local disruption within the triple helix. At 34 °C, the local structure further unfolded to allow cleavage after the arginine residue in the exon 24-deleted chain(s). The resulting ⅓ and ⅔ segments retained a native triple helix, so explaining the products of trypsin digestion identified on SDS-PAGE (Fig. 4*A*). Molecules assembled from three normal α1(II) chains are not cleaved at 24 or 34 °C, as seen for the control type II collagen. If heterotrimers of short and normal chains had folded as an uninterrupted zipper into a continuous triple helix from the COOH to the NH_2 terminus through the deletion site (36–38), then trypsin would not be expected to have cleaved at the bonds observed. The data, therefore, strongly support the loop-out explanation and a mechanism of triple-helix folding that can accommodate a relatively large deletion of 18 residues. This is consistent with the concept that rather than being a uniform rod, the triple helix consists of a series of alternating domains of high and low stability that can fold cooperatively but somewhat independently as helix formation proceeds from initial registration and nucleation at the COOH terminus (39). Indeed, regions of micro-unfolding can be observed in preparations of normal types I and II collagens as cleavage products when pepsin is used at acid pH above 4 °C (40). But such products were not a relevant feature of our starting collagen preparation made at 4 °C.

The fibrillar matrix of hyaline cartilage is a cross-linked copolymer of collagen types II, IX, and XI (41, 42). Type II collagen is the main component. Types IX and XI together represent about 20% of the total collagen in fetal cartilage and decrease to <5% in mature articular cartilage (43). In mature cartilage collagen, the main intermolecular cross-links are trivalent pyridinoline residues, which link type II collagen molecules in fibrils at two head-to-tail sites (27). Two COOH-telopeptides link to helix Lys-87, and two NH_2-telopeptides link to helix Lys-930. The present analyses for cross-links in Kniest cartilage and the occupancy of cross-linking sites in the isolated type II collagen show no gross effect on intermolecular cross-linking of the short α1(II) chains. There may be subtle effects, for example in restricting the potential number of cross-linking interactions between nearest neighbor molecules packed in the polymer. Thus, if molecules containing short α1(II) chains are shorter than normal molecules and become part of the fibril polymer, then not all telopeptide-to-helix interaction sites may be aligned correctly, so that the normal spatial pattern of intermolecular cross-linking may be disturbed. It may be relevant in this respect that the known mutations causing Kniest dysplasia appear to be mostly clustered about one region of the NH_2-terminal half of the molecule. One exception is a deletion of the COOH-terminal six residues of the triple-helix (26). Conceivably, shortening here could affect the positioning of the nearby COOH-telopeptides relative to their normal interaction sites in the helical domains of adjacent molecules in a fibril. The initial physical interactions responsible for the assembly of monomers into a type II collagen fibril, which may require specific binding domains along the triple helix (44), could also be affected.

The susceptibility of collagen molecules from Kniest tissue to helical domain cleavage by trypsin raises another potential source of cartilage morbidity. Matrix proteases that are involved in extracellular remodeling that do not normally attack collagen might damage Kniest fibrils. Thus, the collagen network might be prone to progressive proteolytic damage at the sites of the helical domain imperfections. This could explain the degenerate, vacular appearance of the matrix. The presence of abnormal type II collagen molecules might also disturb the copolymeric assembly of types IX and XI collagens into the fibril architecture. Finally, since the collagen type XI molecule normally includes one α1(II) chain (34), incorporating a short α1(II) chain could negatively affect collagen type XI function.

The exceptions to the emerging pattern of full or partial exon-skipping mutations underlying Kniest dysplasia are two G to D substitutions (Ref. 7).[2] They are close to each other (G103D, G127D), and one of them (G103D) is within the 7 amino acid sequence at the COOH terminus of exon 12 that is deleted in two other, unrelated Kniest cases (6). Therefore, if a G to D substitution is particularly disruptive, as collagen type I mutations in osteogenesis imperfecta suggest (36, 45), it might also result in a looping out of normal chains in this domain of the heterotrimer so that the net effect on the molecule and fibril may be the same as a short deletion. We suggest this unifying hypothesis to explain in part how different mutations might act through a common molecular mechanism to produce characteristic features of the Kniest phenotype.

Acknowledgments— We thank Mary Ann Priore and Sheila Levin of the International Skeletal Dysplasia Registry at Cedars-Sinai Medical Center for assisting with sample collection and Kae Pierce for manuscript preparation.

REFERENCES

1. Rimoin, D. L., and Lachman, R. S. (1996) in *Principles and Practice of Medical Genetics* (Emery, A. E. H., and Rimoin, D. L., eds) 3rd Ed., pp. 2779–2815, Churchill Livingstone, Edinburgh, UK
2. Horton, W. A., and Hecht, J. T. (1993) in *Connective Tissue and Its Heritable Disorders* (Royce, P. M., and Steinmann, B., eds) pp. 641–675, Wiley-Liss, Inc., New York
3. Spranger, J., Winterpacht, A., and Zabel, B. (1994) *Eur. J. Pediatr.* **153,** 56–65
4. Kniest, W. (1952) *Z. Kinderheilkd* **70,** 633–640
5. Rimoin, D. L. (1975) *Adv. Hum. Genet.* **5,** 1–118
6. Bogaert, R., Wilkin, D., Wilcox, W. R., Lachman, R., Rimoin, D., Cohn, D. H., and Eyre, D. R. (1994) *Am. J. Hum. Genet.* **55,** 1128–1136
7. Wilkin, D. J., Bogaert, R., Lachman, R. S., Rimoin, D. L., Eyre, D. R., and Cohn, D. H. (1994) *Hum. Mol. Genet.* **3,** 1999–2003

8. Spranger, J., Winterpacht, A., and Zabel, B. (1997) *Am. J. Med. Genet.* **69,** 79–84
9. Lachman, R. S., Rimoin, D. L., Hollister, D. W., Dorst, J. P., Siggers, D. C., McAlister, W., Kaufman, R. L., and Langer, L. O. (1975) *Am. J. Roentgenol. Radium Ther. Nucl. Med.* **123,** 805–814
10. Miller, E. J. (1972) *Biochemistry* **11,** 4903–4909
11. Eyre, D. R., and Muir, H. (1975) *Biochem. J.* **151,** 595–602
12. Bateman, J. F., Mascara, T., Chan, D., and Cole, W. G. (1986) *Anal. Biochem.* **54,** 338–344
13. Eyre, D. R. (1987) *Methods Enzymol.* **144,** 115–139
14. Laemmli, U. K. (1970) *Nature* **227,** 680–685
15. Eyre, D. R., Koob, T. J., and Van Ness, K. P. (1984) *Anal. Biochem.* **137,** 380–388
16. Stegemann, H. (1958) *Z. Phys. Chem.* **311,** 41–45
17. Bogaert, R., Tiller, G. E., Weis, M. A., Gruber, H. E., Rimoin, D. L., Cohn, D. H., and Eyre, D. R. (1992) *J. Biol. Chem.* **267,** 22522–22526
18. Chomczynski, P., and Sacchi, N. (1987) *Anal. Chem.* **162,** 156–159
19. Edwards, A., Hammond, H. A., Jin, L., Caskey, C. T., and Chakraborty, R. (1992) *Genomics* **12,** 241–253
20. Baldwin, C. T., Reginato, A. M., Smith, C., Jiminez, S. A., and Prockop, D. J. (1989) *J. Clin. Invest.* **81,** 521–528
21. Poole, A. R., Pidoux, I., Rosenberg, L., Hollister, D., Murray, L., and Rimoin, D. (1988) *J. Clin. Invest.* **81,** 579–589
22. Winterpacht, A., Hilbert, M., Schwarze, U., Mundlos, S., Spranger, J., and Zabel, B. (1993) *Nat. Genet.* **3,** 323–326
23. Fernandes R. J., Weis, M. E., Cohn, D. H., Rimoin, D. L., and Eyre, D. R. (1995) *Trans. Ortho. Res. Soc.* **20,** 30 (abstr.)
24. Tiller, G. E., Weis, M. A., Polumbo, P. A., Gruber, H. E., Rimoin, D. L., Cohn, D. H., and Eyre, D. R. (1995) *Am. J. Hum. Genet.* **56,** 388–395
25. Winterpacht, A., Schwarze, U., Mundlos, S., Menger, H., Spranger, J., and Zabel, B. (1994) *Hum. Mol. Genet.* **3,** 1891–1893
26. Winterpacht, A., Superti-Furga, A., Schwarze, U., Stoss, H., Steinmann, B., Spranger, J., and Zabel, B. (1996) *J. Med. Genet.* **33,** 649–654
27. Wu, J. J., and Eyre, D. R. (1984) *Biochemistry* **23,** 1850–1857
28. Horton, W. A., and Rimoin, D. L. (1979) *Pediatr. Res.* **13,** 1266–1270
29. Chen, L., Yang, W., and Cole, W. G. (1996) *J. Orthop. Res.* **14,** 712–721
30. Byers, P. H. (1993) in *Connective Tissue and Its Heritable Disorders* (Royce, P. M., and Steinmann, B., eds) pp. 317–350, Wiley-Liss, Inc., New York
31. Kuivaniemi, H., Tromp, G., and Prockop, D. J. (1991) *FASEB J.* **5,** 2052–2060
32. Mundlos, S., Chan, D., Weng, Y. M., Sillence, D. O., Cole, W. G., and Bateman, J. F. (1996) *J. Biol. Chem.* **271,** 21068–21074
33. Chipman, S. D., Shapiro, J. R., McKinstry, M. B., Stover, M. L., Branson, P., and Rowe, D. W. (1992) *J. Bone Miner. Res.* **7,** 793–805
34. Lee, B., Vissing, H., Ramirez, F., Rogers, D., and Rimoin, D. (1989) *Science* **244,** 978–980
35. Bächinger, H. P., Morris, N. P., and Davis, J. M. (1993) *Am. J. Med. Genet.* **45,** 152–162
36. Wallis, G. A., Kadler, K. E., Starman, B. J., and Byers, P. H. (1992) *J. Biol. Chem.* **267,** 25529–25534
37. Willing, M. C., Cohn, D. H., Starman, B., Holbrook, K. A., Greenberg, C. R., and Byers, P. H. (1988) *J. Biol. Chem.* **263,** 8398–8404
38. Chessler, S. D., and Byers, P. H. (1992) *J. Biol. Chem.* **267,** 7751–7757
39. Prockop, D. J. (1990) *J. Biol. Chem.* **265,** 15349–15352
40. Sokolov, B. P., Sher, B. M., Kozlov, E. A., Tsvetkova, T. A., and Del'vig, A. A. (1988) *Biokhimiya* **53,** 1828–1836
41. Wu, J. J., and Eyre, D. R. (1995) *J. Biol. Chem.* **270,** 18865–18870
42. Diab, M., Wu, J. J., and Eyre, D. R. (1996) *Biochem. J.* **314,** 327–332
43. Eyre, D. R., Wu, J. J., and Niyibizi C. (1990) in *Calcium Regulation and Bone Metabolism* (Cohn, D., Glorieaux, F. H., and Martin, T. J., eds) pp. 188–194, Elsevier Science Publishers B.V., Amsterdam
44. Fertala, A., Holmes, D. F., Kadler, K. E., Sieron, A. L., and Prockop, D. J. (1996) *J. Biol. Chem.* **271,** 14864–14869
45. Lightfoot, S. J., Atkinson, M. S., Murphy, G., Byers, P. H., and Kadler, K. E. (1994) *J. Biol. Chem.* **269,** 30352–30357