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Expression, in Cartilage, of a 7-Amino-Acid Deletion in Type II Collagen from Two Unrelated Individuals with Kniest Dysplasia

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Summary

Kniest dysplasia is a heritable chondrodysplasia that severely affects skeletal growth. Recent evidence suggests that the etiology is based on mutations in COL2A1, the gene for collagen type II. We report the detection and partial characterization of an identical defect in type II collagen in two unrelated patients with Kniest dysplasia. Analysis of cyanogen bromide (CB)–digested cartilage samples from both probands by SDS-PAGE revealed an abnormal band for peptide α1(II)CB12. The peptide was purified and digested with endoproteinase Asp-N. Fragments unique to the Kniest tissues were identified by reverse-phase high-pressure liquid chromatography and by sequence analysis. The results established a deletion of amino acids 102–108 of the α1(II) triple-helical domain, which disrupted the (gly-X-Y)n repeat needed for helix formation. This was confirmed by sequence analysis of DNA amplified from both probands, revealing the molecular basis to be a single nucleotide mutation at a CpG dinucleotide (GCG→GTG) in the codon for alanine 102. The mutation created a new splice donor site, which would account for the absence of the last seven amino acids from the 3′ end of exon 12 in α1(II)CB12. Light and electron micrographs of the probands' cartilage showed the perilacunar foamy matrix ("Swiss cheese") characteristic of Kniest dysplasia and chondrocytes containing dilated rough endoplasmic reticulum, which earlier studies had shown were filled with type II procollagen. These two cases strengthen the concept that Kniest dysplasia is based on mutations of COL2A1 and belongs within the broad spectrum of chondrodysplasias caused by type II collagenopathies.

Introduction

Type II collagen is a homotrimeric molecule found in hyaline cartilage, intervertebral disks, and tissues of the eye. Mutations in the type II collagen gene, COL2A1, have been identified in certain chondrodysplasias. These heritable disorders of skeletal growth show a wide range of clinical phenotypes (Rimoin and Lachman 1990). Those based on COL2A1 mutations commonly express skeletal and ocular anomalies, but individual characteristics and clinical severity presumably depend on the degree of disruption of collagen structure, assembly, secretion, and fibrillogenesis. The current list of clinical forms includes achondrogenesis (Eyre et al. 1986; Vissing et al. 1989), hypochondrogenesis (Bogaert et al. 1992; Horton et al. 1992), Kniest dysplasia (Wilkin et al. 1993; Winterpacht et al. 1993; Spranger et al. 1994), spondyloepiphyseal dysplasia congenita (SEDc) (Lee et al. 1989; Tiller et al. 1990), spondyloepimetaphyseal dysplasia (SEMD) (Vikkula et al. 1993; Tiller et al. 1993), familial osteoarthritis/late-onset SED (Ala-Kokko et al. 1990; Katzenstein et al. 1992; Williams et al. 1993; Winterpacht et al., in press), Stickler syndrome (Ahmad et al. 1991, 1993), and Wagner syndrome (Körkkö et al. 1993). These diseases span an extremely wide range of clinical severity, from the Stickler and Wagner syndromes at the mild end, which are characterized by ocular pathology and arthropathy, to the perinatal lethal forms of achondrogenesis and hypochondrogenesis.

Kniest dysplasia is characterized clinically by disproportionate dwarfism, cleft palate, myopia, progressive conductive hearing loss, arthropathy, and scoliosis. Radiographically, Kniest dysplasia exhibits progressive skeletal changes, including splayed epiphyses and metaphyses, platyspondyly, and narrowed joint spaces (Taybi and Lachman 1990). Histologically, cartilage from Kniest dysplasia patients demonstrates a unique extracellular matrix of sparse fibrils with a vacuolar appearance, referred to as "Swiss-cheese" cartilage (Horton and Rimoin 1979). Recent reports suggest that Kniest dysplasia may be caused by deletions or point mutations in COL2A1, which result in abnormal mRNA splicing and deletions in type II collagen (Winterpacht et al. 1993; Spranger et al. 1994).

In two sporadic cases of Kniest dysplasia, we have identified a deletion of 7 amino acids within a proportion of the α-chains of type II collagen present in cartilage. The deletion in both probands resulted from the same mutation, which occurred at a CpG dinucleotide in exon 12.

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The mutation created a preferred splice donor, effectively truncating the exon.

Subjects and Methods

Clinical Summary

Each of the two patients studied was the only affected individual within her respective family. The two families were of different ethnic backgrounds and from different parts of the United States. We therefore presume that they were unrelated.

Proband 1.—This patient (our reference number R77-019A) was initially seen by one of us (D.R.) when she was 23 years old. Physical examination showed her trunk to be quite short, with a high dorsal lumbar lordosis. Her elbows were held in a fixed position, with extension limited to almost 90°. Shoulder movement was also limited to about 90°. Her fingers were long, with prominent interphalangeal joints. There was genu valgum and some knobbiness around the knees, and her feet were held in a varus position. She had a repaired cleft palate, severe myopia, and hearing loss. Examination of X-rays (fig. 1, *upper left*) defined her diagnosis as Kniest dysplasia. Neither parent was affected.

Proband 2.—This patient (reference number R77-017) was initially referred to one of us (D.R.) at 12 years of age. Her medical history revealed that, at birth, she had a cleft palate, bilateral varus deformity of the tibias, bilateral hip dislocation, and a heart murmur. During early childhood, deformities of the arms, legs, and spine developed, as did high-frequency hearing loss and myopia. Physical examination revealed significantly short stature, a flattened face with prominent eyes, and maxillary hypoplasia. There was a repaired cleft palate, and speech was markedly palatal. There was thoracolumbar lordosis, with a prominence at the lumbar-sacral junction. All joints of the fingers were prominent and had limited flexibility. There was also limited extension of the elbows and external rotation of the hips. Her knees appeared knobby, with severe genu valgum, and there was varus deformity of the feet. The radiographic phenotype was compatible with the diagnosis of Kniest dysplasia (fig. 1, *upper right, lower left,* and *lower right*). Neither parent was affected.

Protein Analysis

Pepsin-solubilized collagen.—Samples of articular and costal cartilages from both patients were 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 (Miller 1972). Articular cartilage from a 24-year-old male was processed as a control. SDS-PAGE was carried out by the method of Laemmli (1970) on 6% and 15% gels.

Peptide analyses.—Articular and costal cartilage samples were digested with cyanogen bromide (CB) in 70% (w/v) formic acid for 24 h at room temperature (Eyre and Muir 1975). Peptides were fractionated by molecular-sieve high-performance liquid chromatography (HPLC) (Toso-Haas G3000SW, 7.5 mm × 60 cm, two columns in tandem; eluant, 140 mM Na phosphate, pH 6.8, 30% [v/v] acetonitrile) and then by reverse-phase HPLC (Bateman et al. 1986; Eyre 1987). The pool containing α1(II)CB12 (identified by SDS-PAGE) was dried and digested with endoproteinase Asp-N (sequencing grade; Boehringer Mannheim), and the peptides were fractionated by reverse-phase HPLC (Eyre 1987). Individual peptide yields were estimated by integrated 220-nm absorbance of peaks and PTH–amino acid recovery on Edman sequence analysis.

Protein microsequencing.—Peptides were covalently bound to Sequelon-AA membranes (Millipore) activated by carbodiimide. Sequencing was carried out on a Porton 2090E instrument equipped with on-line HPLC analysis of PTH–amino acids, using a modified manufacturer's program that resolved derivative peaks for 4-hydroxyproline, hydroxylysine, and hydroxylysine glycosides.

Collagen cross-linking analysis.—Cartilage was acid hydrolyzed (6 M HCL, 110°C, 24 h), and pyridinoline cross-linking residues were quantified by reverse-phase HPLC and fluorometry (Eyre et al. 1984). Hydroxyproline was assayed as an aliquot of the hydrolysate colorimetrically (Stegemann 1958). Pyridinoline content was expressed as moles per mole of collagen.

Histology

Cartilage from patient 2 was prepared for light and electron microscopy, as described elsewhere (Bogaert et al. 1992).

COL2A1 Analysis

DNA was isolated from white blood cells by standard procedures. The forward primer 5′-CTCTGTGCTCTG-AACACCTCC-3′ (from intron 10) and reverse primer 5′-GCTAGTTCCACTGAGCTCCAC-3′ (from intron 12) were synthesized and used in the PCR (Saiki et al. 1988) to amplify a 374-bp DNA fragment containing exons 11 and 12. Thirty-five PCR cycles of 1 min at 94°C, 1 min at 62°C, and 1 min at 72°C were performed in a DNA thermal cycler (Perkin Elmer Cetus), under conditions recommended by the manufacturer. The final 72°C step was 10 min. The PCR product was purified by agarose gel electrophoresis and was isolated from the gel by using Gene Clean (Bio 101). DNA was directly sequenced, using the cycle sequencing kit as recommended by the manufacturer (Promega), using a primer homologous to COL2A1 exon 11 (5′-GCTCGTGGTTTCCCAGGAAC-3′). The sequences were analyzed by denaturing gel electrophoresis (Sambrook et al. 1989).

AciI RFLP

A primer homologous to sequences within COL2A1 exon (5′-GTTATCCAGGCCTGGACGGT-3′) was de-

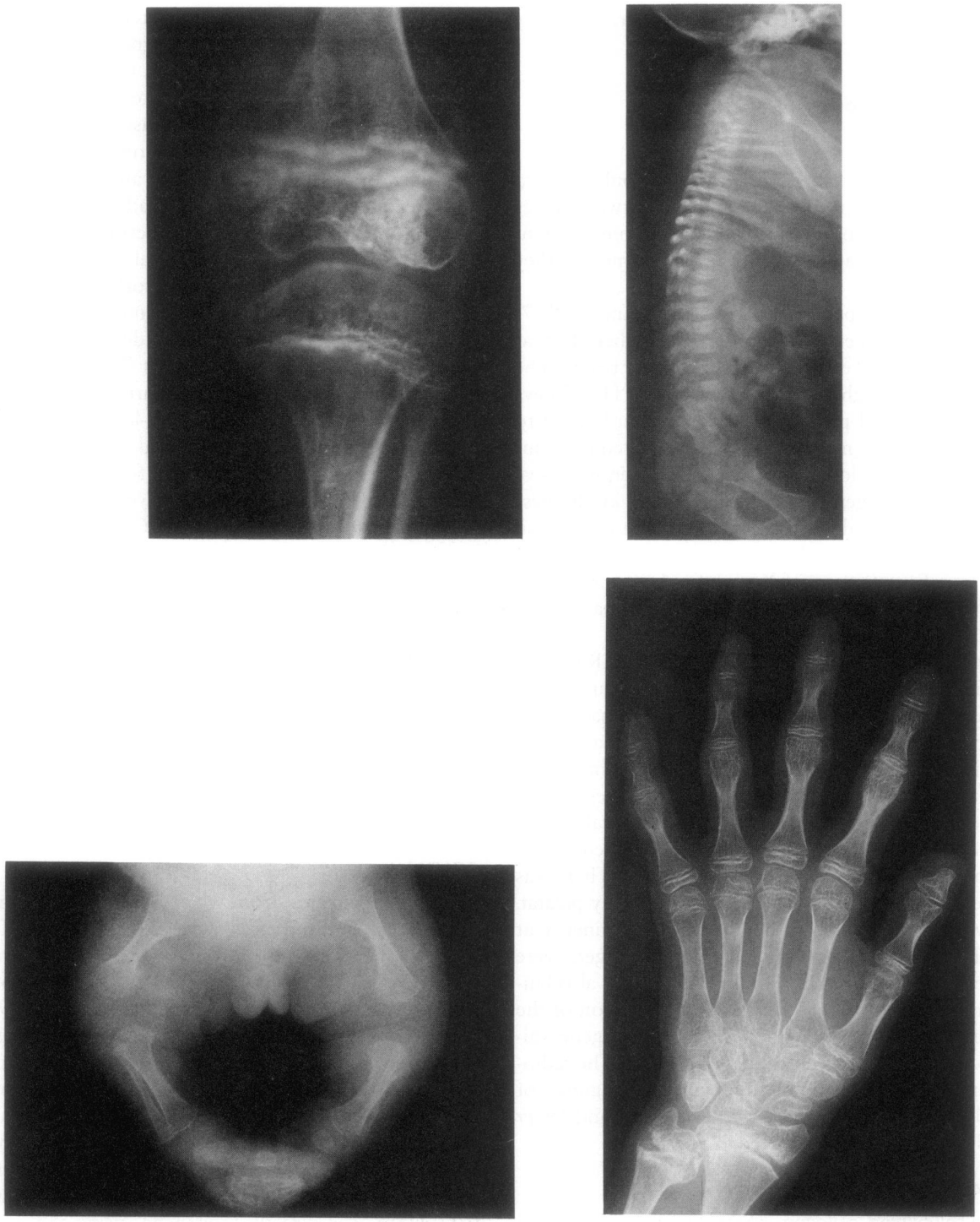

Figure 1 *Upper left,* Proband 1: anterior-posterior view (AP) of the knee at 6 years of age, showing megaepiphyses and widened metaphyses. *Upper right,* Proband 2: lateral film of the spine in the newborn period, showing platyspondyly and coronal clefts at L2 and L4. *Lower left,* Proband 2: AP of the lower extremities in the newborn period, revealing "dumbbell" femora (and tibiae) with epiphyseal delay at the knees. *Lower right,* Proband 2: AP film of the hand and wrist at 12 years of age, showing epiphyseal ossification delay and joint space narrowing with interphalangeal bulbous enlargement.

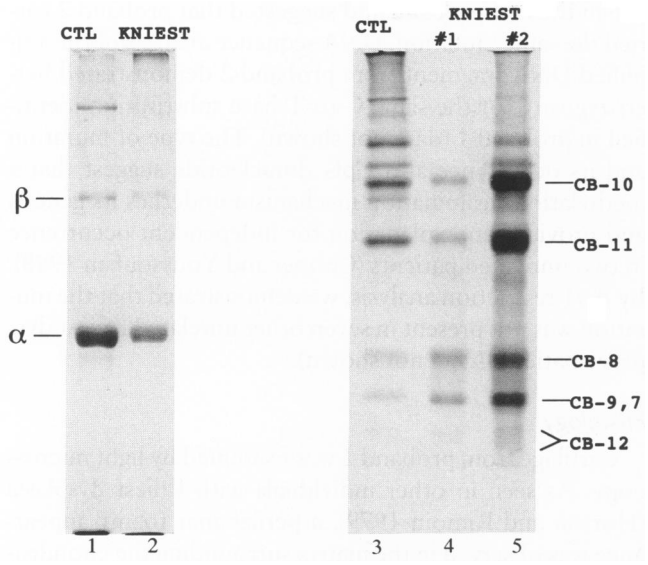

Figure 2 SDS-PAGE of pepsin-solubilized collagen (lanes 1 and 2) and CB-derived peptides (lanes 3–5) from human control and Kniest dysplasia cartilage. Pepsin-solubilized α1(II) chains are slightly retarded in the Kniest sample (lane 2; proband one) compared with the control (lane 1). Peptide α1(II)CB12 is abnormally broad in the Kniest tissue (lanes 4 and 5), compared with the control tissue (lane 3).

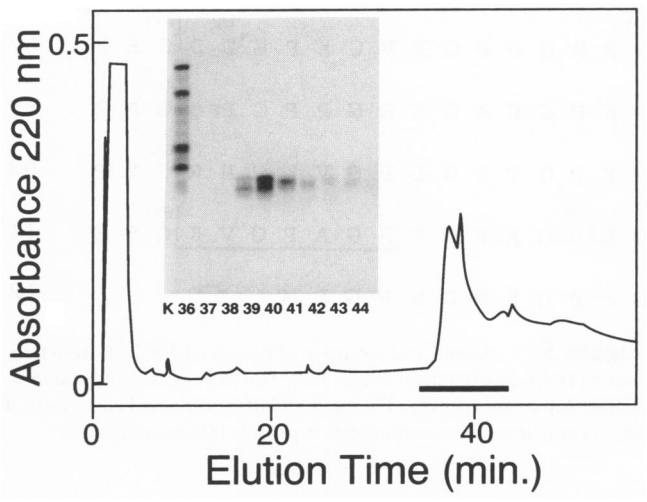

Figure 3 Reverse-phase HPLC of peptide CB12 (from fig. 2). The inset shows the results of SDS–15% PAGE on fractions across the peak, as indicated by the bar. A doublet is evident, the lower band being partially resolved slightly earlier in the chromatogram. Fractions 38–41 were pooled for proteolysis (endoproteinase Asp-N) and peptide mapping. Lane K shows total CB peptides from the Kniest tissue.

signed and used with the COL2A1 intron 12 reverse primer described above, to amplify a 150-bp fragment containing the mutation. DNA was digested with *Aci*1, as recommended by the manufacturer (New England BioLabs). Products were analyzed by 6% PAGE and staining with ethidium bromide.

Results

Protein Analysis

Electrophoresis (SDS-PAGE) of pepsin-extracted type II collagen from cartilage of proband 1 consistently showed marginally slower α1(II) chains, compared with controls (fig. 2). Similarly, peptides α1(II) CB10 and CB11 from both Kniest dysplasia patients tended to be marginally slower than control CB10 and CB11 on SDS-PAGE (fig. 2). Close inspection of the CB12 peptide from the Kniest dysplasia tissue showed a broad smear, with a portion running faster than the control CB12 peptide, suggesting an abnormality in this domain (fig. 2). Sufficient CB12 for peptide mapping and sequence analysis was isolated by sequential molecular sieve (data not shown) and reverse-phase HPLC (fig. 3). SDS-PAGE of reverse-phase HPLC fractions revealed a doublet, with the lower component eluting slightly earlier than the upper component (fig. 3), suggesting a peptide with a deletion.

A pool containing total α1(II)CB12 was digested with endoproteinase Asp-N, and the resulting peptide profile on reverse-phase HPLC revealed features unique to the Kniest dysplasia cartilages. Reverse-phase HPLC of Asp-N–digested peptides demonstrated a single peptide peak for control cartilages, D3 (fig. 4, *top*). The Kniest dysplasia samples gave two versions of peptide D3 (D3 and D3′), and a new peptide ΔD3 (fig. 4, *bottom*). Microsequencing of peptide D3 from control and Kniest dysplasia tissues yielded mostly galactosylhydroxylysine at cycle 4 and a mixture of galactosylhydroxylysine and hydroxylysine at

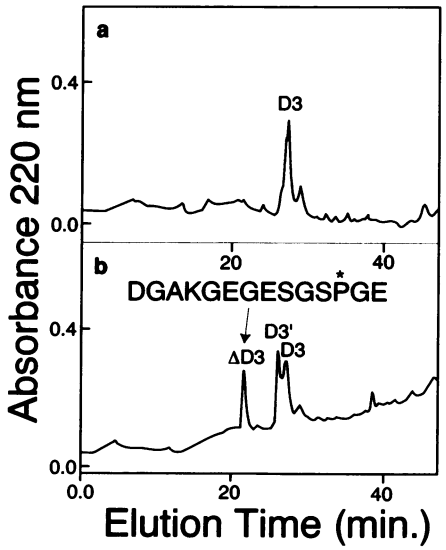

Figure 4 Elution profile on reverse-phase HPLC of endoproteinase Asp-N peptides from the CB12 peptide fragment of α1(II) from (*a*) human control cartilage and (*b*) Kniest cartilage. Peptide ΔD3 harbored the 7-amino-acid deletion.

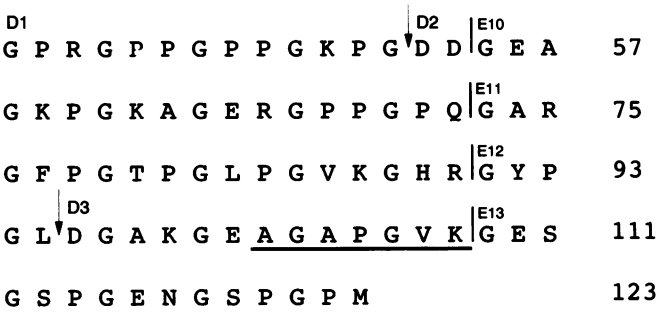

Figure 5 Amino acid sequence of human α1(II)CB12, from published cDNA data (Baldwin et al. 1989). The cleavage sites by endoproteinase Asp-N are arrowed. The exon boundaries are marked by vertical lines. The deleted seven amino acids in peptide D3 are underlined.

cycle 13. Peptide D3′ proved to be an overmodified version of D3, with predominantly glucosylgalactosylhydroxylysine at both cycles 4 and 13. The new peptide, ΔD3, revealed a 7-amino-acid deletion (α1(II) residues 102–108; fig. 5) and exclusively glucosylgalactosylhydroxylysine at cycle 4. The ratio of peptides D3, D3′, and ΔD3 was ~1:1:1. In yield, therefore, mutant α1(II) chains accounted for about one-third of total type II collagen in the cartilage. The results of peptide mapping and microsequencing of peptide CB12 were essentially identical for both Kniest dysplasia cases.

The measured concentration of hydroxylysylpyridinoline cross-links in collagen of the Kniest dysplasia cartilages was 1.2 mol/mol for proband 1 and 1.3 mol/mol for proband 2, compared with a mean of 1.5 ± 0.2 (SD) mol/mol of collagen for control adult human cartilage (Eyre et al. 1984).

COL2A1 Analysis

To determine the structure of the mutation that resulted in the peptide deletion identified at the protein level, we amplified a genomic DNA fragment from proband 1 containing exon 12 and its flanking sequences. DNA sequence analysis showed that the patient was heterozygous for a C-to-T transition within exon 12 (fig. 6, *top*). This sequence change created a potential new splice-donor site. Splicing at this site would account for the absence of the last 7 amino acids of exon 12 from the type II collagen chains encoded by the mutant allele (fig. 6, *bottom*).

The mutation abolished an *Aci*1-restriction-endonuclease cleavage site. To confirm the location of the mutation, and to determine if proband 2 carried the same defect, we used *Aci*1 digestion to analyze an amplified 150-bp genomic DNA fragment containing the mutation. When digested with *Aci*1, amplified DNA from the control individuals, including the mother of proband 1, produced fragments of 118 and 32 bp, whereas a portion of the amplified DNA from both probands remained undigested (fig. 7). These data confirmed the presence of a base change at this site in the first proband and suggested that proband 2 carried the same mutation. DNA sequence analysis of the amplified DNA fragment from proband 2 demonstrated heterozygosity for the same C-to-T base substitution identified in proband 1 (data not shown). The type of mutation and its occurrence at a CpG dinucleotide suggest that a methylation-deamination mechanism underlies its genesis and provides an explanation for independent occurrence in two unrelated patients (Cooper and Youssoufian 1988). By *Aci*1 restriction analysis, we demonstrated that the mutation was not present in seven other unrelated Kniest dysplasia patients (data not shown).

Histology

Cartilage from proband 2 was examined by light microscopy. As seen in other individuals with Kniest dysplasia (Horton and Rimoin 1979), a perilacunar foamy appearance was observed in the matrix surrounding the chondrocytes (fig. 8). On transmission-electron microscopy, chondrocytes contained swollen, rough endoplasmic reticulum filled with a granular material (data not shown). At the light-microscopic level, earlier studies (Poole et al. 1988)

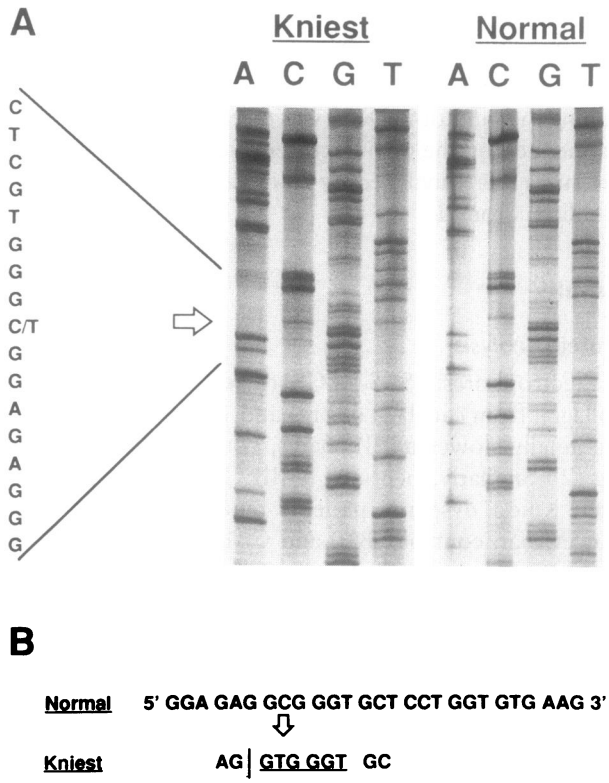

Figure 6 Sequence analysis of COL2A1 exon 12. *A*, Sequencing of COL2A1 exon 12 from amplified genomic DNA, which demonstrated heterozygosity for a C-to-T transition (*arrow*) in proband 1 (Kniest) compared with the normal DNA. *B*, Newly created splice donor sequence, shown in the Kniest dysplasia samples (*underlined*). Splicing occurs at the position marked by a vertical line.

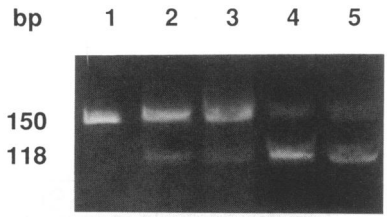

Figure 7 *Aci*1 RFLP analysis. Amplified genomic DNA was digested with *Aci*1 restriction endonuclease, as described in Subjects and Methods. Lane 1, Undigested DNA. Lane 2, Proband 2, Lane 3, Proband 1. Lane 4, Mother of proband 1. Lane 5, Unrelated, unaffected control. The *Aci*1 restriction digestion results in 118-bp and 32-bp bands. The 32-bp band is not shown. A small percentage of the initial 150-bp DNA remains uncut (lanes 4 and 5). Heterozygosity for a mutation in the restriction sequence abolishes the restriction site on one allele, allowing only 25% of the DNA to be digested at that site (lanes 2 and 3).

showed that the chondrocytes from this same patient stained with antibodies to type II procollagen. Similar histological results were found for proband 1 (data not shown).

Discussion

The results established that two unrelated individuals with typical Kniest dysplasia were heterozygous for the same mutation in the type II collagen gene. The mutation, a C-to-T transition, created a splice donor that resulted in truncation of exon 12. Although the mutation also changed the codon for alanine[102] (GCG) to valine (GTG), protein analysis showed absence of a valine-containing peptide. The mutation thus resulted exclusively in use of the new splice donor in the product of the mutant allele. This appears to be the first example of a splicing mutation that produces a deletion in the collagen triple helix and that also disrupts the gly-X-Y repeat.

Specific effects on the posttranslational chemistry of the cartilage type II collagen molecule were also evident. Peptide sequencing clearly showed that the lysines at residues 99 and 108 were overmodified in the normal α1(II) chain and at residue 99 in the mutant chain of the Kniest dysplasia cartilages, compared with the control tissue α1(II) chain. There was also slight retardation on SDS-PAGE of the α1(II) CB10 and CB11 peptides, suggesting a minor degree of overmodification (fig. 2), but much less marked than that observed, for example, with the gly853→glu substitution, which resulted in a hypochondrogenesis phenotype (Bogaert et al. 1992).

In the interpretation of these findings, current concepts of triple-helix formation and the known effects of mutations that disrupt (gly-X-Y)n must be considered. The triple helix is believed to fold from the carboxyl-terminal end of class 1 collagen molecules (types I–III). Substitutions for glycine in the triple helix of type I collagen result in marked posttranslational overmodification of those molecules containing one or more abnormal chains, amino-terminal to the mutation site (Byers 1993). Both the mutant and the normal chains are believed to be overmodified, because the mutation results in retarded helix formation, which affects all three chains equally. On this basis, the 1:1:1 ratio of the three forms of peptide D3 (figs. 4 and 5) from Kniest dysplasia cartilage can be interpreted as follows. One-third of the type II collagen molecules in the matrix were normal homotrimers (no overmodification). Two-thirds of the molecules contained one or more mutant chains (both mutant and normal being overmodified), with an overall ratio of mutant:normal chains of ~1:1 in this pool. For this to occur, there would have to be preferential retention in the cell and/or degradation of molecules containing mutant chains. For example, failure of all mutant homotrimers and of two-thirds of heterotrimers to leave the cell would account for the observed peptide ratios and would result in only three-eighths of theoretically possible molecules being deposited in the matrix.

The observation of both type II procollagen in dilated, rough endoplasmic reticulum of chondrocytes in cartilage of proband 2 (Poole et al. 1988) and sparsely distributed collagen staining in the matrix (Horton and Rimoin 1979) are consistent with the above interpretation of poorly secreted type II collagen bearing mutant chains. In addition to poor secretion and, presumably, some degradation in the cell, of molecules that contain mutant chains, abnormal molecules that are secreted and incorporated into the extracellular fabric may be preferentially degraded during normal matrix remodeling. Nevertheless, it is clear that the phenotype results, in part, from the deposition of a significant amount of mutant protein into the cross-linked fabric of the extracellular matrix.

The deletion disrupted the gly-X-Y triple-helical repeat of the type II collagen molecule. In considering how heterotrimeric molecules may assemble from shortened and

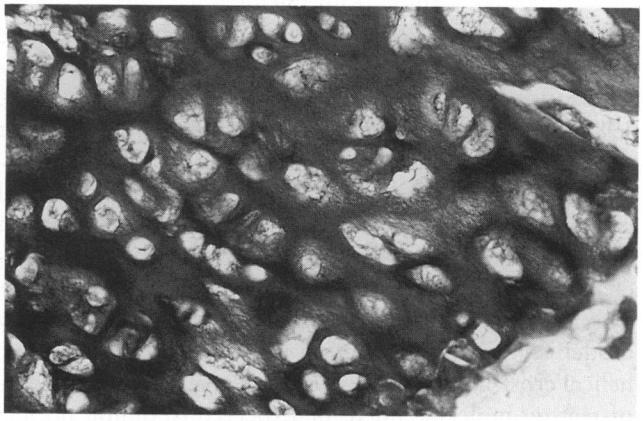

Figure 8 Morphology of cartilage from proband 2. Light microscopy shows "Swiss-cheese" cartilage. Notice the perilacunar foamy appearance.

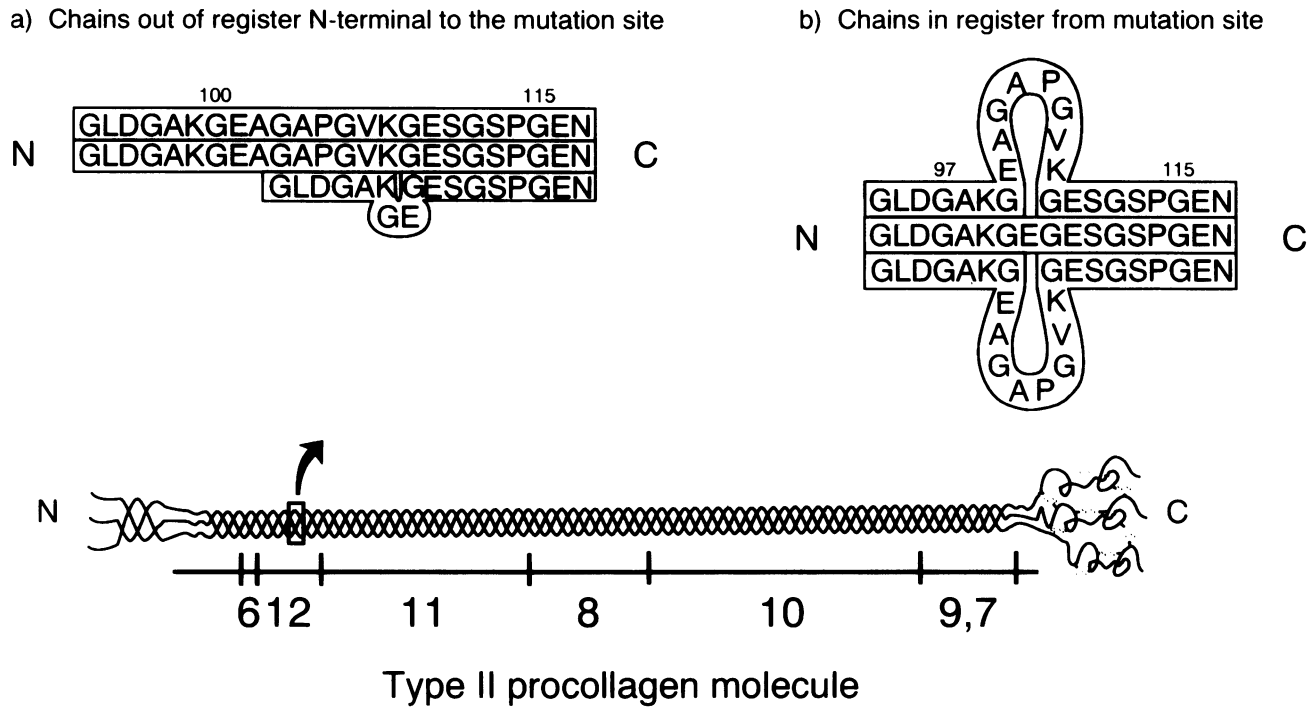

Figure 9 Divergent molecular concepts of heterotrimeric molecules containing one mutant α1(II) (Δ102-108) chain and two normal chains. In proposal a, chains are out of register amino-terminal to the mutation site; in proposal b, chains are in register in both directions away from the disrupted mutation site.

normal chains, it is assumed that deletions that preserve the (gly-X-Y)n structure would result in out-of-register chains amino-terminal to the deletion site (Willing et al. 1988; Chessler and Byers 1992; Wallis et al. 1992). The mutation described here is unusual in that it deletes a peptide segment and disrupts the (gly-X-Y)n triple-helical repeat, so this theory of helix assembly may not apply. Figure 9 illustrates two possible consequences of the deletion described in this study. In model a, assuming the triple-helix forms from the carboxyl-terminus to the amino-terminus in a zipper-like fashion (Kuivaniemi et al. 1991), α-chains resume helix formation out-of-register from a hiatus at the mutation site. This potentially could interfere with amino-propeptide removal by the propeptidase (Vogel et al. 1988). However, the lack of pN-α-chains in denaturant extracts of the Kniest dysplasia cartilage (not shown) is evidence against this model. In model b, mutant and normal chains resume an in-register triple helix from the mutation site, accommodated by a looping out of the normal α-chain sequence. This would result in shorter molecules. Though the results do not allow conclusions on which model is correct, the deletion is close to one of the two helical cross-linking sites in α1(II) at residue 87, so an out-of-register molecule might be expected to inhibit normal cross-linking. However, cross-linking of the matrix collagen was essentially normal, albeit at the low end of the range.

In another case of Kniest dysplasia, Winterpacht et al. (1993) identified a 28-bp deletion across the exon 12/intron 12 boundary in a 2-year-old girl. This mutation completely removed the splice-donor site in intron 12 and produced mRNA that lacked the exon 12 coding sequence (Spranger et al. 1994). Kniest dysplasia is not exclusively produced, however, by mutations in the exon 12 region of COL2A1. We have identified a mutation in a splice-donor consensus sequence, which resulted in the skipping of exon 24 and produced typical Kniest dysplasia (Wilkin et al. 1993). A 6-amino-acid deletion at the carboxyl-terminus of α1(II) and a 6-amino acid deletion in exon 21 have been identified in additional Kniest dysplasia patients (Spranger et al. 1994). While the evidence indicates that most cases of Kniest dysplasia may result from deletion mutations, we cannot rule out the possibility that glycine substitutions can also produce the phenotype. Furthermore, deletions within the type II collagen triple helix do not exclusively produce Kniest dysplasia. Deletion of exon 48 from genomic DNA (Lee et al. 1989) and an exon skipping mutation that resulted in deletion of exon 20 (G. E. Tiller, M. A. Weiss, P. A. Polumbo, H. E. Gruber, D. L. Rimoin, D. H. Cohn, and D. R. Eyre, unpublished data) both produced mild forms of SED. However, inheritance of the latter mutation resulted in Kniest-like radiographic findings in an affected fetus.

The mutation described here appears to lead to the de-

letion by creating a preferred splice donor, thereby truncating the exon. The new splice donor, 5′-GTGGGT-3′ (fig. 6), is identical to the splice donor of COL2A1 intron 27 (Ala-Kokko and Prockop 1990) and is closer to the consensus splice-donor sequence of higher animals (5′-GT(A/G)AGT-3′) than is the normal intron 12 donor sequence (5′-GTGAGA-3′). Both the presence of a high proportion of type II collagen chains containing the deletion and the absence of a significant level of α1(II) translated by the normally spliced mRNA encoded by the mutant allele (which would contain valine at residue 102) suggest that the splice donor created by the mutation is preferentially used. However, we cannot rule out the possibilities that either a normally spliced mRNA containing the point mutation or an α1(II) chain containing the A102V substitution is unstable.

In conclusion, the present findings support the concept that Kniest dysplasia primarily results from mutations that alter the triple-helical domain of type II collagen. The phenotype includes a disordered cartilage that results both from the deposition of abnormal type II collagen molecules in the extracellular matrix and from the accumulation of defective type II procollagen in chondrocytes (Poole et al. 1988).

Acknowledgments

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