Procollagen with Skipping of α1(I) Exon 41 Has Lower Binding Affinity for α1(I) C-telopeptide, Impaired \textit{in Vitro} Fibrillogenesis, and Altered Fibril Morphology\textsuperscript{*}

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Previous \textit{in vitro} data on type I collagen self-assembly into fibrils suggested that the amino acid 776–796 region of the α1(I) chain is crucial for fibril formation because it serves as the recognition site for the telopeptide of a docking collagen monomer. We used a natural collagen mutation with a deletion of amino acids 766–801 to confirm the importance of this region for collagen fibril formation. The proband has type III osteogenesis imperfecta and is heterozygous for a COL1A1 IVS 41 A→C substitution. The intronic mutation causes splicing of exon 41, confirmed by sequencing of normal and shorter reverse transcriptase-PCR products. Reverse transcriptase-PCR using RNA from proband dermal fibroblasts and clonal cell lines showed the mutant cDNA was about 15% of total α1(I) cDNA. The mutant transcript is translated; structurally abnormal α chains are demonstrated in the cell layer of a proband fibroblast by SDS-PAGE. The proportion of mutant chains in the secreted procollagen was determined to be 10% by resistance to digestion with MMP-1, since chains lacking exon 41 are missing the vertebral collagenase cleavage site. Secreted procollagen was used for analysis of kinetics of binding of α1(I) C-telopeptide using an optical biosensor. Telopeptide had slower association and faster dissociation from proband than from normal collagen. Purified proband pC-collagen was used to study fibril formation. The presence of the mutant molecules decreases the rate of fibril formation. The fibrils formed in the presence of 10–15% mutant molecules have strikingly increased length compared with normal collagen, but are well organized, as demonstrated by D-periodicity. These results suggest that some collagen molecules containing the mutant chain are incorporated into fibrils and that the absence of the telopeptide binding region from even a small portion of the monomers interferes with fibril growth. Both abnormal fibrils and slower remodeling may contribute to the severe phenotype.

Type I collagen is the predominant protein in the extracellular matrix of skin, bone, and tendon. In tissue, the type I collagen heterotrimer is organized into heterotypic fibrils along with types III and V collagen and non-collagenous proteins of matrix, such as decorin (1–4). Collagen fibrils in a wide range of different tissues have distinct diameter distributions (5), supporting a process for strict control over fibril growth and assembly. Structural abnormalities of type I collagen are the cause of significant medical disorders such as osteogenesis imperfecta, a genetic disorder of connective tissue characterized by bone fragility, and two of the types of Ehlers-Danlos Syndrome, in which joint and skin findings predominate (6–8). Mutant collagen may interfere with proper assembly of fibrils, which may contribute to the pathogenesis of the disorders. Conversely, studies on assembly of mutant collagens may shed new light on normal assembly processes in extracellular matrix.

Collagen molecules have the ability to self-assemble into fibrils with the well described quarter-staggered array. The \textit{in vitro} process has been the subject of investigations for more than two decades. Early studies with collagen extracted from tissues showed that assembly was a multistep process with monomers nucleating during a lag phase and then assembling both laterally and longitudinally into the fibril until attaining equilibrium with collagen monomer (9–10). These early studies also noted that removal of the nonhelical telopeptides at each end of the helical region from the collagen monomer by pepsin digestion increases assembly time 10-fold (11). These telopeptides have lengths of about 11–26 aa\textsuperscript{1} per chain. Addition of a small amount of intact collagen increased the rate of nucleation of collagen from which the telopeptides had been removed (12). In the late 1980s, a more physiological \textit{in vitro} fibrillogenesis system was developed which involved the isolation of an intermediate in the processing pathway, pC-collagen. In this system, fibrillogenesis is initiated by incubation of the intermediate with purified procollagen C-proteinase (13). Using this system, the growth of collagen I fibrils was demonstrated to be unidirectional and to occur from their tapered end in a C- to N-terminal direction (14).

More recently, the role of the telopeptide in fibrillogenesis has been further explored. Using synthetic peptides, the telopeptides were shown to bind to specific sites on the collagen monomers (15). The binding region was localized to the CB7 peptide of α1(I), covering aa 552–822. By digesting type I

\textsuperscript{1} The abbreviations used are: aa, amino acid(s); PBS, phosphate-buffered saline; MMP-1, matrix metalloproteinase-1.
Deletion of Telopeptide Binding Site Impairs Fibrogenesis

MATERIALS AND METHODS

Case Report—The patient in whom this mutation occurs is now a 6.5-year-old boy with severe type III osteogenesis imperfecta. He was the product of a 39-week gestation to a 29-year-old mother and 30-year-old father. Short bowed femurs were noted prenatally by ultrasound. He was delivered by C-section for breech and weighed 7 lbs at birth. In the perinatal period, he was noted to have blue sclerae, significant osteoporosis, and three intrauterine fractures of lower long bones.

He has subsequently had about 35 long bone fractures, only 4 of which were comminuted. He has had 9 orthopedic procedures, and has intramedullary rods in bilateral humeri, left ulna, bilateral femurs, and tibiae. Radiographically, he has severe osteoporosis with metaphyseal flaring of long bones and popcorn formation at the growth plates. He has severe compressions of his vertebral bodies since 1 year of age. Bone density of L1–L4 by DEXA has yielded a Z score of –6 to –7 S.D. below age matched normal children for the last 3 years. He has recently developed a 20° thoracic scoliosis.

His gross motor development was delayed. Currently, he can stand 10 min and take a few steps with a walker. Mobility is by scooting and wheelchair. Fine motor and intellectual development are normal. Neuropsychological exam is within normal limits; CT of skull base shows only mild platybasia without impingement of the brainstem.

His fine motor impairment at 6 years old, his length is 50th percentile for an 18-month old boy and his head circumference is relatively macrocephalic at 50th percentile for a 9-year-old boy. His bone age is appropriate for chronological age.

Medically, he has been in general good health. He had one pneumonia before 1 year of age and currently has normal pulmonary function tests. He has crumpling brown dentinogenesis imperfecta of his teeth. He had chronic otitis media as a toddler, which was responsive to myringotomy and currently has a minimal low frequency conductive hearing loss. He received a bone marrow transplantation from an HLA matched sibling at St. Jude’s Hospital at age 2.5 years as part of an experimental protocol for mesenchymal precursor therapy for OI (16).

He received 2 subsequent transfusions of cells at around age 5 years.

Cell Culture—Skin fibroblast cultures were established from dermal punch biopsies of the proband and his parents. Fibroblasts were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 2 mM glutamine in an atmosphere of 5% CO2. Clonal cell lines of fibroblasts were obtained by plating diluted p8–10 cells in 96-well plates. Wells containing single cells were identified on the following day. Clonal lines were cultured in P10 media (Invitrogen, Grand Island, NY), supplemented with 20% fetal bovine serum, and harvested at confluence in 6-well plates or 25-cm2 flasks.

Osteoblast primary cultures were established from surgical bone chips of the proband and from a 4-year-old normal child. In brief, osteoblasts were released from bone chips by digesting for 2 h at 37 °C with 0.3 units/ml collagenase P in serum-free medium, and grown in a 1:1 mixture of low calcium Dulbecco’s modified Eagle’s medium and low calcium Ham’s F12K medium (Biofluids, Rockville, MD), supplemented with 25 μg/ml ascorbate and 10% fetal bovine serum (17). Only first- or second-passage osteoblasts were used in these studies.

Identification of Proband COL1A1 Intronic and cDNA Mutations—Leukocyte genomic DNA was isolated from the proband and his parents using the Puregene DNA Isolation Kit (Genta Systems, Minneapolis, MN). Conformation sensitive gel electrophoresis was performed on PCR amplified products from each exon and its surrounding intronic sequences as previously described (18) to identify sequence variations. Products with abnormal CSGE were directly sequenced in an ABI Prism 377 DNA Sequencer (Foster City, CA).

To confirm the genomic mutation, a 597-bp region of COL1A1 genomic DNA was amplified by PCR (19) using 500 ng of genomic DNA in the presence of 0.1 mM dNTP, 1.0 units of AmpliTaq, and 0.15 μM of each primer. The sense primer used for normal and cDNA using 5′-GGGAACCGCCTGCCTGGTGG-3′ and normal cDNA using 5′-GGGAACCGCCTGCCTGGTGG-3′ and 32P-labeled RT-PCR was performed with total RNA from fibroblasts grown at 37 °C and the primers listed above, using 94 °C for 1 min, 63 °C for 2 s, and 72 °C for 2 min for 20 cycles of PCR. A 32P-labeled standard curve was prepared by PCR of mixtures of subclones containing mutant and normal cDNA using 5 μl of 111 TBq/mmol [α-32P]dUTP. PCR products were electrophoresed on a 5% polyacrylamide gel. The percent of mutant PCR products/total PCR products was determined by densitometry and normalized to the standard curve.

Protein Analysis—To label procollagens, proband and control (ATCC 3349) cells were plated at 30 or 37 °C, grown to confluence, and incubated for 2 h in serum-free medium containing 50 μg/ml ascorbic acid, followed by incubation with 325 μCi of 3,86 TBq/mmol L-2,3,4,5,6- p-Glucosamine (Amersham, Buckinghamshire, UK, 300 mCi/mL) for 16 h. Procollagen was harvested from media and cell layer and precipitated with ammonium sulfate. Collagen samples were treated with pepsin digestion (55 μg/ml) of procollagen samples (27).

Thermal denaturation curves of osteoblast collagen were obtained by trypsin digestion (2 min, 20 °C) of collagen samples after equilibration at the indicated temperatures in a PerkinElmer Life Science 2400 thermogravimeter, as previously described (28). Relative amounts of collagen used to plot melting curves of α1(I) chains were obtained by densitometry of radiograms. Thermal denaturation curves of fibroblast collagen were obtained by a 2-min trypsin (100 μg/ml) and chymotrypsin (250 μg/ml) digestion of procollagen samples at the temperature at which they had been preincubated for 5 min (29). Trypsin inhibitor was added and the samples were immediately denatured by boiling. The proteins were separated on SDS-PAGE and visualized with Colloidal Brilliant Blue G (Sigma). The gels were analyzed by densitometry.

For digestion with MMP-1 (Calbiochem), the enzyme was first activated at 37 °C for 4 h in the presence of p-aminophenylmercuric acetate, according to the manufacturer’s directions. Then 2 μg of proband or normal collagen was digested with MMP-1 (0.675 μg of enzyme/μg of collagen) for 24 h at 30 °C. The reaction buffer was 50 mM Tris-HCl, pH 7.0, 300 mM NaCl, 5 mM CaCl2, 1 mM ZnCl2, 0.05% Brij 35, 0.02% NaN3. The reaction was terminated by adding EDTA to a final concentration of 10 mM.

Preparation of Proband Secreted Collagens—Proband fibroblasts were grown to confluency at 30 or 37 °C. Culture medium was removed, reduced to half volume, and desalted with a Sephadex G-25 column (26). Reduced fibroblast medium was supplemented with 2 mM glutamine and 50 μg/ml ascorbate was added to the cell cultures. Fresh ascorbate (50 μg/ml) was added each day until media was harvested on the fourth day. Medium was buffered with 100 mM Tris-HCl, pH 7.4, and cooled to 4 °C. Protease inhibitors were added to obtain the final concentration of 250 μM EDTA, 0.2% NaN3, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 10 mM N-ethylmaleimide (30). Procollagen was precipitated by gradual addition of 1 volume of saturated ammonium sulfate to 0.5 volume of the supernatant and stored at −80 °C. The precipitated collagen was collected by centrifugation. The pellets were dissolved in 0.1 M Tris-HCl, pH 7.4, 0.1 M EDTA, 0.5 M NaCl, and 5% PMSF, and dialyzed against Tris-HCl, pH 7.4, 0.1 M EDTA, 0.5 M NaCl, and 5% PMSF, and dialyzed against 0.1 M Tris-HCl, pH 7.4, 0.1 M EDTA, 0.5 M NaCl, and 5% PMSF.
tion of ammonium sulfate to a final concentration of 176 mg/ml, incubation at 4 °C overnight, then centrifugation at 12,000 × g for 2 h.

**Telopeptide Binding Studies**—Normal control or proband collagens were immobilized onto the surface of carboxyl chips (lasys; Affinity Sensors, UK). The biosensor surface (carboxylate groups) was activated by use of a 0.1 M mixture of 0.1 M N-hydroxy succinimide and 0.4 M N-ethyl-3-(3-dimethylaminopropyl carbodiimide) (Pierce). Collagen dissolved in PBS at a concentration of 100 μg/ml was allowed to bind to the activated surface until a response plateau was reached. The residual active groups were blocked by an injection of 200 μl of 1 M ethanolamine-HCl, pH 8.5. Nonspecific binding sites were blocked by an injection of 200 μl of 10% bovine serum albumin in PBS. Excess bovine serum albumin was removed by washing the cuvette with PBS containing 0.05% of Tween 20 (PBST), followed by three successive washes with 0.1 M HCl. A control cuvette with immobilized bovine serum albumin was prepared using the same procedure.

A 200-μl sample containing C1-telopeptide at concentrations ranging from 2.5e-05 to 1.75e-03 μ, dissolved in PBST was added to the cuvette, and the peptide was allowed to interact with the immobilized collagen for 10 min (association phase). Subsequently, the sample was removed and PBST without peptide was added to the cuvette for 5 min (dissociation phase). After each assay, the surface of a cuvette was regenerated by washing with 0.1 M HCl, followed by equilibration with PBST. Data from the biosensor were analyzed by the global fitting method described by Myszka and Morton (31). For each assay, the koff and koff were obtained, and the Kd values were calculated from the koff/kon ratio. Mean values and the standard error of the mean were derived from three independent binding studies. The Student’s t test was employed to evaluate statistical significance of observed differences (unpaired t test was performed using GraphPad prism version 3.0 for Windows, GraphPad Software, San Diego, CA).

**Fibrillogenesis Studies**—To generate pC-collagen, procollagen purified from culture media by ion exchange chromatography (32) was cleaved with procollagen N-proteinase purified from chick tendons, as described by Hajima et al. (33). The reaction mixture contained 500 μg of procollagen and 200 units of N-proteinase in 25 mM Tris-HCl, pH 7.5, 7 mM CaCl2, 0.1 M NaCl, 0.015% Brij, and 0.02% NaN3. One unit of procollagen and 200 units of N-proteinase in 25 mM Tris-HCl, pH 7.5, 7 mM CaCl2, 0.1 M NaCl, 0.015% Brij, and 0.02% NaN3. One unit of enzyme is defined as the amount needed to cleave 1 μg of substrate in 1 h at 35 °C. Enzymatic cleavage was performed at 35 °C for 4 h. The pC-collagen product was purified by size exclusion chromatography (G 3000 SW, Tosohhaa, Japan). Protein fractions were analyzed by electrophoresis on a 7.5% SDS-polyacrylamide gel. Peak fractions were combined and concentrated by ultrafiltration.

pC-collagen and chick embryo procollagen C-proteinase were dialyzed separately against fibril formation buffer (34) and stored in paraffin-sealed tubes at –20 °C under an atmosphere of 10% CO2 and 90% air. To initiate fibril formation, pC-collagen and procollagen C-proteinase were mixed at 4 °C in a 250-μl polypropylene tube in a total volume of 40 μl. In a first series of experiments, both normal and mutant pC-collagen were used at 50 μg/ml. Because mutant collagen did not form fibrils at this concentration, it was increased to 150 μg/ml. The procollagen-C-proteinase-collagen ratio was 1 unit of enzyme/5 μg of pC-collagen. After preparing the mixtures, the tubes were briefly flushed with water-saturated 10% CO2 and 90% air. The tubes were incubated from 0.5 to 24 h at 37 °C. The fibrils which formed were separated by centrifugation at 15,000 × g for 20 min. The supernatant and pellet fractions were analyzed by electrophoresis on a 7.5% SDS-polyacrylamide gel. The gels were stained with Colloidal Brilliant Blue G (Sigma), and the relative amounts of α chains were assayed by densitometry.

**Microscopy of Fibrils**—For dark-field light microscopy, mixtures of pC-collagen and procollagen C-proteinase (40 μl final volume) were placed in sealed glass chambers (14). The samples were incubated at 37 °C for 24 h. Fibrils were photographed using a light microscope (Zeiss, Model 9801) equipped with a Fuji digital camera. For electron microscopy, the pelleted fractions containing collagen fibrils were suspended in 10 μl of the fibril formation buffer. Subsequently, the sample was transferred to a carbon-coated electron microscopic grid and stained negatively with 1% phosphotungstate. The grids were examined with a transmission electron microscope (Hitachi Model 7000) at a magnification of ×3000–5000.

**Analysis of Collagen Extracted from Dermal Skin Biopsy**—A 4-mm punch biopsy from the proband’s skin was frozen in liquid nitrogen and then dried. The sample was suspended in 0.5 M acetic acid. Collagen was extracted by digestion of the non-collagenous proteins with 100 μg/ml pepsin for 24 h at 4 °C. The supernatant was collected from the centrifuged sample and neutralized with NaOH to inactivate pepsin. The extracted collagen was then cleaved with MMP-1 and analyzed by electrophoresis as described earlier. Collagen extracted from a foreskin sample was used as control.

**RESULTS**

Identification of Proband’s COL1A1 Mutation and Characterization of Mutant Transcript—Using leukocyte DNA from the proband, the genes coding for both the α1(1) and α2(1) chains of type I collagen were screened on an exon by exon basis for the mutation causing his severe type III osteogenesis imperfecta. Individual exons and flanking intron sequences were amplified by PCR. PCR products were examined by CSGE and all products with electrophoretic variations were sequenced. CSGE indicated a sequence variation in the fragment containing the proband’s COL1A1 exon 41 and flanking intronic sequences. Sequencing of this fragment (Fig. 1) revealed a heterozygous A → C substitution at the fourth nucleotide of the exon 41 splice donor site (4+ position). This IVS 41 A+4 → C transversion, indicated by the arrow, was not seen in the parental genomic DNA samples or in over 100 other alleles analyzed.

Since the +4 position of the splice donor site is not invariant, it was necessary to determine whether and to what extent skipping of exon 41 would occur. RT-PCR of total RNA isolated from proband and control fibroblast cultures was used to amplify the IVS 41–43 region of cDNA. In addition to the expected 384-bp product, both smaller and larger PCR products were detected (Fig. 2). The 276-bp smaller product has the size expected from skipping of exon 41 and represents only a minor portion of the total RT-PCR products. The larger product was a heteroduplex of normal and shorter products, as determined by sequencing of subclones. Alternative products were about twice

![Fig. 1. Proband genomic DNA mutation in COL1A1.](http://www.jbc.org/ Downloaded from http://www.jbc.org/ by guest on July 24, 2018)
as abundant in total RNA from fibroblasts grown at 30°C than at 37°C. Treatment of cultures with cycloheximide did not affect the abundance of the alternative splicing products (Fig. 2).

RT-PCR products were subcloned and sequenced. Two sets of PCR products were demonstrated: one with normal allele sequences and one with skipping of exon 41 (Fig. 3).

Synthesis and Secretion of Collagen with Structurally Abnormal \( \alpha_1(1) \) Chain—We analyzed the steady state collagen synthesized by proband dermal fibroblasts grown at 30 and 37°C (Fig. 4), to determine whether the \( \alpha_1(1) \) transcripts with skipping of exon 41 were translated into stable \( \alpha \) chains and whether the chains could be incorporated into type I collagen heterotrimer.

In the cell layer collagen fraction of fibroblasts grown at 30°C, we distinguished a more rapidly migrating population of \( \alpha_1(1) \) chains. In fibroblasts and osteoblasts grown at 37°C, we detected a more slowly migrating component of both \( \alpha_1(1) \) and \( \alpha_2(1) \), indicating overmodification of a portion of the heterotrimers resulting from interference with the normal rate of helix formation. These cell layer results demonstrate that the mutant \( \alpha_1(1) \) transcript is translated into stable \( \alpha \) chains, which are incorporated into triple helices.

Mutant chains could not be clearly distinguished electrophoretically in media samples from cultured cells (Fig. 4). As an alternative method of demonstrating the presence of mutant chain in secreted proband collagen, we determined the \( T_m \) of collagen helices secreted by fibroblasts and osteoblasts. The osteoblast collagen \( T_m \) was determined to be 4°C lower than the control samples but the fibroblast collagen had normal stability (data not shown).

Quantitation of Mutant mRNA and Protein—Since the proband does not synthesize equimolar amounts of normal \( \alpha_1(1) \) chain and \( \alpha_1(1) \Delta E41 \) in dermal fibroblast cultures, we distinguished between two possibilities for mutation distribution and efficiency: (a) inefficient skipping of exon 41 in all cells, and (b) proband dermal mosaicism for an efficient exon skipping mutation that was present in only a small proportion of cells.

Eight clonal fibroblast lines were isolated from proband fibroblasts. RT-PCR of total RNA extracted from each of the clonal lines showed the same low proportion of product with exon 41 skipping as in the mixed cell population (Fig. 5). There were, however, no clonal lines that synthesized only normal product and none that had equimolar normal and exon 41 skipping products, as would be expected if mosaicism were present. This data confirms that the \( 41 +4 \) mutation is present in all cells but causes alternative splicing from only a small proportion of the mRNA transcribed from the mutant allele.

The proportion of mutant product was quantitated for both mRNA and secreted protein (Fig. 6). \( \text{[3H]} \)-Labeled RT-PCR was used to compare the proportion of mutant product with a standard curve prepared from mixtures of normal and mutant cDNA subclones (Fig. 6, Panel A). Mutant product was about 15% of the \( \alpha_1(1) \) mRNA in fibroblasts grown at 30°C. These were the cells used for preparation of pC-collagen for fibrillogenesis experiments. In fibroblasts grown at 37°C, mutant mRNA was less abundant than in 30°C.
abundant and comprised about 8% of the total α1(I) mRNA.

The protein quantitation is in good agreement with mutant mRNA levels. Mutant α1(I) chains in proband secreted procollagen were quantitated by digestion with mammalian collagenase (MMP-1). The MMP-1 cleavage site in α1(I) collagen is located at aa 775–776 in exon 41 (7) and is missing in mutant monomers, which are resistant to digestion. Approximately 10% of the proband’s total α1(I) chains was resistant to enzyme digestion (Fig. 6, Panel B). Because these mRNA and protein measurements permit only moderate precision, we estimate the mutant product to comprise 10–15% of total α1(I) chains synthesized by proband fibroblasts.

**Binding of Synthetic Telopeptide to Proband Collagen**—The telopeptide binding site in the triple helix of type I collagen had been previously mapped to α1(I) as 776–796 by a combination of binding studies and molecular modeling (15). The effect of the natural exon 41 (aa 766–801) skipping mutation on telopeptide binding was investigated by comparing binding of synthetic C1-telopeptide (aa 1015–1040) to proband and control collagen using a biosensor. The presence of only 10% mutant collagen in the proband sample was sufficient to cause significant alterations in telopeptide binding (Table I). Telopeptide association with proband collagen, 4.29 × 10^4 M^{-1} s^{-1}, was approximately half as fast as with control, 7.4 × 10^4 M^{-1} s^{-1}. Dissociation is altered more than association, and is five times faster from proband than from control collagen, 5.85 × 10^{-1}/s and 1.17 × 10^{-1}/s, respectively. The slower association and faster dissociation combine to produce an overall affinity of telopeptide for proband collagen that is about an order of magnitude lower than for control. As indicated in Table I, the observed changes were statistically significant.

![Figure 5. RT-PCR of total RNA from fibroblast clonal lines. Lane 1, 100-bp ladder; lanes 2–9, total RNA from proband fibroblast clonal lines was amplified by RT-PCR; lanes 10 and 11, RT-PCR products from total RNA isolated from proband fibroblasts incubated with or without cycloheximide. Normal (384 bp) and mutant (276 bp) RT-PCR products were quantitated by densitometry. The percent mutant product in the individual clonal lines was equivalent to the percent mutant product in the total cell population.](http://www.jbc.org/)

![Figure 6. Quantitation of mutant mRNA and protein from proband fibroblasts. A, mutant mRNA quantitation by 32P-labeled RT-PCR.](http://www.jbc.org/)  

### Table I  
**Biosensor analysis of the binding of synthetic C1 telopeptide to type I collagen**

Collagen was covalently immobilized on the surface of a biosensor. Synthetic C1 telopeptide was added to the biosensor’s cuvette at concentrations ranging from 2.5e-05 to 1.75e-03 M and association and then dissociation phases were analyzed. Data were analyzed by global fitting. Altered on- and off-rates were observed in assays with proband collagen during binding of C-telopeptide. Peptide association with proband collagen was slower than with control and dissociation from proband collagen is faster than from control. Thus, the overall affinity of the synthetic telopeptide for proband collagen is lower.

| Target protein          | $k_{on}$ (S.E.M.$^a$) M^{-1} s^{-1} | $k_{off}$ (S.E.M.$^b$) s^{-1} | $K_{d}$ (S.E.M.$^c$) M^{-1} |
|-------------------------|-----------------------------------|-------------------------------|----------------------------|
| Normal collagen I       | 7.40 × 10^4 (6.9 × 10^4)          | 1.17 × 10^{-1} (3.1 × 10^{-3})| 1.58 × 10^{-8} (2.7 × 10^{-8}) |
| Proband collagen I      | 4.29 × 10^4 (3.3 × 10^4)          | 5.85 × 10^{-3} (3.6 × 10^{-3})| 1.36 × 10^{-7} (6.5 × 10^{-7}) |

$^a$ $k_{on}$ association rate constant.  
$^b$ $k_{off}$ dissociation rate constant.  
$^c$ $K_{d}$ equilibrium dissociation constant. $K_{d}$ values are means of a ratio of $k_{off}/k_{on}$ derived from three independent experiments.
Effect of Absence of Telopeptide Binding Site on in Vitro Fibrillogenesis—Fibril formation by the collagen mixture containing 10% chains with exon 41 skipping was studied using the self-assembly system developed by Kadler et al. (35, 36). The intermediate pC-collagen was first purified and then fibril self-assembly was initiated by incubation with procollagen C-proteinase. Under these conditions, the control collagen yields a substantial proportion of fibrils by 4 h and is essentially fully incorporated into fibrils by 8 h (Fig. 7, upper panel). In contrast, only a minimal amount of fibrils is seen in the proband sample at 10 h and the majority of the sample is still in monomer form at 24 h (Fig. 7, lower panel). Thus, the monomers that lack exon 41 are causing a dramatic decrease in the rate of in vitro fibril assembly. The proband sample has assembled in 24 h the same proportion of fibrils that the control assembles in about 2 h. Furthermore, the critical concentration for fibril formation was higher in the presence of monomers with exon 41 deleted; the critical concentration was about 5 µg/ml for normal collagen and about 140 µg/ml for proband collagen.

To determine the effect of an α1(I) exon 41 deletion on the morphology of fibrils, the samples were examined by dark-field microscopy (Fig. 8). Proband fibrils were substantially longer than control fibrils. Fibril diameter and D-periodicity were essentially indistinguishable from controls.

Deposition of Mutant Collagen into Matrix in Vivo—Since the proband’s mutant collagen lacked an MMP-1 cleavage site, it was possible that the mutant forms could accumulate proportionately in connective tissue because they were resistant to digestion. Collagen was extracted from a dermal punch biopsy of the proband and digested with MMP-1. No resistant α1(I) chain was detected above the background level seen in control (data not shown).

DISCUSSION
We have delineated here the structural and functional properties of a special exon-skipping mutation in the α1(I) chain of type I collagen. The mutation occurs in a 6.5-year-old boy with severe type III osteogenesis imperfecta. On one COL1A1 allele, the child has an IVS 41 A→T single nucleotide change. The mutation causes skipping of exon 41 from a small portion of the mRNA transcribed from that allele. Exon skipping products were demonstrated by RT-PCR and shown to occur with equal proportion in a mixed dermal fibroblast population and in 8 clonal fibroblast lines. As estimated by RT-PCR, exon 41 is skipped in about 15% of total α1(I) mRNA from cells grown at 30 °C and 8% of total α1(I) mRNA from cells grown at 37 °C. This was consistent with a protein determination using MMP-1 to quantitate chains from which the MMP-1 cleavage site had been deleted; about 10% of α1(I) chains secreted at 30 °C were not digested with MMP-1. This inefficient skipping of exon 41 may result from the mutation itself, in that the +4 position of the donor site is not invariant, or it may also be influenced by the rate of intron removal of adjacent introns (37). If intron 40 is rapidly spliced out and there is no cryptic donor in exon 41, a higher proportion of product will retain exon 41 than if the
The deletion of 36 amino acids in the \( \beta \) chains. This exon contains both the cleavage site for MMP-1 and the putative telopeptide binding site for fibrillogenesis. The disproportionate effect is consistent with a role for this portion of monomers being incorporated into fibrils after 24 h.

The deleterious effect on fibrillogenesis may be due to reduced trypsin-chymotrypsin resistance of the mutant monomer. This is likely that deletion of amino acids 766–801 of the \( \alpha(1) \) chain significantly alters the nucleation process and the rate of lateral growth of collagen fibrils rather than the rate of longitudinal growth.

These experiments demonstrate the critical importance of the sequences encoded by exon 41 for in vitro fibrillogenesis. Indeed, it is striking that a disproportionate effect of mutant chain on telopeptide binding, fibrillogenesis kinetics, and fibril structure was seen, relative to the amount of mutant chain in the collagen mixture. There was a substantial increase in the time required for fibril formation, resulting in only a small portion of monomers being incorporated into fibrils after 24 h. The disproportionate effect is consistent with a role for this binding region in the generation of higher-order structures. The presence of a small percent of mutant monomers may “poison” the assembly process by sterically hindering the binding of additional monomers to the growing fibril.

The exon 41 skipping mutation in this OI patient also provides insight into turnover of collagen by MMP-1 in tissues. Since the mutant chains lack the MMP-1 site, they might persist for a longer time in the tissues, accumulating to a higher level than in the secreted sample from cultured cells. The proband does have severe OI but neither his phenotype nor his radiographs are distinctive compared with other patients with type III OI. Extraction of collagen directly from a dermal punch biopsy did not yield a higher level of MMP-1 resistant \( \alpha(1) \) chain than in the normal control. As previously seen in the murine model with an altered MMP-1 site, turnover of collagen in human ECM may be a redundant system with multiple enzymes (40, 41). The exon 41 sequences are appar-

exon 40 donor site was available for a prolonged time as an alternative donor site.

The ultimate consequence is that the collagen synthesized by these cells has a deletion of exon 41 from 10–15% of its \( \alpha(1) \) chains. This exon contains both the cleavage site for MMP-1 and the putative telopeptide binding site for fibrillogenesis. The deletion of 36 amino acids in the \( \alpha \) chain most likely has an effect on the folding of the collagen triple helix. Because the mutant protein is secreted from the proband’s fibroblasts and is trypsin-chymotrypsin resistant at 37 °C, it must be folded into a collagen triple helix. Previous studies on type II collagen with a deletion of a fragment of 18 residues, demonstrated that to accommodate a shortened \( \alpha \) chain and to preserve the structure and proper alignment of the propeptides, the normal length \( \alpha \) chains must form a loop (38). In the case presented here, there is a deletion of 36 amino acids in the \( \alpha(1) \) chain. It is possible that the mutant chains do not co-assemble with normal \( \alpha 1 \) and \( \alpha 2 \) chains because this large a deletion would require the normal \( \alpha \) chains in a triple helix to assume an energetically unfavorable structure. Instead, the mutant chains could theoretically fold into a shorter \( \alpha 1 \) homotrimer. This would be consistent with our observation that trypsin-chymotrypsin digestion of mutant collagen did not produce the intermediate digestion products that were previously detected in digestion of type II collagen containing a trypsin-chymotrypsin sensitive loop (38). The present data do not allow us to distinguish experimentally between incorporation of mutant chain into heterotrimer or homotrimer.

The telopeptide binding site on the helical portion of the collagen monomer had previously been mapped using binding of biotinylated synthetic telopeptides (15). Mutating the synthetic peptides altered their binding to collagen triple helices and thus abolished their inhibitory effect on fibrillogenesis. However, the complementary investigations involving the triple helical telopeptide binding region have not been done. The natural collagen mutation delineated in this child with type III OI provides an excellent model for these studies. Even though the mutant chain comprised only 10–15% of total \( \alpha 1 \) chains and could be theoretically distributed among 20–25% of type \( \alpha 1 \) trimers, it had a significant effect on binding of synthetic C1-telopeptide. The slower association constant and faster dissociation constant are additive to an affinity constant that is an order of magnitude less in the proband than in control. In vitro fibrillogenesis of the collagen from the proband resulted in a dramatic delay of fibril formation, with the majority of the sample still in monomers at 24 h. The fibrils that were formed by 24 h were substantially longer than in control. Furthermore, the critical concentration of collagen was 140 \( \mu \)g/ml for the proband collagen compared with 5 \( \mu \)g/ml for control.

In a previously described model for helical growth of microfibrils from paraboloidal tips (39), two specific binding steps were required. One binding step, governed by rate constant \( k_1 \), is proposed to be critical for assembly of monomers in a 1D-stagger to form a Smith-type microfibrillar core and to regulate longitudinal growth of the fibril. The model required a second binding step, governed by a smaller rate constant \( k_2 \), to initiate growth of each new layer of helical sheets of monomers on the microfibrillar core. This rate constant thus regulates lateral growth of the core. Because the length-to-diameter ratio is higher in the fibrils assembled in the presence of the proband’s mutant collagen, it is likely that deletion of amino acids 766–801 of the \( \alpha(1) \) chain significantly alters the nucleation process and the rate of lateral growth of collagen fibrils rather than the rate of longitudinal growth.

FIG. 8. Dark-field images of fibrils formed in vitro from proband and control collagen. Fibrils formed after 24 h of in vitro fibrillogenesis were imaged. Fibrils formed from proband collagen (B) are significantly longer than control fibrils (A). Magnification \( \times \sim \) 300. Insets in each panel are EM images showing expected D-period banding (bars = 100 nm).
Deletion of Telopeptide Binding Site Impairs Fibrillogenesis

...ently less important for collagen turnover in vivo than for fibrillogenesis.

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Procollagen with Skipping of α1(I) Exon 41 Has Lower Binding Affinity for α1(I) C-telopeptide, Impaired in Vitro Fibrillogenesis, and Altered Fibril Morphology
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