

# New Molecular Mechanism for Ullrich Congenital Muscular Dystrophy: A Heterozygous In-Frame Deletion in the *COL6A1* Gene Causes a Severe Phenotype

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Recessive mutations in two of the three collagen VI genes, *COL6A2* and *COL6A3*, have recently been shown to cause Ullrich congenital muscular dystrophy (UCMD), a frequently severe disorder characterized by congenital muscle weakness with joint contractures and coexisting distal joint hyperlaxity. Dominant mutations in all three collagen VI genes had previously been associated with the considerably milder Bethlem myopathy. Here we report that a de novo heterozygous deletion of the *COL6A1* gene can also result in a severe phenotype of classical UCMD precluding ambulation. The internal gene deletion occurs near a minisatellite DNA sequence in intron 8 that removes 1.1 kb of genomic DNA encompassing exons 9 and 10. The resulting mutant chain contains a 33–amino acid deletion near the amino-terminus of the triple-helical domain but preserves a unique cysteine in the triple-helical domain important for dimer formation prior to secretion. Thus, dimer formation and secretion of abnormal tetramers can occur and exert a strong dominant negative effect on microfibrillar assembly, leading to a loss of normal localization of collagen VI in the basement membrane surrounding muscle fibers. Consistent with this mechanism was our analysis of a patient with a much milder phenotype, in whom we identified a previously described Bethlem myopathy heterozygous in-frame deletion of 18 amino acids somewhat downstream in the triple-helical domain, a result of exon 14 skipping in the *COL6A1* gene. This deletion removes the crucial cysteine, so that dimer formation cannot occur and the abnormal molecule is not secreted, preventing the strong dominant negative effect. Our studies provide a biochemical insight into genotype-phenotype correlations in this group of disorders and establish that UCMD can be caused by dominantly acting mutations.

## Introduction

Congenital muscular dystrophy (CMD) comprises a heterogeneous group of disorders characterized by muscle weakness at birth or in early infancy (Voit 1998; Tubridy et al. 2001; Muntoni et al. 2002a). Mutations in the extracellular matrix protein laminin  $\alpha 2$  chain, a subunit of laminin 2 in the muscle basement membrane, account for a substantial proportion of CMD cases without mental retardation. An emerging group of CMD affects O-mannose-linked glycosylation of  $\alpha$ -dystroglycan, an important laminin-2 receptor in muscle, causing a variable secondary deficiency in laminin  $\alpha 2$  (Muntoni et al. 2002b). Recently, another extracellular matrix protein, collagen

type VI, has been shown to cause a distinct type of laminin  $\alpha 2$ -positive CMD (Bertini and Pepe 2002), initially described as “atonic-sclerotic muscular dystrophy” by Otto Ullrich (Ullrich 1930) and now known as “CMD type Ullrich” (MIM 254090). The signature clinical feature of the Ullrich congenital muscular dystrophy (UCMD) phenotype is congenital muscle weakness with proximal joint contractures and coexisting marked distal joint hyperlaxity (Nonaka et al. 1981; De Paillette et al. 1989). Cases of variable severity have been described; however, in the classical most severe presentation, independent walking is not achieved or is achieved briefly and then lost, owing to progression of weakness and contractures (Bertini and Pepe 2002).

Collagen type VI is a ubiquitous connective tissue component, present primarily in the stroma and also close to the basement membrane of most tissues (Timpl and Engel 1987; Timpl and Chu 1994). It consists of three separate chains, the  $\alpha 1$ (VI),  $\alpha 2$ (VI) and  $\alpha 3$ (VI) collagen chains, encoded by the *COL6A1*, *COL6A2*, and *COL6A3* genes, respectively. The  $\alpha 1$ (VI) and  $\alpha 2$ (VI) collagen chains are similar in size (140 kDa),

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and their genes are linked in a head-to-tail orientation on chromosome 21q22.3 (Weil et al. 1988; Heiskanen et al. 1995). The  $\alpha 3(\text{VI})$  collagen chain is much larger (260–330 kDa) and is encoded by the gene located on chromosome 2q37 (Weil et al. 1988). The three chains share the same core structure: a relatively short triple-helical domain of 335–336 amino acids with repeating Gly-Xaa-Yaa amino acid sequences flanked by globular domains made up of motifs of 200 amino acids each that are homologous to von Willebrand factor type A (vWF A) domains (Chu et al. 1988, 1989, 1990). The three chains fold into triple-helical monomers, which are then assembled in an antiparallel manner into dimers with the N-terminal globular domains protruding (Furthmayr et al. 1983; Chu et al. 1988). The dimers associate laterally into tetramers, which are subsequently secreted into the extracellular space and associate, end-to-end, into double-beaded collagen VI microfibrils.

Collagen VI mutations in UCMD reported to date are exclusively recessive mutations in either the *COL6A2* or *COL6A3* genes (Camacho Vanegas et al. 2001; Higuchi et al. 2001; Demir et al. 2002; Ishikawa et al. 2002) (table 1), whereas dominant mutations in all three collagen genes have been associated with Bethlem myopathy (MIM 158810) (Jöbsis et al. 1996; Pan et al. 1998), a milder childhood-onset disorder characterized by muscle weakness and later manifesting multiple joint contractures (Bethlem and Wijngaarden 1976). In three

families, a form of autosomal dominant limb girdle muscular dystrophy has also been attributed to heterozygous mutations in the *COL6A1* and *COL6A2* genes (Scacheri et al. 2002). All of the *COL6A2* mutations described in UCMD so far lead to translational frameshifts and subsequent premature stop codons (Camacho Vanegas et al. 2001; Higuchi et al. 2001; Ishikawa et al. 2002). In two of these patients with UCMD, the *COL6A2* mRNA has been shown to be nearly absent because of nonsense-mediated mRNA decay (Zhang et al. 2002), resulting in a severe deficiency of collagen VI protein in the muscle. Of the three *COL6A3* homozygous mutations in UCMD reported more recently, two are nonsense mutations—one in the N-terminal globular domain and one in the triple-helical domain—and the third one leads to skipping of an exon encoding the triple-helical domain (Demir et al. 2002). The patients with the *COL6A3* gene mutations exhibited complete or partial deficiency of collagen VI protein in the muscle, but the biochemical mechanisms underlying the gene mutations have not yet been investigated.

Here we characterized two patients affected with CMD. One patient displays a classical, severe phenotype of UCMD, whereas the other patient exhibits a relatively milder CMD phenotype with distal joint hyperlaxity in the absence of contractures. We show that both patients carry de novo heterozygous in-frame deletions of different sizes and locations in the triple-helical domain of the *COL6A1* mRNA. We provide biochemical

**Table 1****Summary of *COL6* Mutations in UCMD**

Patient and Location of Mutation	Mutation	Consequence	Status	Reference
1: <i>COL6A2</i> exon 13	Ins C	Frameshift	Homozygous	Camacho Vanegas et al. 2001
2: <i>COL6A2</i> intron 17	–2a→g	Del 28 bp	Compound heterozygous	Camacho Vanegas et al. 2001
<i>COL6A2</i> intron 23	–1g→a	Exon 24 (46 bp) skipping	Compound heterozygous	Camacho Vanegas et al. 2001
3 <sup>a</sup> : <i>COL6A2</i> intron 17	–2a→g	Del 28 bp	Compound heterozygous	Camacho Vanegas et al. 2001
4: <i>COL6A2</i> exon 18	Del 26 bp	Frameshift	Homozygous	Higuchi et al. 2001
5: <i>COL6A2</i> intron 14	–1g→c	Ins 53 bp or 247 bp from intron sequence	Compound heterozygous	Ishikawa et al. 2002
<i>COL6A2</i> intron 23	–3c→g	Exon 24 (46 bp) skipping	Compound heterozygous	Ishikawa et al. 2002
6: <i>COL6A3</i> intron 29	+5a→g	In-frame del of 17 amino acids in the triple-helical domain	Homozygous	Demir et al. 2002
7: <i>COL6A3</i> exon 5	Nonsense	Alternatively spliced variant	Homozygous	Demir et al. 2002
8: <i>COL6A3</i> exon 31	Nonsense	Premature termination	Homozygous	Demir et al. 2002

NOTE.—Ins = insertion; del = deletion.

<sup>a</sup> Only one mutation has been found in this presumably compound heterozygous patient.

and immunohistochemical evidence for a genotype/phenotype correlation and show that the severe UCMD phenotype can arise from a heterozygous mutation in collagen VI through a dominant negative mechanism.

## Subjects and Methods

### *Patients and Their Families*

Two patients (UC-1 and UC-4) affected with CMD associated with distal joint hyperlaxity were studied, as were their unaffected family members. Both families were from Brazil. Dermal fibroblast cultures were established from skin biopsies of the patients, and genomic DNA was extracted from peripheral blood samples from their families. Control fibroblasts and DNA samples were obtained from unaffected volunteers. The genetic and clinical studies were performed in accordance with institutional review board-approved protocols and informed consent.

### *Cell Culture*

Dermal fibroblasts were grown in Dulbecco's modified Eagle medium with 10% fetal bovine serum (Life Technologies) in 5% CO<sub>2</sub> at 37°C. For immunoblotting, immunoprecipitation, and immunofluorescence staining, cells were cultured in the presence of 50 µg/ml L-ascorbic acid phosphate (Wako).

### *Immunostaining of Fibroblasts*

Fibroblasts were grown in the presence of L-ascorbic acid phosphate until 4 d postconfluency. Cells were fixed in 3.7% formaldehyde and were incubated at 25°C with a 1:1,000 dilution of a polyclonal antibody specific for the  $\alpha 1$ (VI) collagen chain (antibody 1026) (Tillet et al. 1994) for 1 h. The antibody labeling reaction was detected with Cy3-conjugated goat anti-rabbit IgG (1:1,000 dilution; Jackson ImmunoResearch Laboratories), and nuclei were counterstained with DAPI (4',6'-diamidino-2-phenylindole hydrochloride). Images were obtained using a Zeiss Axioskop epifluorescence microscope with a Toshiba 3CCD camera.

### *RNA Extraction and Northern Blot Analysis*

Total RNA was isolated from confluent fibroblasts with the RNeasy Mini Kit (Qiagen). The total RNA (5 µg) was separated on a 1% agarose gel containing formaldehyde, was transferred to Duralon-UV membrane (Stratagene), and was hybridized with cDNA probes labeled with [<sup>32</sup>P]dCTP by the Random Prime Labeling system (Amersham Pharmacia) in Ultrasensitive Hybridization Buffer (Ambion), following protocols suggested by the suppliers. The probes were a mixture of cDNA clones encoding the  $\alpha 1$ (VI),  $\alpha 2$ (VI), and  $\alpha 3$ (VI) collagen

chains (clones F157, F225, and FO19) (Chu et al. 1989, 1990).

### *Mutational Analysis by RT-PCR and DNA Sequencing*

Total RNA isolated from fibroblasts was reverse transcribed using Superscript II reverse transcriptase (Life Technologies) and oligo(dT) primer for 1 h at 42°C. The resulting cDNA was used as a template for PCR amplification of the entire coding regions of the COL6A1, COL6A2, and COL6A3 mRNA transcripts (GenBank accession numbers NM\_0018489, NM\_001849, and X52022, respectively) with AmpliTaq Gold polymerase (Applied Biosystems). The COL6A1, COL6A2, and COL6A3 transcripts were amplified with 5, 4, and 14 primer pairs, respectively, yielding overlapping cDNA fragments 400–1,200 bp long. The PCR conditions were 10 min at 94°C; 35 cycles of 94°C for 30 s, 58–60°C for 30 s, and 72°C for 1 min; followed by a final extension of 72°C for 7 min. PCR products were subjected to DNA sequencing with an ABI377 DNA Sequencer (Applied Biosystems). The primer pairs revealing the COL6A1 deletions were as follows: forward, 5'-AACG-TTGAGCAAGTGTGCTG-3'; and reverse, 5'-TTCACC-AGGGTCTCCTCTTG-3'.

### *Characterization of the COL6A1 Gene Mutations*

Genomic DNA isolated from patient UC-1 and his family was PCR amplified with a forward primer in intron 6 (5'-GACTCGTCTCCATGCTTTCC-3') and a reverse primer in intron 11 (5'-CATGGGGACTGACAGTGATG-3'), through use of the Expand Long Template PCR system (Roche). The PCR conditions were 94°C for 2 min; 30 cycles at 94°C for 10 s, 58°C for 30 s, and 68°C for 8 min; followed by a final extension at 68°C for 7 min. The PCR products were analyzed on 1% agarose gels. PCR products yielded from patient UC-1 were cloned into the pCR-TOPO vector (TOPO TA Cloning Kit; Invitrogen) and were subjected to DNA sequencing.

Genomic DNA isolated from patient UC-4 and his family was PCR amplified with a forward primer located in the boundary of exon 12/intron 12 (5'-AGGGCTACAA-GGTGAGCGTG-3') and a reverse primer in intron 15 (5'-CAGGACTTGCTTCCAGGAGA-3'), through use of AmpliTaq Gold polymerase (Applied Biosystems) and the cycling conditions described above for RT-PCR. The PCR products from UC-4 and his family were subjected to DNA sequencing.

### *Protein Analysis by Immunoblotting and Immunoprecipitation*

Immunoblot analysis of culture medium and cell layers from confluent fibroblasts were performed using 1026 antibody specific for the  $\alpha 1$ (VI) collagen chain (Til-

let et al. 1994), as described elsewhere (Pepe et al. 1999; Camacho Vanegas et al. 2002). For immunoprecipitation of collagen VI protein, confluent fibroblasts were metabolically labeled with [<sup>35</sup>S]cysteine overnight and were precipitated with 1014 antibody specific for the  $\alpha 3(\text{VI})$  collagen chain (Specks et al. 1992), as described elsewhere (Camacho Vanegas et al. 2001). Samples were electrophoresed on 3%–8% SDS-polyacrylamide gels under reduced conditions (25 mM dithiothreitol [DTT]) or on composite 0.5% agarose, 2.4% polyacrylamide gels without reduction with DTT (Lamandé et al. 1998). Reduced (230 and 400 kDa) and unreduced (900 kDa) forms of laminin were used as the molecular mass standard on the composite gel.

#### *Immunostaining of Muscle Biopsies*

Biopsies were cut in 10- $\mu\text{m}$  sections through use of a Microm HM505E cryostat and were mounted onto microscope slides before being stored at  $-80^{\circ}\text{C}$  until further use. After allowing the slides to acclimate to room temperature, they were fixed with methanol at room temperature for 20 s and were briefly washed with ice-cold  $1 \times \text{PBS}$  (0.01 M  $\text{KH}_2\text{PO}_4$ , 0.1 M  $\text{Na}_2\text{HPO}_4$ , 1.37 M NaCl, 0.027 M KCl, pH 7.4). The sections were preincubated in vehicle (10% fetal bovine serum,  $1 \times \text{PBS}$ , 0.1% Triton X-100) at room temperature for 1 h. Primary antibodies at the appropriate dilution in vehicle were applied to the sections and allowed to incubate overnight at  $4^{\circ}\text{C}$ . A mouse monoclonal antibody recognizing collagen type VI (MAB3303; 1:2,500) was obtained from Chemicon International. The rabbit polyclonal antibody against perlecan domain III (1030; 1:2,000) was graciously provided by R. Timpl of the Max Planck Institute for Biochemistry, Germany. Slides were washed in  $1 \times \text{PBS}$  (3  $\times$  20 min) prior to applying the secondary antibodies in vehicle at room temperature for 1 h in the dark. Alexa Fluor 488–conjugated goat anti-mouse IgG and Alexa Fluor 568–conjugated goat anti-rabbit IgG (Molecular Probes) were used as secondary antibodies at 1:500 dilution. The slides were washed in  $1 \times \text{PBS}$  and were post-fixed at room temperature for 8 min in the dark, using cold methanol ( $-25^{\circ}\text{C}$ ). The slides were briefly washed with  $1 \times \text{PBS}$  before being coverslipped with either Vectashield or Biomedica Gel/Mount media mount.

Images were obtained on a Leica Inverted DM IRE2 HC fluo TCS 1-B-UV microscope coupled to a TCS SP2 spectral confocal system/UV with a scan head containing an AOBs acousto-optical tunable filter. The green HeNe (543 nm/1.2 mW) and blue argon (488 nm/20 mW) laser lines were used to visualize the fluorophores. The images were captured at  $1,024 \times 1,024$  resolution under a  $40 \times$  oil lens.

## Results

### *Patients UC-1 and UC-4 Display Severe and Mild Symptoms of CMD, Respectively*

Patient UC-1, age 12 years, had congenital-onset muscular hypotonia and weakness, followed by severely delayed motor development. He never stood or walked without support, and his maximal motor ability was sitting without support. He was first evaluated by us at age 6 years, when he presented with severe muscular hypotonia and weakness. The muscle weakness was graded as 3/5 MRC (Medical Research Council) proximally and distally, with minimal bilateral facial weakness. There was marked generalized muscle atrophy. The tendon reflexes were absent or depressed. Severe joint contractures were prevalent, affecting preferentially proximal but also distal joints in the finger flexors of the hands while maintaining small joint hyperlaxity at the same time. He had protruding calcanei. His cognitive development had been completely normal. Since age 11 years, he had suffered from respiratory insufficiency, necessitating ventilatory assistance. His creatine kinase (CK) level was normal, and electromyography (EMG) results were interpreted as showing a myopathic pattern. Results of a CT scan of the brain, cardiac evaluation, and ophthalmological evaluation were all normal. A muscle biopsy, which had been obtained at age 9 years 5 mo, showed moderate proliferation of endomysial and perimysial connective tissue and variation of muscle fiber diameter, as well as scattered degenerating fibers. Patient UC-1 thus displays a classical severe UCMD phenotype.

Patient UC-4, age 6 years, also presented with hypotonia since birth. However, he was able to walk since age 1 year and able to climb stairs, but he got up from the floor with a Gowers' maneuver and suffered frequent falls. Muscle weakness was moderate, at  $\sim 4+/5$  MRC, with some proximal predominance. The patient had generalized hyperextensibility of his joints in the upper and lower extremities, in particular in the small joints. There were slight asymmetric contractures of the Achilles tendons, but no other contractures were found. CK level was normal. EMG demonstrated a myopathic pattern, and muscle biopsy exhibited a mildly dystrophic pattern, with variation in muscle fiber diameter, moderate increase in endomysial connective tissue, and occasional degenerating fibers.

### *Collagen VI Microfibrils Are Absent in Fibroblasts from UC-1 and Markedly Reduced in Fibroblasts from UC-4*

Fibroblasts from patients UC-1 and UC-4 were immunostained with an antibody specific to the  $\alpha 1(\text{VI})$  collagen chain. Whereas the control fibroblasts exhibited an extensive collagen VI microfibrillar network in the

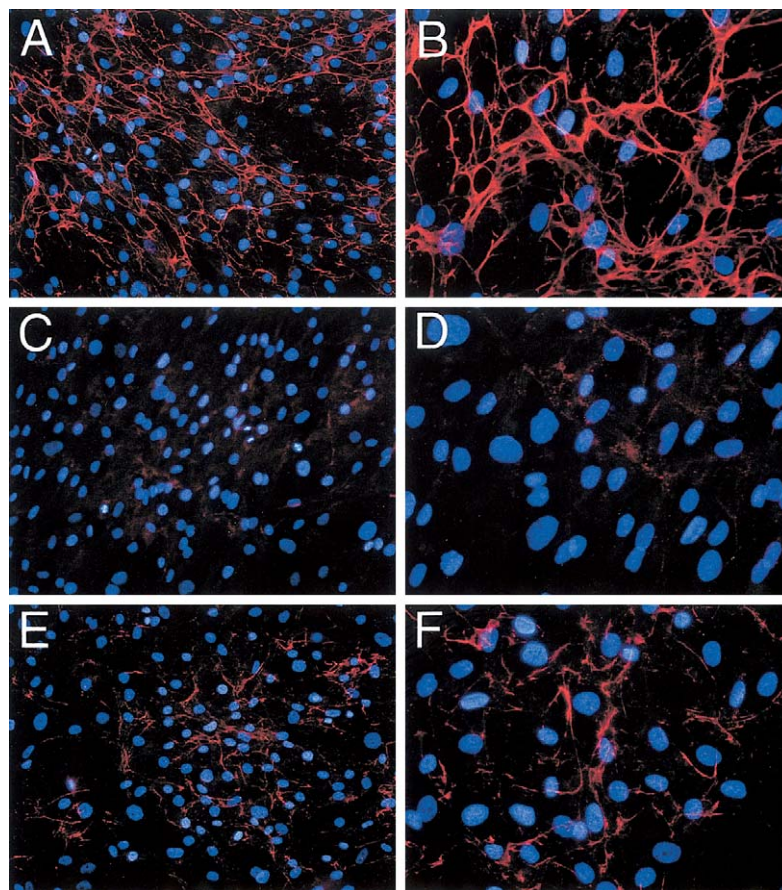
extracellular matrix, fibroblasts from UC-1 were found to deposit few, if any, collagen VI microfibrils (fig. 1). In contrast, fibroblasts from UC-4 deposited collagen VI microfibrils in markedly reduced amounts as compared with the control fibroblasts. Similar immunostaining results were observed when the  $\alpha 3(\text{VI})$  collagen chain-specific 1014 antibody was used (data not shown). The fibroblasts from both UC-1 and UC-4 deposited amounts of type I collagen fibrils similar to that deposited by the control fibroblasts (data not shown). The results suggested that both patients might carry collagen VI mutations.

*The COL6A1 Transcripts in UC-1 and UC-4 Each Contain a Heterozygous In-Frame Deletion within the Triple-Helical Domain*

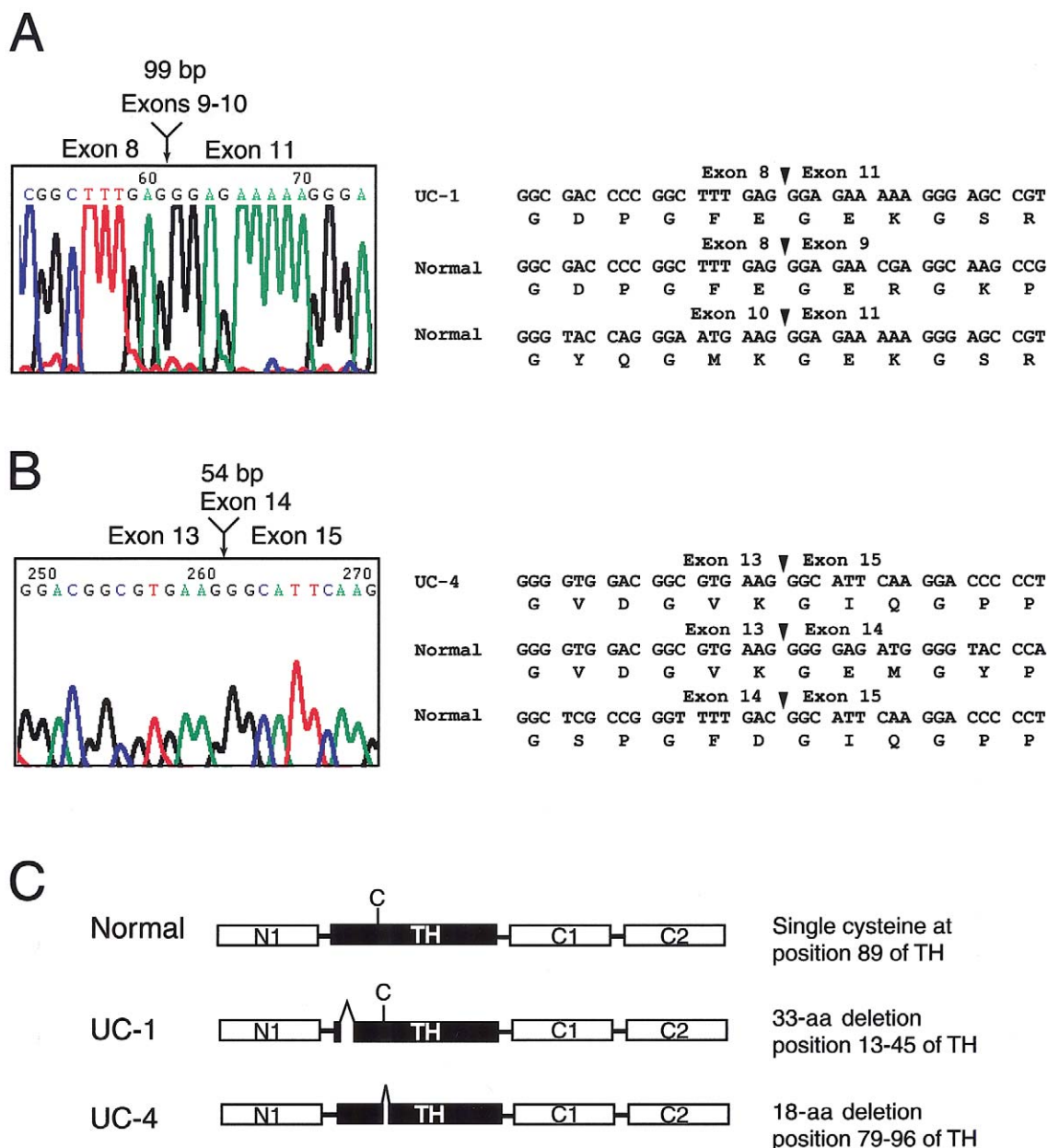
To search for possible collagen VI mutations in UC-1 and UC-4, the coding regions of the COL6A1, COL6A2, and COL6A3 mRNA transcripts were am-

plified from fibroblast RNA by RT-PCR. A primer pair flanking the COL6A1 triple-helical coding region amplified two products of equal intensity in both patients, one of which was the expected size, whereas the other one was slightly smaller (data not shown). DNA sequencing revealed that the shorter product from UC-1 contained a deletion of 99 bp, extending from nucleotide 805 to 903, encoded by exons 9 (54 bp) and 10 (45 bp) of the COL6A1 gene (fig. 2). The shorter product from UC-4 harbored a smaller deletion, of 54 bp, that was located farther downstream and corresponded to nucleotides 1003–1056, encoded by exon 14. DNA sequences of the larger PCR products from both patients showed no deviations from the reference COL6A1 cDNA sequence. The results indicated that UC-1 and UC-4 carry heterozygous COL6A1 in-frame deletions of different sizes located at different positions within the triple-helical domain.

Because all previously reported patients with UCMD



**Figure 1** Deposition of collagen VI microfibrils in the extracellular matrix of dermal fibroblasts. Fibroblasts from an unaffected control individual (A and B), patient UC-1 (C and D), and patient UC-4 (E and F) were grown in the presence of 50  $\mu\text{g}/\text{ml}$  L-ascorbic acid phosphate for 4 d postconfluency and were stained with the  $\alpha 1(\text{VI})$  collagen-specific antibody. The antibody reaction was detected with Cy3-conjugated goat anti-rabbit IgG (red), and cells were counterstained with DAPI (blue) to visualize nuclei. Original magnifications  $\times 20$  (A, C, and E) and  $\times 40$  (B, D, and F).



**Figure 2** In-frame deletions in the triple-helical domain of the *COL6A1* mRNA in patients UC-1 and UC-4. A and B, DNA sequence chromatograms showing a deletion of 99 bp encoded by exons 9 and 10 in UC-1 (A) and a deletion of 54 bp encoded by exon 14 in UC-4 (B). DNA and amino acid sequences of the normal and mutant *COL6A1* mRNA surrounding the mutations are shown. Arrowheads mark exon borders. C, Schematic diagram of normal and mutant  $\alpha 1(\text{VI})$  collagen chains. The normal  $\alpha 1(\text{VI})$  collagen chain consists of a triple-helical domain (TH) of 336 amino acids flanked by N- and C-terminal globular domains made up of vWF-A-like motifs (N1, C1, and C2). The TH domain contains a single cysteine residue (depicted as "C") at position 89, which is important for dimer assembly. Patient UC-1 contains a 33-amino acid deletion near the N-terminus of the triple-helical domain, whereas patient UC-4 contains an 18-amino acid deletion somewhat downstream, which removes the cysteine residue.

carry homozygous or double heterozygous mutations, it was possible that a second mutation in one of the collagen VI genes could be present in UC-1 and UC-4 in a compound heterozygous state with the identified mutations. Therefore, the entire coding regions of the

*COL6A1*, *COL6A2*, and *COL6A3* transcripts were analyzed by DNA sequencing. The analyses yielded a number of silent nucleotide polymorphisms and single-nucleotide changes that resulted in amino acid substitutions (table 2). All of the amino acid substitutions were found



**Table 2****Polymorphisms in Collagen VI Genes That Result in Amino Acid Changes**

Patient, Gene, and Exon	Nucleotide Change	Amino Acid Change	Status
UC-1:			
COL6A1:			
35	2669C→T	S889L	Heterozygous
COL6A2:			
26	2039A→G	H680R	Heterozygous
28	2803G→A	G935R	Heterozygous
COL6A3:			
25	6653C→T	P2218L	Heterozygous
28	6860C→G	P2287R	Homozygous
41	9203C→T	T3068I	Homozygous
UC-4:			
COL6A2:			
18	1466G→A	R489Q	Heterozygous
26	2039A→G	H680R	Homozygous
COL6A3:			
4	872G→A	R291K	Heterozygous
36	7292T→A	V2431D	Homozygous
39	8491G→C	D2831T	Homozygous
40	8780C→T	T2927M	Heterozygous
40	8870-8871insTGC	2957insA	Homozygous
40	8959G→A	V2987M	Homozygous
41	9203C→T	T3068I	Heterozygous

in genomic DNA from control individuals and thus were not pathological mutations.

*An Internal COL6A1 Gene Deletion in UC-1 Removes Exons 9 and 10, Whereas a Splice Donor Site Mutation in UC-4 Results in Skipping of Exon 14*

To delineate the mRNA mutations at the genomic level and determine whether the mutations were inherited, genomic DNA from the patients and their families was PCR amplified and DNA sequenced. Primers in introns 6 and 11 were used to amplify genomic DNA from UC-1 and his family (parents, elder brother, and sister) (fig. 3A). The genomic region was found to be highly variable in length, since two major PCR products, ranging in size from 2.6 to 3.2 kb, were amplified from DNA of the family members and unaffected controls (fig. 3B). The size variation was due to the presence of a minisatellite near the 5' end of intron 8. In the reference sequence (GenBank accession number NT\_011515), the minisatellite consists of 32 tandem repeats of the sequence ccagatggaggga(t/c)ggcggg(a)gt. In UC-1, a 2.6-kb PCR product and a substantially smaller 1.8-kb fragment were obtained. DNA sequencing indicated that the 1.8-kb DNA fragment contained a deletion of 1,095 bp that removed the genomic region encompassing exons 9 and 10 and the intervening intron (fig. 3A). The proximal deletion breakpoint was located in intron 8 at 17 bp downstream of the last repeat in the minisatellite sequence (fig. 3C). The distal breakpoint was in intron 10

at 5 bp downstream of exon 10. At the distal breakpoint, a 15-bp inserted sequence (tcctcccccatttcc) was found, of which the sequence tccccatttcc was a direct duplication of adjacent intron 10 sequence. The DNA sequence of the 2.6-kb fragment corresponded well with the reference COL6A1 genomic sequence. Comparison of the sizes of the PCR products from UC-1 and his family indicated that the genomic deletion in UC-1 was not present in his parents and two siblings (fig. 3B) and, hence, had arisen de novo. It also indicated that the normal 2.6-kb COL6A1 allele in UC-1 was inherited from the father; therefore, the novel genomic deletion in UC-1 had occurred on his maternal COL6A1 allele.

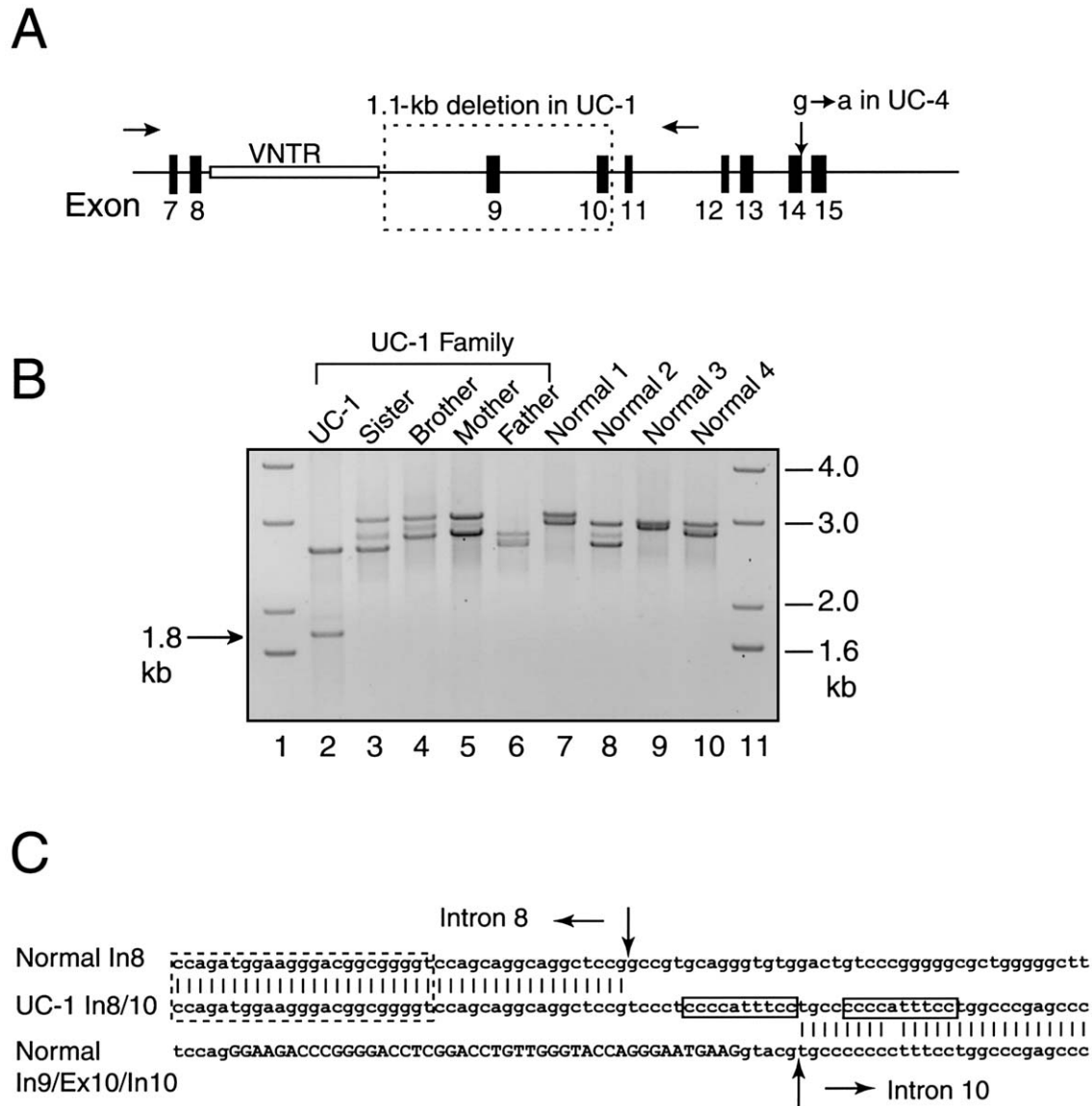
Genomic DNA from UC-4 and his family (parents, elder brother and sister) was PCR amplified with a primer pair flanking exon 14, which yielded a single PCR product of 854 bp in all family members. DNA sequencing of the fragments showed a heterozygous G→A transition at +1 position of the splice donor site in intron 14 in UC-4 (fig. 3A). The mutation was not found in his parents and siblings and therefore also had arisen de novo.

*Mutant  $\alpha 1(VI)$  Collagen Chain Is Secreted Extracellularly in UC-1 but Not in UC-4*

The effects of the COL6A1 gene mutations on collagen VI mRNA and protein synthesis were studied. Analysis of total RNA from UC-1 and UC-4 fibroblasts by northern blot hybridization showed that the steady-state levels of collagen VI mRNA for all three chains in UC-1 and UC-4 were comparable to those in the control (fig. 4A). The results suggest that the mutations do not significantly affect transcription and stability of the COL6A1 mRNA, unlike previous findings of other patients with UCMD (Zhang et al. 2002).

To assess the synthesis and secretion of the mutant  $\alpha 1(VI)$  collagen chains in fibroblasts from UC-1 and UC-4, cell layers and culture media were analyzed by immunoblotting using the antibody specific for the  $\alpha 1(VI)$  collagen chain. In the cell layers of UC-1 and UC-4, approximately equal amounts of normal and shorter  $\alpha 1(VI)$  collagen chains were observed (fig. 4B, lanes 1–3), indicating that the mutant chains in both patients were synthesized efficiently and were stable. Because of posttranslational modifications, the  $\alpha 1(VI)$  collagen chains that were secreted extracellularly migrated more slowly than those found in the cell layers. Both normal and shorter chains could be detected in the medium of UC-1, whereas only the normal chain was seen in the medium of UC-4. Quantification of three separate immunoblots with a densitometer revealed that the amount of mutant chain secreted by the fibroblasts from UC-1 was 53% of the normal counterpart.

Fibroblasts from UC-1 and UC-4 were metabolically

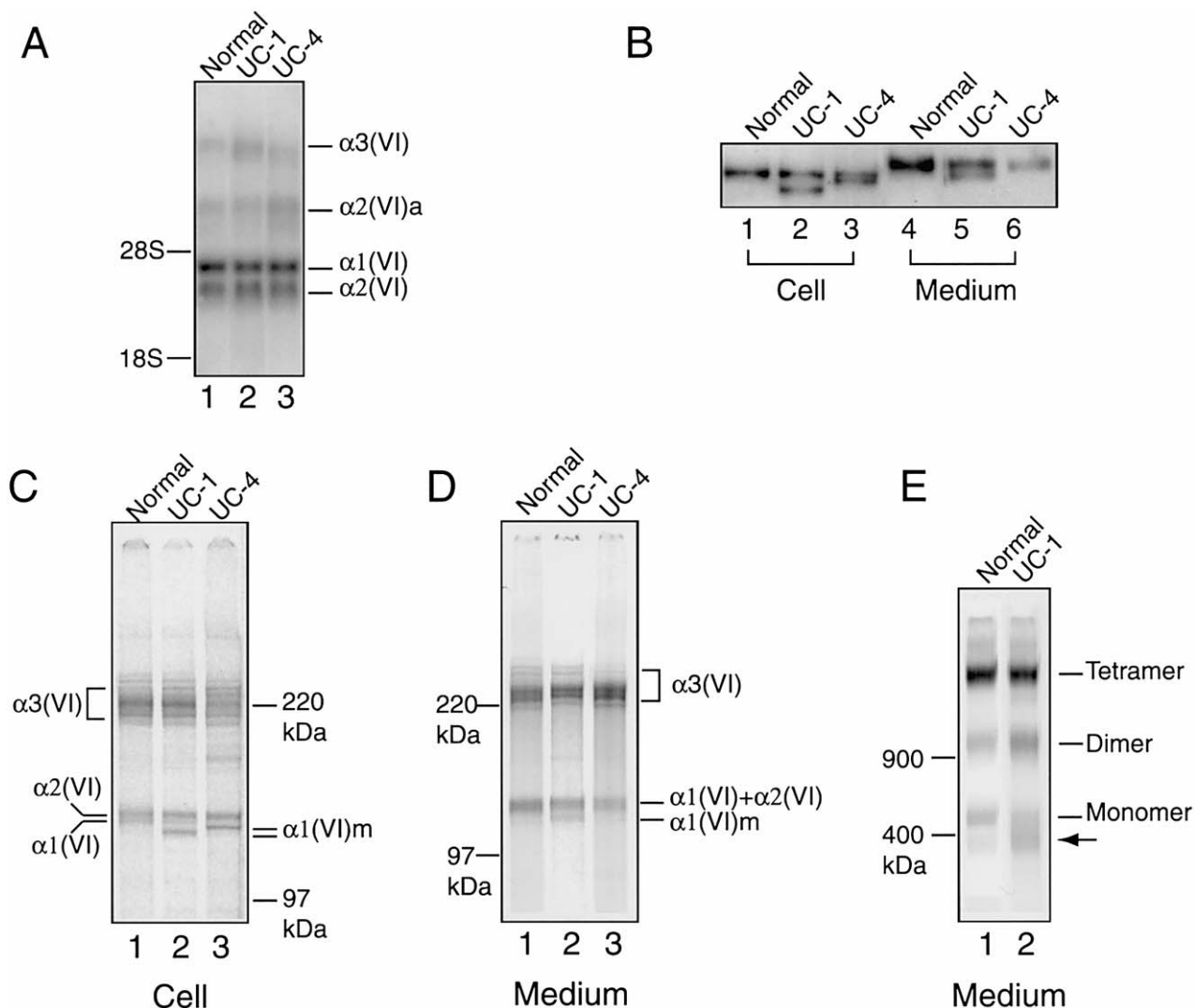


**Figure 3** Mutational analysis of the *COL6A1* gene. **A**, Schematic diagram of the *COL6A1* genomic region including exons 7–15 (blackened boxes). Exon 8 encodes the beginning of the triple-helical domain, and exons 9–15 each encode discrete numbers of Gly-Xaa-Yaa repeats in the triple-helical domain. A minisatellite (unblackened box labeled “VNTR”) is present at the 5′ end of intron 8. In patient UC-1, one of the *COL6A1* alleles contains a 1.1-kb gene deletion (dotted box) extending from intron 8 to intron 10. Patient UC-4 carries a heterozygous G→A transition at the +1 position of intron 14. **B**, PCR amplification of genomic DNA from patient UC-1 and his family (lanes 2–6) and from four unaffected control individuals (lanes 7–10) with primers in introns 6 and 12 (arrows in **A**). Lanes 1 and 11 contain DNA size markers. **C**, DNA sequence of the 1.8-kb PCR product from UC-1, showing the breakpoint of the internal gene deletion (middle line) and its alignment with the normal *COL6A1* genomic sequence of intron 8 (top line) and of intron 9–exon 10–intron 10 (bottom line). Exon sequences are in capital letters. Between the arrows are 15-bp inserted sequences in the deletion junction, which contains an 11-bp direct duplication of the sequence in intron 10 (boxed). The dotted box depicts the last repeat of the minisatellite sequence in intron 8. In = intron; Ex = exon.

labeled with [<sup>35</sup>S]cysteine and were immunoprecipitated with the antibody against the  $\alpha 3(\text{VI})$  collagen chain. The antibody would precipitate free  $\alpha 3(\text{VI})$  collagen chain as well as  $\alpha 1(\text{VI})$  and  $\alpha 2(\text{VI})$  collagen chains that are folded together with the  $\alpha 3(\text{VI})$  collagen chain in a triple helix. In the cell layers of fibroblasts from both UC-1

and UC-4, the antibody precipitated both the normal and shorter  $\alpha 1(\text{VI})$  collagen chains (fig. 4C), indicating that the mutant chains from both patients were able to incorporate into triple helices. As predicted from the sizes of the deletions, the mutant chain in UC-1 migrated faster than that in UC-4. In contrast, the mutant  $\alpha 1(\text{VI})$





**Figure 4** Analysis of collagen VI mRNA and protein in fibroblasts from UC-1 and UC-4. A, Northern blot analysis of total RNA (5 µg) from normal fibroblasts and those from UC-1 and UC-4, with a mixture of [<sup>32</sup>P]dCTP-labeled cDNA probes for the three collagen VI mRNA. The probes hybridized with the α1(VI), α2(VI), and α3(VI) collagen mRNA of 4.2, 3.5, and 9–10 kb, respectively, plus an alternatively spliced α2(VI) collagen mRNA, α2(VI)a, of 6.0 kb. B, Immunoblot analysis of cell layer extracts (lanes 1–3) and culture media (lanes 4–6) from normal fibroblasts (lanes 1 and 4) and those from UC-1 (lanes 2 and 5) and UC-4 (lanes 3 and 6), with the antibody specific for the α1(VI) collagen chain. Lanes 1–3 contain 10 µg of total protein from the cell layers, and lanes 4–6 contain 100 µl of culture medium precipitated with 0.9 ml of 100% EtOH. C–E, Immunoprecipitation of cell layers (C) and culture media (D, E) from normal fibroblasts (lane 1) and those from UC-1 (lane 2) and UC-4 (lane 3), with the antibody specific for the α3(VI) collagen chain. Fibroblasts were labeled with [<sup>35</sup>S]cysteine overnight. Samples in B, C, and D were reduced with 25 mM DTT and separated on 3%–8% polyacrylamide gels. Samples in E were separated on a composite agarose polyacrylamide gel in the absence of 25 mM DTT. The mutant α1(VI) collagen chains are depicted as α1(VI)m. The arrow indicates a protein present in higher amounts in fibroblasts from UC-1 compared with the normal control fibroblasts.

collagen chains could be detected only in the medium of UC-1 but not of UC-4 (fig. 4D), consistent with the results of the immunoblot analysis above.

To determine whether the shorter α1(VI) collagen chains secreted by fibroblasts from UC-1 were present in dimers and tetramers, the immunoprecipitated material was analyzed on a composite agarose/polyacryl-

amide gel without reduction using DTT. The majority of the secreted collagen VI chains in UC-1 were found in tetramers similar to the normal control fibroblasts (fig. 4E). This implied that the mutant α1(VI) collagen chain secreted extracellularly was most likely incorporated into the tetramers. It was noted that there were fewer monomers in UC-1 than in the control, whereas a pro-

tein complex that migrated below the monomers was present in higher amounts in UC-1 as compared with the control. This was a consistent finding in two separate experiments. The nature of this faster-migrating protein band is unclear; its apparent molecular mass appears larger than that of a mutant monomer containing a shorter  $\alpha 1(\text{VI})$  collagen chain.

#### *Collagen VI Protein Is Aberrantly Localized in the UC-1 Muscle but Reduced in the UC-4 Muscle*

Muscle biopsies from UC-1 and UC-4 were immunostained with antibodies against collagen VI and perlecan simultaneously (fig. 5). In normal muscle, collagen VI colocalized with perlecan in the basement membrane surrounding muscle fibers. In the UC-1 muscle, collagen VI protein was not seen in the basement membrane, even though the basement membrane appeared intact, as shown by the uniform perlecan staining. Abundant collagen VI protein, however, was found in the interstitial and perivascular regions between muscle fibers. In contrast, some collagen VI protein was present in the basement membrane of the UC-4 muscle, but at a markedly reduced level relative to the staining with the perlecan antibody.

#### **Discussion**

Here we demonstrate that a patient affected with the severe phenotype of UCMD (UC-1) carries a heterozygous in-frame deletion in the *COL6A1* gene but no additional mutations in the *COL6A1*, *COL6A2*, and *COL6A3* genes. The gene deletion is not present in the unaffected family members and has arisen de novo. The findings indicate that the heterozygous *COL6A1* gene deletion is the underlying cause of the severe UCMD phenotype in this patient.

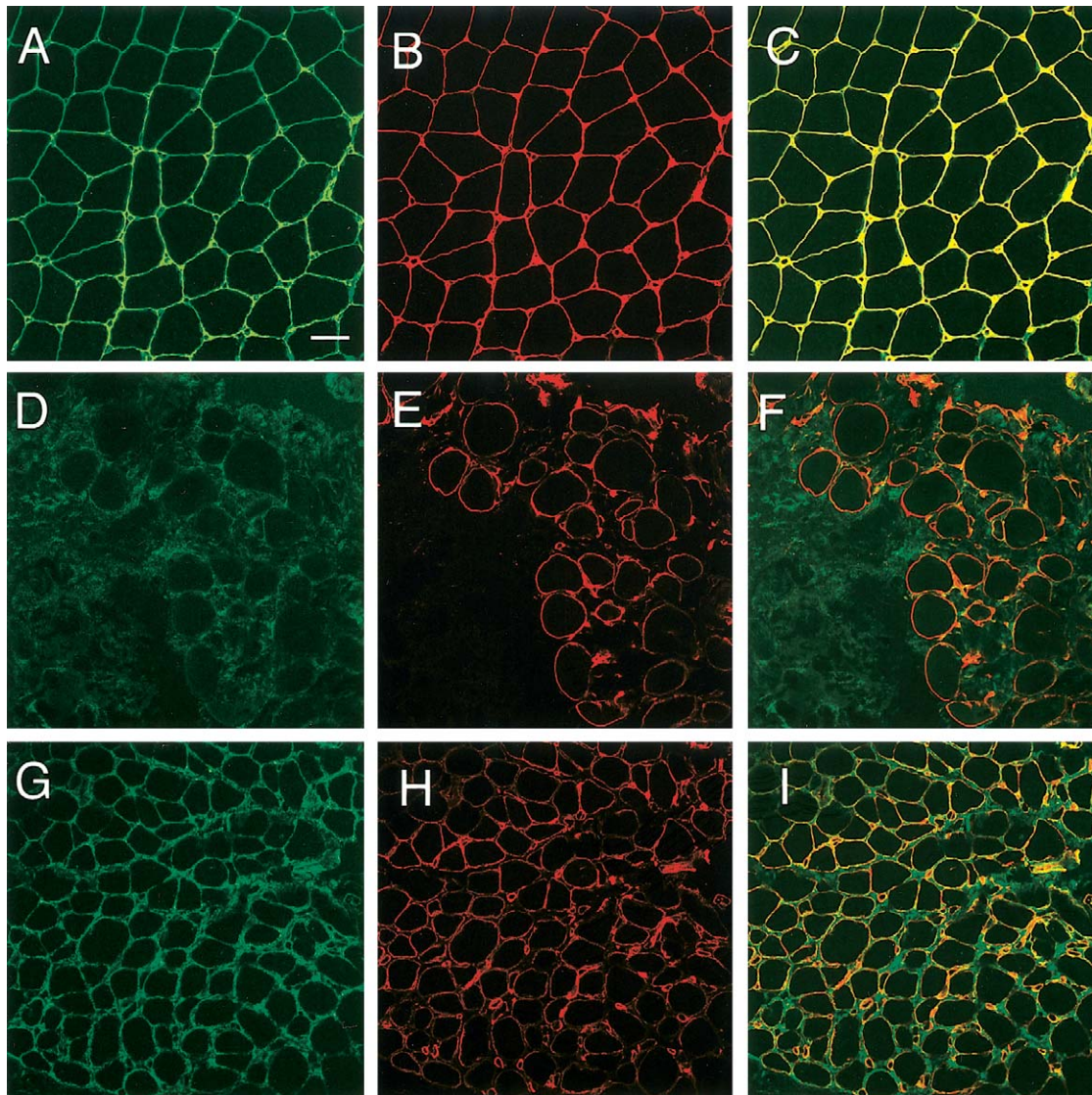
The deletion in UC-1 is located very close to the minisatellite in intron 8 of the *COL6A1* gene, suggesting a role for the minisatellite sequence in the gene deletion. PCR analysis of the *COL6A1* minisatellite alleles in the UC-1 family and 15 unaffected control individuals indicates that the length of this minisatellite varies greatly (fig. 3B and data not shown). This result supports previous findings that minisatellites can be found in the vicinity of recurrent translocation breakpoints and constitute chromosomal fragile sites (Vergnaud and Denoed 2000). Minisatellites have, in addition, been shown to affect transcription and intron splicing (Kennedy et al. 1995; Turri et al. 1995). For instance, an expansion of an intronic minisatellite in the *USH1C* gene has been correlated with the Usher syndrome type 1C in the Acadian population (Verpy et al. 2000). It remains to be tested whether the variation in the size of the minisatellite in intron 8 of the *COL6A1* gene

interferes with intron splicing and/or affects the steady-state mRNA level.

The milder CMD phenotype of patient UC-4 is caused by a heterozygous skipping of exon 14 in the *COL6A1* transcript. This is a recurring mutation in the *COL6A1* gene, since skipping of exon 14 has previously been found in two unrelated patients with Bethlem myopathy resulting from mutations in either the first or second nucleotide of the splice donor site in intron 14 (Lamandé et al. 1999; Pepe et al. 1999). Patient UC-4 does not display the significant joint contractures characteristic of patients with Bethlem myopathy at this time. However, he is still young and may develop contractures in the future; therefore, his phenotype at this age could well be compatible with sporadic Bethlem myopathy. It has been pointed out that Bethlem myopathy may present as a mild congenital muscular dystrophy (Jöbsis et al. 1999).

The deletion in UC-1 removes 11 contiguous Gly-Xaa-Yaa repeats in the  $\alpha 1(\text{VI})$  collagen chain very close to the N-terminus of the triple-helical domain (amino acids 13–45 of the triple-helical domain). On the other hand, UC-4 contains a smaller deletion of six Gly-Xaa-Yaa repeats at amino acids 79–96, which is also located at the N-terminal portion of the triple-helical domain, but somewhat downstream. For fibrillar collagens, the triple-helical folding starts from the C-terminal non-collagenous domains and propagates in a zipperlike fashion toward the N-terminus (Bachinger et al. 1981; Engel and Prockop 1991). If we assume that this is also the case for collagen VI, deletions in the N-terminal portion of the triple-helical domain such as those found in UC-1 and UC-4 would not prevent the mutant chains from folding into a triple helix. Indeed, immunoprecipitation of the cell layers from the fibroblasts of UC-1 and UC-4 indicates that the shorter  $\alpha 1(\text{VI})$  collagen chains in both patients are incorporated into triple-helical monomers (fig. 4C). The mutant monomers presumably would contain shorter triple-helical domains, because the deletions in the mutant chain are likely to be accommodated by looping out the corresponding regions of the normal chains in a manner similar to a triple-helical deletion in type II collagen described elsewhere (Weis et al. 1998).

The two different deletions, however, have distinct effects on the subsequent step of collagen VI supramolecular assembly. Unlike most collagens, the triple-helical collagen VI monomers are assembled into dimers and tetramers prior to secretion into the extracellular space (fig. 6A) (Timpl and Engel 1987; Timpl and Chu 1994). Dimers are formed by the antiparallel, staggered assembly of two monomers, and the single cysteine located at amino acid 89 of the triple-helical domains in the  $\alpha 1(\text{VI})$  or  $\alpha 2(\text{VI})$  collagen chains is responsible for the assembly/stability of the dimers (Furthmayr et al.



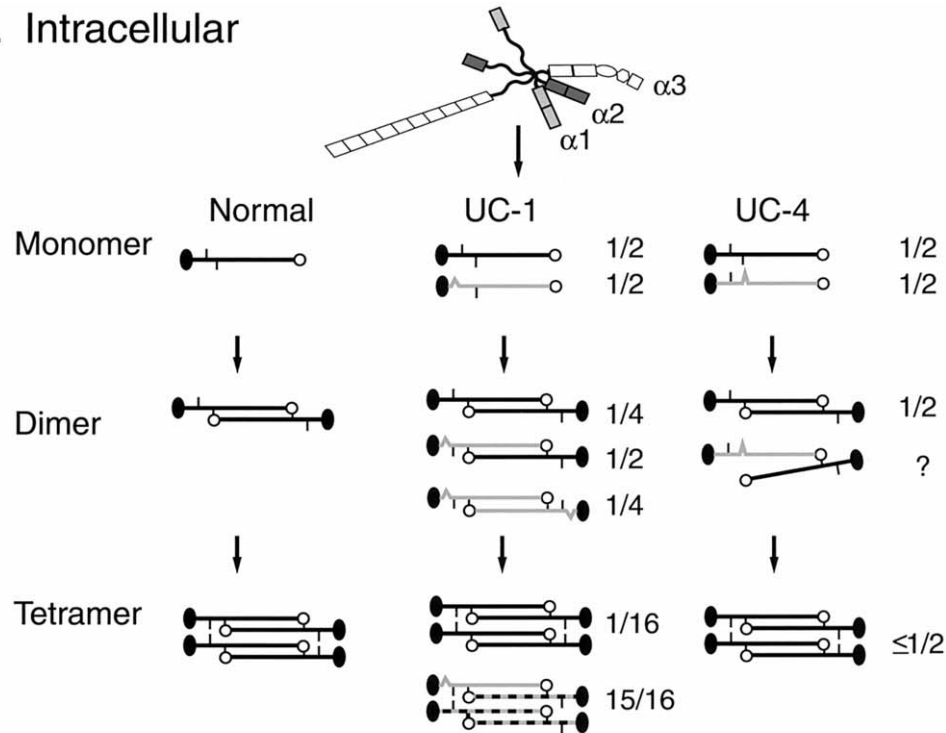
**Figure 5** Immunostaining of muscle biopsies from UC-1, UC-4, and an unaffected control individual. Sections were stained with the monoclonal antibody against collagen VI (*green* in A, D, and G) and the polyclonal antibody against perlecan (*red* in B, E, and H). C, F, and I show a merge of green and red fluorescence. In normal muscle (A, B, and C), collagen VI and perlecan colocalize in the basement membrane. In the muscle from UC-1 (D, E, and F), collagen VI is absent in the basement membrane surrounding muscle fibers; instead, it is accumulated in the interstitial and perivascular space between muscle fibers. In the muscle from UC-4 (G, H, and I), collagen VI is present in the basement membrane but appears patchy and significantly reduced relative to the perlecan staining. Collagen VI staining is increased in the interstitial and perivascular space.

1983; Chu et al. 1988). Tetramers are assembled by the lateral association of two dimers, whereby the single cysteine residue at amino acid 50 in the triple-helical domain of the  $\alpha 3(\text{VI})$  collagen chain is involved in tetramer formation/stability. The critical regions involved in dimer and tetramer formation are all retained in the mutant chain of UC-1; thus, the mutant chain should be able to assemble into dimers and tetramers and be secreted. By contrast, cysteine 89 is deleted in the mutant chain of UC-4, which would prevent the abnormal

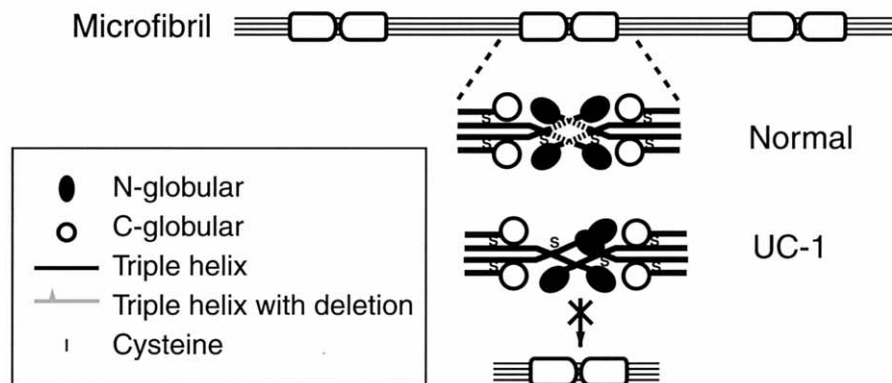
monomer from assembling into dimers. Consequently, only normal dimers and tetramers will be formed.

The immunoprecipitation and immunoblot analyses indeed confirm that the shorter  $\alpha 1(\text{VI})$  collagen chain in UC-1 is incorporated into tetramers and secreted (fig. 4B, 4D, and 4E). Theoretically, equal amounts of the normal and mutant chains synthesized by UC-1 would result in assembly of equal amounts of the normal and mutant monomers. However, because the mutant chain can be incorporated in the subsequent assembly steps,

## A. Intracellular



## B. Extracellular



**Figure 6** Schematic diagram showing the effects of two different internal deletions on collagen VI assembly. *A*, Intracellular events. Normally (*left*), the  $\alpha 1(\text{VI})$ ,  $\alpha 2(\text{VI})$ , and  $\alpha 3(\text{VI})$  collagen chains presumably fold from the C-terminal end into a triple-helical monomer. Two key cysteine residues (*vertical lines*) in the N-terminal portion of the triple helix are critical for subsequent supramolecular assembly. Dimers are formed by the staggered antiparallel association of monomers with the N-terminal globular domains protruding at both ends. The outer cysteines contributed by either the  $\alpha 1(\text{VI})$  or  $\alpha 2(\text{VI})$  collagen chains are important for the dimer assembly. The dimers associate laterally into tetramers, which are stabilized by the outer cysteines contributed by the  $\alpha 3(\text{VI})$  collagen chain. In UC-1 (*middle*) and UC-4 (*right*), equal amounts of normal and mutant (*gray*)  $\alpha 1(\text{VI})$  collagen chains assemble into equal amounts of normal and mutant monomers. Because the cysteines critical for dimer and tetramer formation are preserved in UC-1, both normal and mutant monomers can assemble into dimers and tetramers in any combination. Thus, only 1/4 of the dimers and 1/16 of the tetramers are entirely composed of normal chains, whereas 15/16 of the tetramers contain a mixture of normal and mutant chains (*gray striped lines*). The deletion in UC-4 removes the cysteine in the triple-helical domain of the  $\alpha 1(\text{VI})$  collagen chain, which prevents the abnormal monomers from assembling into dimers. Therefore, only normal tetramers are formed and secreted. The mutant chain in UC-4 may interfere with the assembly of the normal dimers, resulting in the formation and secretion of normal tetramers at a level  $\leq 1/2$  that of the control. *B*, Extracellular event. Normally, tetramers are secreted and associated end-to-end into double-beaded microfibrils (*top*). The microfibrillar assembly involves interactions of the N- and C-globular domains and triple-helical domain. The deletion in UC-1 is located very close to the N-terminal end of the triple-helical domain (*broken thick lines*). Presumably, the triple helix in the mutant monomer is shortened because the normal  $\alpha 2(\text{VI})$  and  $\alpha 3(\text{VI})$  collagen chains are looped out at the region corresponding to the deletion (*bottom*). As a result, the mutant tetramers cannot properly align with normal tetramers to form the double-beaded structure. Because the majority of the secreted tetramers are abnormal, long microfibrils cannot be assembled.

the effect of the mutation would be greatly amplified. It is expected that only 1/4 of the dimers and only 1/16 of the tetramers would be entirely composed of normal chains, whereas 15/16 of the tetramers would be abnormal, with 1–4 mutant chains (fig. 6A). Experimentally, however, it appears that certain types of abnormal tetramers may not be formed and/or secreted, because immunoblot analysis shows that only 53% of the shorter  $\alpha 1(\text{VI})$  collagen chains are secreted (fig. 4B). The effect of the deletion likely is further amplified in the microfibrillar assembly step after the mutant tetramers are secreted. Since microfibrils are assembled by end-to-end association of the tetramers via the N-terminus, mutant tetramers with structurally abnormal N-termini cannot correctly align with their neighboring tetramers and thus would drastically impair microfibrillar assembly (fig. 6B).

In contrast, the shorter  $\alpha 1(\text{VI})$  collagen chain in UC-4 cannot be detected in the culture medium under different electrophoretic conditions (fig. 4 and data not shown). This observation is consistent with previous findings in the two patients with Bethlem myopathy who had the same deletion (Lamandé et al. 1999; Pepe et al. 1999). However, it should be noted that, owing to posttranslational modifications of the secreted chains and relatively small size of the deletion, the possibility remains that the mutant chain may be secreted but cannot be distinguished from the normal chain experimentally. The mutant chain may, in addition, form a partially assembled dimer with its normal counterpart intracellularly (fig. 6). In either case, the mutant chain might exert a slight dominant negative effect. Therefore, the deletion in UC-4 would lead to assembly of collagen VI microfibrils at a level  $\leq 50\%$  that of the control.

It is interesting to consider another in-frame deletion in the triple helical domain reported in a patient with UCMD (table 1). This patient carried a homozygous splice mutation in the *COL6A3* gene, causing skipping of exon 29 and an in-frame deletion of 51 amino acids in the C-terminus of the triple-helical domain (Demir et al. 2002). However, the heterozygous parents were not affected, suggesting that the mutation does not act in a dominant negative fashion. Studies using introduced triple-helical deletions as well as glycine substitutions in the  $\alpha 3(\text{VI})$  collagen chain have shown that the biosynthetic consequences of the mutations depend on their positions in the triple-helical domain (Lamandé et al. 1999, 2002). Whether the mutant  $\alpha 3(\text{VI})$  collagen chain with the C-terminal triple-helical deletion is incorporated into the triple helix to potentially act in a dominant negative way remains an interesting question.

The distinct effects of these two triple-helical deletions on collagen VI supramolecular assembly are supported by immunofluorescent labeling of fibroblasts, showing an almost complete absence of collagen VI

microfibrils in the fibroblasts of UC-1 and a significant reduction in the fibroblasts of UC-4 (fig. 1). The differential effects are also reflected in their muscle biopsies, which display an absence and substantial reduction of collagen VI immunostaining in the muscle basement membrane of UC-1 and UC-4, respectively (fig. 5). This correlates well with the clinical severity of these two patients. The substantial reduction in collagen VI immunofluorescence observed in fibroblasts and muscle biopsy from UC-4 compared with the control suggests that the mutant chain in UC-4 may exert a slight dominant negative effect, either intracellularly or extracellularly.

Of particular interest is the observation that the mutation in UC-1 leads to loss or mislocalization of collagen VI protein in the muscle basement membrane (fig. 5). By immunoelectron microscopy, collagen VI filaments have previously been shown to localize in close juxtaposition to the muscle basement membrane (Kuo et al. 1997). The results from the muscle of UC-1 suggests that only the assembled collagen VI microfibrils can be properly anchored to the basement membrane. It also suggests that the substantial amounts of mutant collagen VI tetramers secreted into the extracellular space are not degraded but instead are aberrantly accumulated in the interstitial and perivascular space. It is not clear what molecule anchors the collagen VI network to the basement membrane. Possible candidates include collagen type IV, perlecan, and fibronectin, all of which are present in the basement membrane and interact with collagen VI in vitro (Tillet et al. 1994; Kuo et al. 1997). The results highlight the critical role of collagen VI in the muscle basement membrane and suggest that mislocalization of collagen VI is a potential pathological mechanism for the severe UCMD phenotype.

Mutations in collagen type VI have now been associated with seemingly distinct types of muscular dystrophy, which vary widely in clinical severity and connective tissue involvement. Previously, heterozygous mutations are found in the milder phenotypes of Bethlem myopathy and a form of dominant limb girdle muscular dystrophy, whereas homozygous or double heterozygous mutations are observed in the severe phenotype of UCMD. The identification of a heterozygous mutation in a severe phenotype of UCMD thus is a significant departure from previous observations. However, this finding should not be entirely unexpected, because heterozygous mutations in the triple-helical domain of collagen genes are known to cause severe or even lethal phenotypes owing to dominant negative effects (procollagen suicide) exerted by structurally defective chains (Prockop 1990; Myllyharju and Kivirikko 2001).

In conclusion, we have established the *COL6A1* gene as the third locus for UCMD and, furthermore, have



identified the first example of a multiexon deletion in collagen VI genes. We have also provided evidence that a heterozygous mutation in collagen VI can cause a severe UCMD phenotype through a dominant negative mechanism and suggested a correlation between mutation location and clinical severity. This finding also has considerable relevance for genetic counseling in UCMD.

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## Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for *COL6A1* [accession numbers NM\_0018489 and NT\_011515], *COL6A2* [accession numbers NM\_001849 and NT\_011515], and *COL6A3* [accession numbers X52022 and NT\_005120])

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for UCMD and Bethlem myopathy)

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