Ullrich scleroatonic muscular dystrophy is caused by recessive mutations in collagen type VI

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Ullrich syndrome is a recessive congenital muscular dystrophy affecting connective tissue and muscle. The molecular basis is unknown. Reverse transcription–PCR amplification performed on RNA extracted from fibroblasts or muscle of three Ullrich patients followed by heteroduplex analysis displayed heteroduplexes in one of the three genes coding for collagen type VI (COL6). In patient A, we detected a homozygous insertion of a C leading to a premature termination codon in the triple-helical domain of COL6A2 mRNA. Both healthy consanguineous parents were carriers. In patient B, we found a deletion of 28 nucleotides because of an A → G substitution at nucleotide −2 of intron 17 causing the activation of a cryptic acceptor site inside exon 18. The second mutation was an exon skipping because of a G → A substitution at nucleotide −1 of intron 23. Both mutations are present in an affected brother. The first mutation is also present in the healthy mother, whereas the second mutation is carried by their healthy father. In patient C, we found only one mutation so far—the same deletion of 28 nucleotides found in patient B. In this case, it was a *de novo* mutation, as it is absent in her parents. mRNA and protein analysis of patient B showed very low amounts of COL6A2 mRNA and of COL6. A near total absence of COL6 was demonstrated by immunofluorescence in fibroblasts and muscle. Our results demonstrate that Ullrich syndrome is caused by recessive mutations leading to a severe reduction of COL6.

Congenital muscular dystrophy (CMD) identifies a heterogeneous group of disorders characterized by muscle weakness and hypotonia at birth, with a muscle pathology reminiscent of muscular dystrophy. This heterogeneous condition varies according to severity, associated symptoms, and outcomes (1). Some genes have already been associated with CMDs, as, for example, integrin receptor a7, fukitin, and the extracellular matrix protein merosin (laminin $\alpha 2$) (2).

In 1930, Ullrich described two unrelated patients with CMD, and the disorder was defined as scleroatonic muscular dystrophy. The peculiar clinical manifestations were proximal joint contractures, striking distal hyperextensibility, and normal intelligence (3, 4). Since then, more than 20 patients with similar clinical manifestations have been reported worldwide (1), and thus recognized as a distinct form of CMD (Online Mendelian Inheritance in Man database no. 254090). Some familial cases show more than one sibling affected and a high incidence of consanguinity among unaffected parents, suggesting an autosomal recessive inheritance (5). Most of the patients die of respiratory failure in the first decade of life. Merosin status is reported as normal in Ullrich CMD (UCMD; ref. 6).

Bethlem myopathy (BM) is transmitted as an autosomal dominant disorder and is associated with mutations in collagen VI (COL6) genes (7, 8). This myopathy was considered as a mild neuromuscular disorder with prominent and multiple joint flexion contractures and slowly progressive proximal muscle weakness. Joint contractures mainly affect neck, elbows, knees (9), and interphalangeal joints and may have a neonatal onset (10). Recent clinical observations have shown that BM is a progressive disorder in adulthood (10). A large number of patients with BM need a wheelchair after the age of 50 years (9), and some die of respiratory failure caused by diaphragmatic paralysis (11).

COL6 is a ubiquitous extracellular matrix protein constituted by three chains, $\alpha 1$, $\alpha 2$, and $\alpha 3$, that form a monomer made up of two globular domains linked by a relatively short triple-helix structure that consists of Gly-Xaa-Yaa amino acid repeat sequences (12). The monomers assemble into dimers and then into tetramers, which associate in an end-to-end fashion to form the final microfilament network. The chains are encoded by COL6A1, COL6A2, and COL6A3 genes. COL6A1 and COL6A2 on chromosome 21q22.3 and COL6A3 on chromosome 2q37 were found to be linked to BM in 1996 (13, 14).

Eight mutations have been characterized so far (8, 15–19), five of which are glycine substitutions. Except for one glycine substitution found in the NH_2-terminal globular domain of the COL6A3 (15), the missense mutations are all localized in the triple helix of the three chains, and most likely exert a dominant-negative effect (8, 16). The other three are splice-site mutations in the COL6A1 gene, leading to a premature termination codon (17) or a skipping of the same exon (18, 19). All three splicing mutations affecting the COL6A1 gene cause haploinsufficiency. These results demonstrate that both the dominant-negative mutation and the haploinsufficiency cause BM.

We observed that UCMD shares some clinical features with BM (4, 7, 10) but displays a more severe course. Therefore, we investigated COL6 (12) in three unrelated patients with UCMD. Here, we demonstrate that recessive mutations in the COL6A2 gene lead to UCMD. In addition, our results widen the clinical spectrum of COL6 defects and disclose another pathogenetic mechanism for CMD.

Materials and Methods

Patients and Their Families. Three Italian families were diagnosed to be affected by Ullrich syndrome. Family A presented one affected son, family B had two affected brothers, and family C presented one daughter who had died at the age of 9 years and one healthy sister. The parents in family A were consanguineous. All parents were healthy and showed no clinical stigma.

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Abbreviations: CMD, congenital muscular dystrophy; UCMD, Ullrich congenital muscular dystrophy; BM, Bethlem myopathy; COL6, collagen VI; RT-PCR, reverse transcription–PCR; CK, creatine kinase; MRC, Medical Research Council.

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Peripheral Blood Samples, Muscle Biopsy, Skin Biopsy, and Fibroblast Cell Culture. All of the biological material was collected after obtaining appropriate informed consent. Peripheral blood samples were obtained from the members of all three families, and genomic DNA was extracted (20). Muscle biopsy was obtained from the proband of family C. Skin biopsies were obtained from the probands of families A and B, and primary fibroblasts were grown from the skin biopsies (16).

RNA Extraction and Reverse Transcription–PCR (RT-PCR). Total RNA was extracted from fibroblasts by using the RNA fast kit (Biotecx Laboratories, Houston) and treated with reverse transcriptase to obtain a pool of cDNAs as described (21).

Heteroduplex Analysis and Direct Sequencing. Heteroduplex analysis of the PCR products corresponding to the triple-helical domains of the three COL6 cDNAs was performed as reported (16). After the first COL6A2 screening (15), the following set of primers was used to amplify the entire COL6A2 cDNA and obtain fragments between 253 and 466 nucleotides:

N-terminal globular domain—T1 (273 nt): 2N1s, GCACGGTGCTCACGCCTGT and 2N2as, ACCATGCAGTCCCCCACGGA; T2 (364 nt): 2N2s, GACTGCCCCATCCACGTG and 2Ex3as1, CGTGGTCATCACCGACGG; and T3 (337 nt): 2Ex3s, GCAAGGGCACCGTCCACT and 2N1as, CCTCTGGCCCCAAGGG.

Triple helical domain—F9 (253 nt): 21s, GCTACCGTGGACAGAAGGG and 24as, TCCCTTGCAGCCAGGAGGT; F10 (312 nt): 26s, AGGGCAAGCTGGGGCGCA and 2as, TGCCTGGGCTGCCCTTGG; F11 (294 nt): 22s, CCAAGGGCAGCCCAGGCA and 26as, CGCCTTTTTCTCCGGGTGC; and F12 (258 nt): 27s, GTGATGCAGGACCCCGTG and 22as, CTCGGGTCCTGGGACTCC.

C-terminal globular domain—F22 (466 nt): 2Ex22s, GGATCCTGGTCCCCCTGG and 2Ex26as, CGGTCGTAGGCAAACTTGAG; F23 (404 nt): 2Ex26s, GAGCTTCAAGGAGGCTGTCA and 2Ex27as, TTTGGCAGGGAAGGTCTG.

The PCR was performed with denaturation at 96°C for 1 min, an annealing temperature between 60°C and 64°C for 1 min and an extension at 72°C for 1 min. PCR fragments displaying heteroduplexes were submitted to direct sequencing, whereas for the PCR fragments carrying deletion/insertion, the alleles were separated, eluted, reamplified, and sequenced as described (21).

Genomic DNA Analysis. Genomic DNA of the patients and their relatives was amplified with the intronic oligonucleotides reported below and the mutations were characterized through heteroduplex analysis and direct sequencing. The C insertion in the parents of family A was confirmed by amplifying a fragment of 240 nts using the primers 2I12s (CCTTCCCCTGCCAAAAGACG) and 2I13as (CATGGCCCAGATGAACAGTC). The amplified fragments underwent direct sequencing analysis. The A → G single nucleotide substitution in intron 17 of family B was detected by amplifying a fragment of 434 nucleotides with the primers 2I17s (CTCAGAAGCCAGGACCTGCT) and 2I18as (GCAGCTGCCTGAGGAGCAG). The presence of the mutation was confirmed by digesting the genomic DNA of family B and that of the parents of family C with *Dde*I. The A → G single nucleotide substitution in intron 23 was detected by amplifying a fragment of 573 nucleotides with the primers 2I22s (TCTGCCCACGGTGGACCCA) and 2Ex25as (CTCCAGTGTGAAGTTGGTGTA).

Northern Blot Analysis. Total RNA samples (15 μg per lane) were separated on a 1% agarose gel containing formaldehyde and transferred to a Hybond N nylon membrane (Amersham Pharmacia) following standard protocols (20). The membrane was hybridized with a mixture of [^{32}P]dCTP-labeled cDNA probes, F157, F225, and FO19 (22, 23) which code for human α1(VI), α2(VI), and α3(VI) collagen chains, respectively. Hybridization and washing were performed under stringent conditions, and signals were visualized by autoradiography.

Immunoprecipitation. Dermal fibroblasts were grown in DMEM with 10% (vol/vol) FBS in 35-mm dishes to confluence and then incubated overnight in the same medium with 50 μg/ml sodium ascorbate. Cells were washed with PBS, incubated with serum-free and methionine-free medium in the presence of 50 μg/ml sodium ascorbate for 2–3 h, which was then replaced with 1 ml of fresh medium containing 100 mCi/ml (1 Ci = 37 GBq) [^{35}S]methionine (ICN) overnight. At the end of the labeling period, 0.8 μl of medium was removed, and 0.2 ml of 5× lysis buffer containing protease inhibitor (24) was added. Immunoprecipitation with 1014 antibody specific for the α3(VI) collagen chain (25) was performed as described (24).

Immunofluorescence of Cultured Fibroblasts. Fibroblast cultures from the controls and from the patients were grown to confluence on coverslips and treated with 0.25 mM ascorbic acid for 5 days (26), washed in PBS, and fixed with cold methanol at −20°C for 7 min. The slides were incubated for 1 h at room temperature with a mouse monoclonal anti-COL6 antibody (MAB 3303; Chemicon) and diluted 1:100 in PBS containing 2% (vol/vol) BSA and 5% (vol/vol) normal goat serum. After several washings with 0.1% Tween-20/PBS, the slides were incubated with secondary fluorescein-conjugated rabbit anti-mouse antibody (Dako) and examined with a Nikon E600 fluorescence microscope.

Immunofluorescence of Muscle Biopsies. Skeletal muscle biopsies were trimmed and quickly frozen in methylbutane with liquid nitrogen. Cryosections (7-mm thick) were air-dried for 1 h and incubated at room temperature for 1 h with the following primary antibodies: mouse monoclonal anti-CTVI, 2C6 antibody, against the light chain (140-kDa polypeptide) of COL6 (described by Hessle and Engvall diluted 1:5, ref. 26), and rat monoclonal anti-merosin (Alexis Co., San Diego) diluted 1:1,000. Sections were washed in 0.1% Tween-20/PBS and then incubated with anti-mouse and anti-rat biotinylated IgG (Amersham Pharmacia), respectively, diluted 1:40 for 30 min. After three washes in Tween-20/PBS, the binding reaction was detected with streptavidin fluorescein (Amersham Pharmacia) at 1:250 dilution for 30 min. All antibody dilutions were made in PBS (pH 7.4) containing 1% BSA (Sigma). Sections were mounted in glycerol/PBS 1:1 and examined under a Zeiss microscope equipped for epifluorescence.

Results

Clinical Studies on Patients and Their Families. In all three families, the neurological examination, muscle testing, and creatine kinase (CK) of parents were normal.

Family A. In family A, the parents, in their fifties and healthy, were second-degree cousins. Their only son is (at the time of the study) 11 years old. He was born at 41 weeks of gestation, had an Apgar score of 6–10, and weighed 3.36 kg. Fetal movements were reduced during pregnancy. At birth he was increased in length (55 cm), showed multiple joint contractures of his knees and elbows, a kyphotic contracture of the spine (Fig. 1*B*), a left hip dislocation, bilateral congenital convex pes valgus, long and slender fingers and toes with adducted thumbs, ogival palatus, micrognatia, and a short neck with torticollis. He also showed marked bilateral distal hyperlaxity of fingers (Fig. 1*A*), toes, calcanei, and wrists. At the age of 2 months, electromyography of the deltoid muscle showed a myopathic pattern, whereas motor and sensory nerve conduction velocity was normal for his age. CK was increased 2-fold. Histology of the left quadriceps

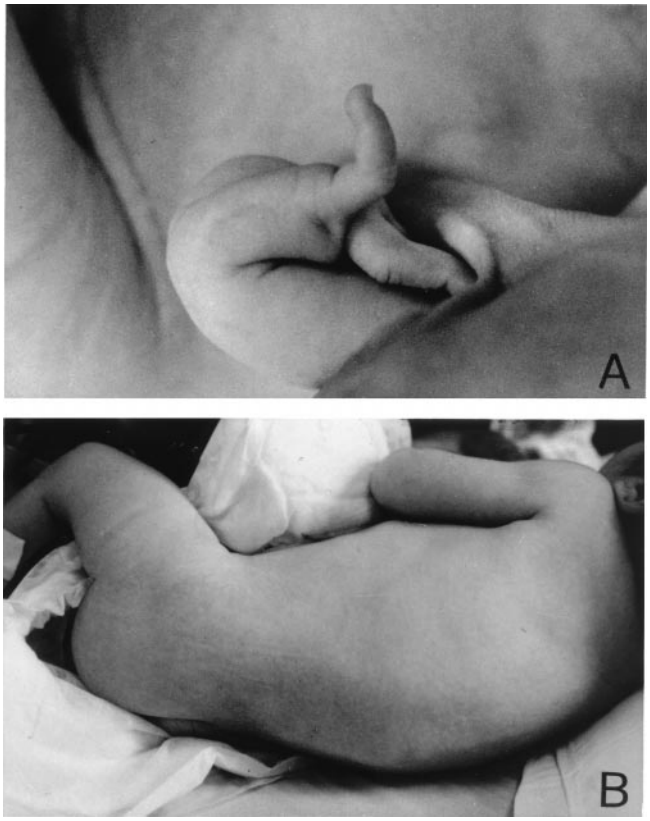

Fig. 1. Clinical aspect during the neonatal period of the patient in family A. (*A*) A remarkable distal hyperlaxity of wrist and fingers. (*B*) A peculiar congenital kyphotic contracture of the spine in the baby.

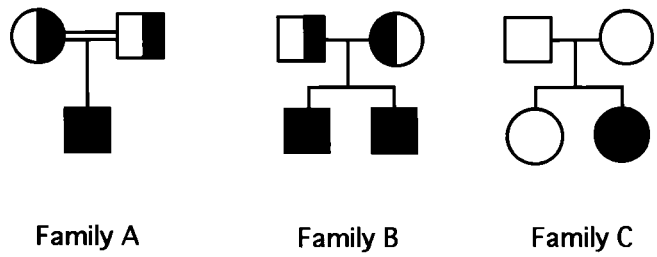

Fig. 2. Pedigrees of UCMD families. Family A shows consanguinity between the parents. Family B displays the presence of two affected sons and two healthy parents formally demonstrating the presence of recessive mutations. In family C, the proband carries a *de novo* mutation identical to one of family B. The second mutation has not been found. We do not know which one of the two parents carries a mutation.

muscle biopsy, performed at age 3 months, showed increased diameter variability, rare sporadic necrotic fibers, and mild endomisial fibrosis. The child walked at the age of 13 months, but generalized muscle weakness persisted thereafter, and progressive scoliosis and respiratory failure developed. He underwent tracheostomy and nocturnal positive pressure mechanical ventilation at the age of 8 years, with signs of diaphragmatic insufficiency. The neurological examination at the age of 9 years showed generalized muscle weakness which was particularly severe in distal limb and neck flexor muscles [Medical Research Council score (MRC) = 3]; the other muscles tested had MRC = 4. Torticollis and elbow contractures persisted. At the age of 11 years, he is still ambulant and has normal intelligence.

Family B. In family B, the proband is 3 years old at the time of the study. He is the offspring of a first pregnancy and was born at term. Both unrelated parents had a normal neurological examination in their thirties, with normal electromyography and normal CK values. Fetal movements were reported as normal, weight was 3.1 kg at 41 weeks of gestation, length was 54 cm, and occipital–frontal circumference was 36 cm. A younger brother had neonatal hypotonia and delayed motor milestones, and walked at 18 months with frequent falls. At birth, he was also hypotonic, showed convergent strabismus, moderate kyphosis with rigidity of the spine, ogival palate, and severe contractures of elbows and knees. At the age of 8 months, he showed marked distal hyperlaxity of toes, fingers, ankles, and wrists, and contractures of both elbows and knees. His CK was 2- to 3-fold higher. Electromyography showed a myopathic pattern, and motor and sensory nerve conduction velocity was normal. Histology of the right quadriceps muscle biopsy performed at age 10 months showed increased diameter variability, rare isolated necrotic fibers, and mild endomisial fibrosis. During the follow-up, joint contractures slowly improved, and the child was able to sit autonomously at age 1 year and walk at the age of 2 years. Clinical examination at age 2 years confirmed distal hyperlaxity, marked weakness of head flexors, and a kyphotic contracture of the spine with an almost complete disappearance of limb joint contractures.

Family C. In Family C, the parents, in their forties and healthy, are both unrelated. The first daughter is healthy and aged 15 years at the time of the study. The proband was the product of a second normal pregnancy and delivery. Her weight at birth was 2.9 kg, and in the neonatal period she was hypotonic with ogival palate, slight micrognatia, kyphotic contracture of the spine, and marked distal hyperlaxity of hands and feet with no joint contractures. She had delayed motor milestones in the first 2 years of life and walked alone with difficulty at the age of 3 years. Her CK was increased 2- to 3-fold. The girl was wheelchair-bound by the age of 4 years, and severe scoliosis, associated with signs of respiratory failure, appeared 1 year later. At age 6 years, the neurological examination showed normal cognitive abilities, generalized muscle wasting and weakness (MRC = 3 in all distal muscles and neck flexor muscles, and MRC = 4 in biceps, triceps, and quadriceps muscles), and elbow joint contractures. Facial mimicry and swallowing was preserved. She had a tracheostomy at age 7 years with positive pressure nocturnal mechanical ventilation and was treated with a Harrington rod for severe scoliosis at the age of 8 years. Histology of the paraspinal muscle biopsy, performed during surgery for scoliosis, showed a striking diameter variability, rare isolated necrotic fibers, and marked endomisial fibrosis. One year later, the girl died of respiratory failure with impairment of diaphragmatic movements.

Mutation Screening Analysis of COL6A1, COL6A2, and COL6A3 cDNAs. We performed RT-PCR amplifications on RNA extracted from fibroblasts of patients A and B or muscle of patient C (Fig. 2). The overlapping PCR fragments, covering the COL6A1, COL6A2, and COL6A3 cDNAs, underwent heteroduplex analysis (16). Direct sequencing was carried out on all heteroduplexes.

Family A. We first identified a homozygous insertion of a C in a stretch of five Cs between nucleotides 1147 and 1151 of the COL6A2 cDNA in the proband of family A, causing a slippage of the reading frame with the formation of a premature termination codon at nucleotides 1347–1349 (Fig. 3*A*). The analysis of the genomic DNA from the proband confirmed the presence of a homozygous C insertion in exon 13 (27). Genomic DNA of both apparently healthy parents showed that they carry the C insertion. The mutation was not present in 50 unrelated controls. The homozygous mutation is positioned in the area corresponding to the amino-terminal one-third of the triple-helical domain

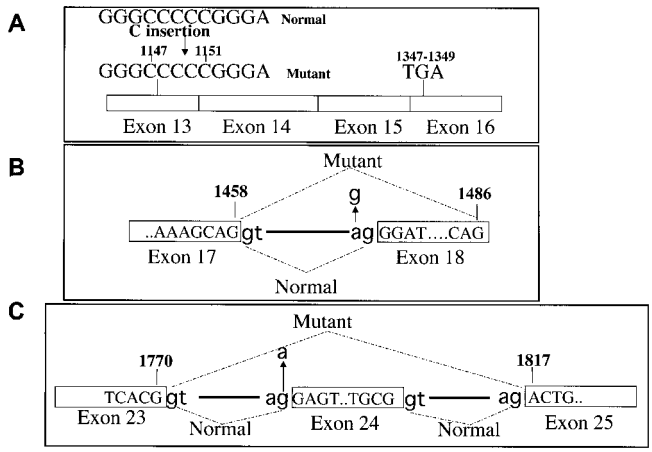

Fig. 3. Diagrammatic representation of mutations in families A, B, and C. (*A*) The proband in family A has a homozygous insertion of a C in a stretch of Cs between nucleotides 1147 and 1151 in exon 13 encoding the triple helix of COL6A2. Both consanguineous parents are heterozygous for this mutation, which causes a frame shift and the formation of a premature termination codon at nucleotides 1347–1349 inside exon 16. (*B*) One of the COL6A2 alleles in both affected sons of family B has an A → G substitution at the conserved splice acceptor site (ag) at position −2 in intron 17, causing the activation of a cryptic acceptor site within exon 18. The aberrant splicing causes the deletion of 28 bp (nucleotides 1459–1486 in exon 18 in the cDNA), the slippage of the reading frame, and the formation of a premature termination codon at nucleotides 1631–1633. Their apparently healthy mother and also the proband in family C are heterozygous for the same mutation. (*C*) The second COL6A2 allele of both affected sons in family B has a G → A substitution at position −1 of intron 23, causing the skipping of the entire exon 24 of 46 bp (from nucleotide 1671 to nucleotide 1716) and the changing of the reading frame thereafter. Their apparently healthy father is heterozygous for this mutation. The nucleotide positions are numbered according to the complete coding sequence of the COL6A2 cDNA (GenBank accession no. AY029208) with nucleotide +1 corresponding to the translation start site.

of the α2(VI) chain, leading to the complete absence of COL6 microfibrils, as shown by immunofluorescence of cultured fibroblasts (Fig. 4*B*).

Family B. In the proband of family B, we detected a heterozygous 28-bp deletion in the COL6A2 cDNA between nucleotides 1459 and 1486. The deletion is caused by a single nucleotide substitution, an A → G, at nucleotide −2 of intron 17 that activates a cryptic acceptor splice site inside exon 18 (Fig. 3*B*). The alternative splicing causes the loss of 9 aa and a frame shift with the formation of a premature termination codon at nucleotides 1631–1633. Also, the younger affected brother and their mother displayed the same mutation in the genomic DNA (data not shown) that abolishes a *Dde*I restriction enzyme site. The mutation was not found in 50 unrelated controls. We also detected another mutation in the second allele of the same gene in the C-terminal coding region; it was a deletion of 46 nucleotides covering the entire exon 24. The deletion in the cDNA is caused by a single nucleotide substitution, a G → A at position −1 of intron 23 that causes the exon skipping (Fig. 3*B*). This mutation is present in the two affected sons and in their father, absent in the mother, and absent in 50 unrelated controls (data not shown).

We also analyzed the expression of COL6 mRNA and protein in fibroblasts from the proband of family B (Fig. 5). Hybridization with cDNA probes for COL6A1, COL6A2, and COL6A3 simultaneously showed an almost total absence of COL6A2 mRNA, although a small amount of COL6A2 mRNA was detected with a prolonged exposure of the autoradiogram (Fig. 5*A*). The low level of COL6A2 mRNA most likely results from a premature termination codon-mediated mRNA degradation in one allele and inefficiency in exon skipping in the other allele. Immunoprecipitation of the fibroblast culture medium with COL6 antibodies showed a marked reduction in the amount of COL6 secreted into the medium (Fig. 5*B*). Presumably, the small amount of COL6 secreted into the medium is composed of the abnormal α2(VI) chains with the in-frame deletion at the C terminus. Immunofluorescence showed the almost total absence of COL6 in cultured fibroblasts (Fig. 4*C*). The muscle biopsy showed the absence of immunolocalization of COL6 (Fig. 6*B*), with normal expression of merosin (Fig. 6*D*)

Family C. The proband of family C showed the same 28-nt deletion in exon 18 of COL6A2 as the first mutation detected in family B and the same A → G substitution at nucleotide −2 of intron 17 in the genomic DNA (data not shown). We did not find the second mutation after direct sequencing of the entire COL6A2 cDNA. We only have RNA and genomic DNA from a muscle biopsy, i.e., no fibroblasts of the proband of family C are available, because the patient died of respiratory failure at the

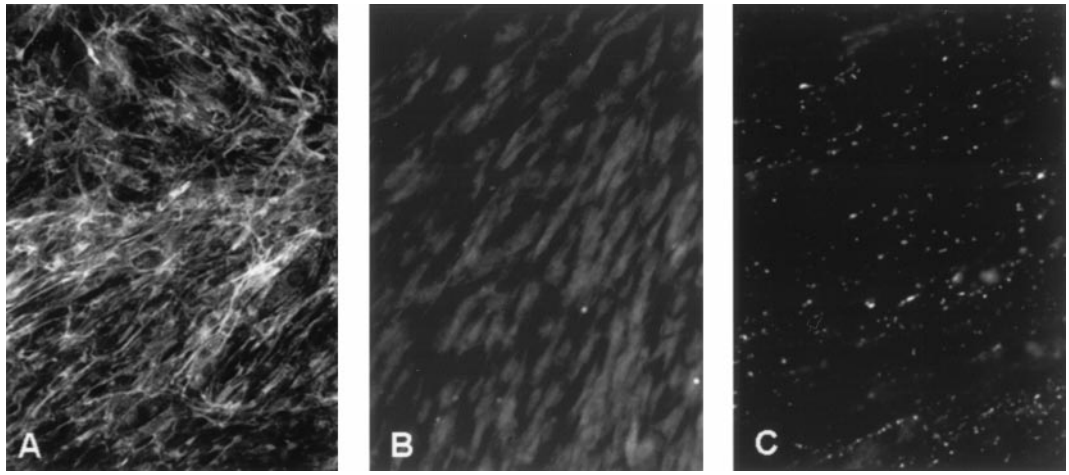

Fig. 4. Immunofluorescence of cultured fibroblasts in proposita of family A and B. (*A*) Immunofluorescence with anti-COL6 (see *Materials and Methods*) of normal fibroblasts. COL6 is secreted in the extracellular matrix and seems organized in a dense three-dimensional network that is entangled with the cells. (*B*) In fibroblasts of propositus of family A, COL6 is not detectable in the extracellular matrix, whereas a moderate labeling is present inside the cytoplasm of the cells. (*C*) In the propositus of family B, COL6 is not detectable inside the cytoplasm of the cells whereas a residual labeling is present in the extracellular matrix as small multiple dots. (Magnification = ×20).

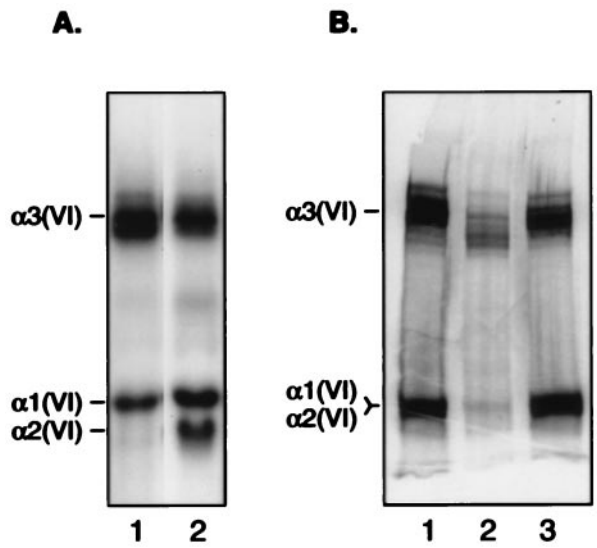

Fig. 5. Analysis of COL6 mRNA and protein in fibroblasts from the proband of family B. (*A*) Northern blot analysis showing little α2(VI) mRNA in the patient's fibroblasts. Fifteen mg of total RNA from fibroblasts of the proband in family B (lane 1) and a control (lane 2) were run on a 1% denaturing agarose gel, transferred to a nylon membrane, and hybridized with ^{32}P-labeled cDNA probes for the α1(VI), α2(VI), and α3(VI) collagen chains. The sizes of the α1(VI), α2(VI), and α3(VI) collagen mRNAs are 4.2, 3.5, and 8–10 kb, respectively. Multiple α3(VI) mRNAs result from alternative splicing of exons encoding the N-globular domain (23). The amount of each splice variant varies in different control fibroblast strains according to the growth state of cells. Therefore, the difference in the intensity of the α3(VI) mRNA splice variants between the control and the patient probably is not a direct consequence of the mutation. (*B*) Immunoprecipitation showing a marked reduction in COL6 secretion. Fibroblasts from a control (lane 1), the proband of family B (lane 2), and a patient with BM (lane 3) were metabolically labeled overnight. The culture media were immunoprecipitated with antibodies specific for the α3(VI) collagen chain (25). The immunoprecipitated samples were reduced with 5% 2-mercaptoethanol and run on a 5% SDS/polyacrylamide gel. The α1(VI) and α2(VI) collagen chains comigrated at 140 kDa, whereas the α3(VI) collagen chains migrated at 260–300 kDa.

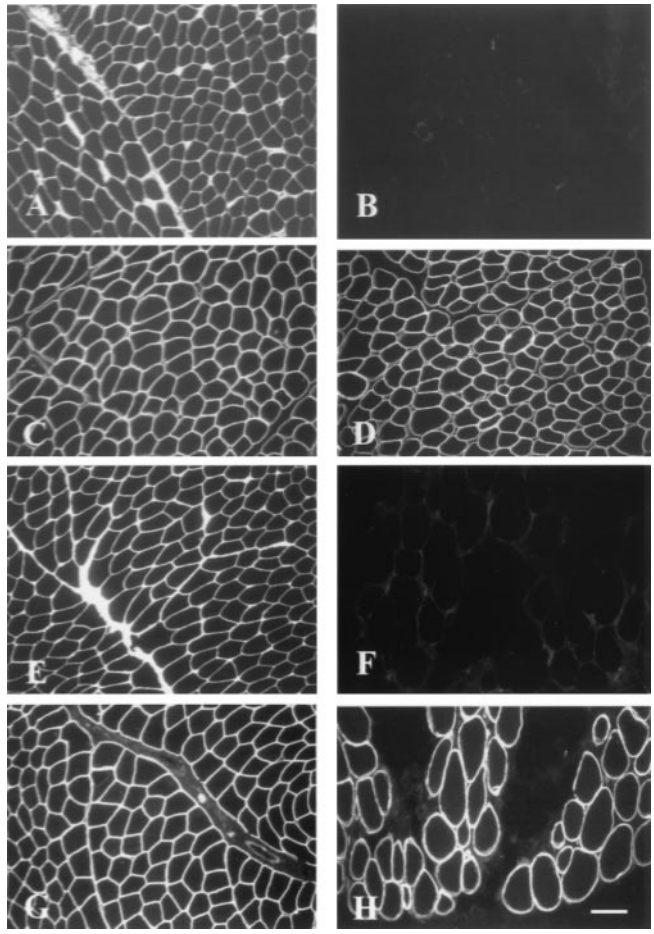

Fig. 6. Immunofluorescence of muscle in the proposita of family B (*A–D*) and C (*E–H*). Immunolocalization of COL6–140 kDa (2C6 antibody; *A*, *B*, *E*, and *F*) and merosin (*C*, *D*, *G*, and *H*) in skeletal muscle from controls (*A*, *C*, *E*, and *G*) and patients (*B*, *D*, *F*, and *H*). The expression of COL6 was almost absent in muscle fibers of the patient of family B (*B*), and drastically reduced in the patient of family C (*F*), whereas merosin-staining seemed to be normal (*D* and *H*). (Bar = 80 μm.) These results show that this recessive disorder can be screened by immunofluorescence using monoclonal antibodies directed against one of the three COL6 subunits.

age of 9 years. As with patient B, the muscle biopsy showed the absence of immunolocalization of COL6 (Fig. 6*F*) with normal expression of merosin (Fig. 6*H*). We obtained some genomic DNA from peripheral blood lymphocytes of the two parents and did not find the A $\rightarrow$ G substitution at nucleotide -2 of intron 17. Thus, the proband has a *de novo* mutation.

Discussion

We report four patients (three males and one female) affected by UCMD showing clinical features of neonatal hypotonia, multiple joint contractures at birth with a kyphotic rigidity of the spine, and marked distal hyperlaxity of hands and feet. The combination of joint contractures and distal hyperlaxity was somewhat evocative of a collagen disorder and reminiscent of milder clinical features already observed in patients with BM (10). In addition, clinical evidence of autosomal recessive transmission had already been reported in UCMD (5). Thus, we decided to investigate the molecular defects, postulating recessive mutations in the COL6 genes.

Altogether, our results indicate that homozygous or compound heterozygous mutations in the COL6A2 gene (leading to a severe impairment of COL6 deposition in the skeletal muscle) cause UCMD. It is known that heterozygous mutations of the COL6 genes cause BM, and these include missense mutations in all three genes as well as mutations in COL6A1 that cause haploinsufficiency (8, 15–19). It is, therefore, somewhat surprising that both parents in family A and the mother of family B, all of whom carry heterozygous COL6A2 mutations leading to a premature termination codon, are clinically healthy. It is noteworthy that all of the three unrelated patients with Ullrich recessive myopathy carry mutations in the COL6A2 gene, whereas the haploinsufficiency of COL6 in BM patients reported thus far is caused by mutations in the COL6A1 gene (17–19). A possible explanation could be that the COL6A1 and COL6A2 genes are regulated differently. For instance, it is possible that up-regulation of the normal COL6A2 allele could compensate for the lack of expression of the abnormal COL6A2 allele, whereas such a compensatory mechanism may not be operative for the COL6A1 gene. In this context, we have shown that two different promoters control the transcription of the COL6A2 gene (28), whereas only a single promoter has been found for the COL6A1 gene (29). Alternatively, the recessive inheritance in UCMD may be caused by the localization of the mutation, to the kind of the mutation, and to the particular COL6 gene involved. It is notable that, as with BM (8), we found the same mutation in two unrelated UCMD patients. According to the results obtained from family B, the proband of family C should also carry another still-undetected mutation, probably in COL6A2.

Because we did not find any pathogenetic or nonpathogenetic mutation by direct sequencing of the entire COL6A2 mRNA, it is possible that the other mutation could affect mRNA degradation or gene transcription, thereby leading to a complete absence of COL6A2 mRNA. However, we cannot exclude a genetic compound caused by mutations in the other two COL6 genes, COL6A1 and COL6A3.

In the follow-up, patients with UCMD characteristically present respiratory failure with diaphragmatic paralysis; it is noteworthy that the diaphragm was particularly involved in the knockout animal model for COL6A1 (30). Moreover, it was also demonstrated to be involved in BM patients (11). It should be noted that the total absence of COL6A1 in mice leads to a mild myopathy phenotype unlike the severe phenotype seen in Ullrich patients with the absence of COL6A2.

Further investigation of the molecular defects in other UCMD families and in other BM families will provide clues to the genotype–phenotype correlation. Additional experiments will also be required to analyze the effects of the mutations on the protein structure and function. Our finding of an association between UCMD and recessive mutations in the COL6A2 gene opens up an important issue on the genetic characterization of other CMD phenotypes with positive merosin, joint contractures, and distal hyperlaxity. Functional studies of the consequences of gene mutations will help in better understanding COL6 function and regulation.

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