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# Effects on Collagen VI mRNA Stability and Microfibrillar Assembly of Three *COL6A2* Mutations in Two Families with Ullrich Congenital Muscular Dystrophy*

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**We recently reported a severe deficiency in collagen type VI, resulting from recessive mutations of the *COL6A2* gene, in patients with Ullrich congenital muscular dystrophy. Their parents, who are all carriers of one mutant allele, are unaffected, although heterozygous mutations in collagen VI caused Bethlem myopathy. Here we investigated the consequences of three *COL6A2* mutations in fibroblasts from patients and their parents in two Ullrich families. All three mutations lead to nonsense-mediated mRNA decay. However, very low levels of undegraded mutant mRNA remained in patient B with compound heterozygous mutations at the distal part of the triple-helical domain, resulting in deposition of abnormal microfibrils that cannot form extensive networks. This observation suggests that the C-terminal globular domain is not essential for triple-helix formation but is critical for microfibrillar assembly. In all parents, the *COL6A2* mRNA levels are reduced to 57–73% of the control, but long term collagen VI matrix depositions are comparable with that of the control. The almost complete absence of abnormal protein and near-normal accumulation of microfibrils in the parents may account for their lack of myopathic symptoms.**

Congenital atonic-sclerotic muscular dystrophy (MIM 254090), first described by Ullrich in 1930 (1) and later referred to as Ullrich syndrome, delineates a distinct subtype of congenital muscular dystrophy, characterized by muscle weakness and hypotonia starting at birth or in early infancy (2). The salient clinical features of Ullrich congenital muscular dystrophy (UCMD)[1] are multiple joint contractures associated with distal hyperextensibility. Patients frequently display additional skeletal deformities, including scoliosis and kyphosis, and suffer from respiratory difficulties early in life. The mode of inheritance is consistent with an autosomal recessive pattern (3). We and others (4–6) have recently shown that a deficiency in collagen type VI, resulting from homozygous or compound heterozygous mutations in *COL6A2*, which encodes one of the three collagen VI chains, causes UCMD. Subsequently, three homozygous mutations in another collagen VI gene, *COL6A3*, have been shown to cause severe and mild forms of UCMD with complete or partial deficiency in collagen VI (7).

Several distinctive clinical features of UCMD are also observed in Bethlem myopathy, a mild, dominantly inherited congenital disorder characterized by muscle wasting and multiple joint contractures (8). Missense and splicing mutations in all three collagen VI genes have been reported in patients with Bethlem myopathy (9–12). In addition, functional haploinsufficiency of the *COL6A1* gene has been demonstrated in three Bethlem myopathy patients (13–15). All the known mutations show a high penetrance. Recently, in three families the dominant limb-girdle muscular dystrophy has also been shown to be caused by missense mutations in the *COL6A1* and *COL6A2* genes (16). Thus, abnormalities in collagen VI can lead to a wide spectrum of clinical phenotypes.

Collagen VI is a ubiquitous extracellular matrix protein comprising three constituent chains encoded by the *COL6A1* and *COL6A2* genes on chromosome 21q22.3 and the *COL6A3* gene on chromosome 2q37 (17). The α1(VI) and α2(VI) collagen chains are similar in size (140 kDa), each of which contains a triple-helical domain flanked by N- and C-terminal globular domains, composed of two von Willebrand factor type A (vWF-A) motifs of ~200 amino acids (reviewed in Ref. 18). In contrast, the α3(VI) collagen chain is much larger (250–300 kDa) with extended N- and C-terminal globular domains. The three constituent chains, however, have similarly sized central triple-helical domains of 335–336 amino acids, which fold together into a triple-helical collagen VI monomer. Prior to secretion, the monomers associate in an antiparallel fashion into dimers, which assemble laterally into tetramers (18). In the extracellular space, the tetramers associate end-to-end forming microfibrils with a periodicity of two beads every 105 nm.

The first four mutations identified in UCMD are all *COL6A2* mutations, which result in frameshifts and subsequent premature stop codons (PTC) at different positions along the mRNA (5, 6). Little is known about the molecular mechanisms underlying the gene mutations. We have shown previously (5) that the *COL6A2* mRNA is almost absent in one of the patients. Given that haploinsufficiency in the *COL6A1* gene causes

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[1] The abbreviations used are: UCMD, Ullrich congenital muscular dystrophy; PTC, premature termination codon; vWF-A, von Willebrand factor A domain; RT, reverse transcriptase; DTT, dithiothreitol.

Bethlem myopathy, it is unclear why the parents of the UCMD patients, who carry heterozygous mutations, are unaffected. In this study, we have performed molecular, biochemical, and ultrastructural analyses of collagen VI mRNA and protein with fibroblasts from the patients and their parents in the two Italian families that we reported previously (5). The patient in family A has a homozygous mutation in the proximal part of the triple-helical domain, whereas the patient in family B has compound heterozygous mutations in the distal part. A complete absence of *COL6A2* mRNA and protein is observed in patient A, whereas very low levels of mutant *COL6A2* mRNA and protein remain in patient B. The resultant mutant $\alpha$2(VI) collagen chains, which lack the normal C-terminal globular domain, can form triple-helical monomers and assemble into abnormal microfibrils, albeit inefficiently. Fibroblasts from all of the parents contain reduced amounts of the *COL6A2* mRNA. However, the amounts of collagen VI protein deposited by the long term fibroblast cultures from the parents are not apparently reduced compared with the normal control.

## EXPERIMENTAL PROCEDURES

*Patients and Their Families*—The clinical data of the patients and their parents in family A and B have been described previously (5). Skin biopsies were collected from the patients and their parents after obtaining appropriate informed consent. Primary fibroblasts were established from the biopsies as described (11). Dermal fibroblasts from normal adult individuals and two patients with Bethlem myopathy were used in this study. One of the Bethlem myopathy patients carries a heterozygous in-frame deletion in the *COL6A1* mRNA (12). The molecular defect of the other Bethlem myopathy patient has not been defined.

*Cell Culture*—Fibroblasts were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum at 37 °C and 5% $CO_2$. For protein analysis, confluent cells were incubated overnight in the same medium with 50 $\mu$g/ml L-ascorbic acid phosphate (Wako, Richmond, VA).

*Northern Analysis and RT-PCR*—Northern blot analysis of collagen VI mRNA has been described previously (5). Total RNA was isolated from confluent fibroblasts with an RNeasy kit (Qiagen). The probes for Northern blots were a mixture of [$^{32}$P]dCTP-labeled cDNA clones (F157, F225, and FO19) encoding the three collagen VI chains (19, 20). Hybridization signals were detected with a PhosphorImager and quantified with the ImageQuant software (Amersham Biosciences).

Total RNA (1 $\mu$g) was reverse-transcribed with Superscript II and oligo(dT) primer at 42 °C for 1 h (Invitrogen). Equal amounts of the cDNA samples were PCR-amplified with AmpliTaq Gold polymerase (Roche Molecular Biochemicals) using primers flanking the deletion in allele 2 of patient B (forward primer, 5′-AGAAAGGAGAGCCTGCGGAT; reverse primer, 5′-CGCCCGCAAGTTGAGGTCAT). PCR conditions were 94 °C for 2 min and 20 cycles at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 10 min.

*Immunoprecipitation and Immunofluorescence Analyses*—Immunoprecipitation and immunofluorescence of fibroblasts with collagen VI antibodies have been described previously (5). Metabolic labeling of cells was performed with [$^{35}$S]cysteine (ICN) in the presence of 50 $\mu$g/ml ascorbate phosphate. Immunoprecipitation was carried out with 1014 polyclonal antibody specific for the $\alpha$3(VI) collagen chain (21). Immunoprecipitates were analyzed on SDS-polyacrylamide gels under reduced conditions (25 mM DTT) or on composite 0.5% agarose, 2.4% polyacrylamide gels without reduction. Reduced (230 and 400 kDa) and unreduced (900 kDa) forms of laminin (Roche Molecular Biochemicals) served as the molecular weight standard on the composite gel (13).

For immunofluorescence analysis, fibroblasts were grown to confluence on coverslips, treated with 0.25 mM ascorbic acid for 5 and 15 days, and then incubated with 3C4 anti-collagen VI monoclonal antibody (Chemicon, monoclonal antibody 1944).

*Immunoelectron Microscopy*—Fibroblast cultures were incubated with the 3C4 monoclonal antibody (22) diluted 1:50 in culture medium at 37 °C overnight. After several washes with culture medium, slides were incubated with an anti-mouse IgG 10-nm gold-conjugated antibody (Amersham Biosciences) diluted 1:50 in the culture medium for 1 h at 37 °C. Negative controls were performed in the absence of primary antibody. The samples were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 1 h at 4 °C and with 1% $OsO_4$ in veronal buffer, and then they were dehydrated in ethanol and critical point dried. Thereafter, the slides were rotary-shadowed with platinum at 45° and coated with carbon at 90° in a Balzers BAF 400D Freeze Fracture apparatus, and finally floated on 10% hydrofluoric acid to detach the replicas. After digestion with commercial bleach, the replicas were washed with distilled water, collected on copper grids, and examined in a Philips EM 400 electron microscope at 100 kV.

## RESULTS

*Two of the Three COL6A2 Mutations Shift the Collagen Reading Frame, and the Third Mutation Causes a Frameshift Immediately after the Triple-helical Domain*—As reported previously (5), the affected son in family A has a homozygous C insertion in exon 13, causing a frameshift at codon 386 (130 amino acids from the beginning of the triple-helical domain) and a subsequent PTC 64 amino acids downstream (Fig. 1). Both parents are heterozygous for this mutation. In family B, two affected sons have compound heterozygous mutations. The first is a point mutation at the splice acceptor site in intron 17, leading to the activation of a cryptic splice site in exon 18 and a resultant 28-bp deletion (allele 1). The mutation causes a frameshift at codon 487 (231 amino acids from the beginning of the triple-helical domain) and a subsequent PTC at 48 amino acids downstream. This mutation is inherited from their mother. The second mutation (allele 2), also present in their father, leads to skipping of the entire 46-bp exon 24, which encodes a short, cysteine-rich region connecting the triple-helical domain and the C-terminal globular domain. The predicted mutant protein contains a complete triple-helical domain of 335 amino acids, but a translational frameshift occurs immediately thereafter, resulting in a PTC 148 amino acids downstream (Fig. 2). Because the mutant protein from allele 2 has a complete triple-helical domain, it can theoretically form a triple helix with the other two chains, but the mutant C-terminal globular domain is much shorter than its normal counterpart of 449 amino acids, which comprises two vWF-A motifs, C1 and C2 (Figs. 1 and 2). The mutant C terminal globular domain is highly basic (pI = 12.4) compared with the normal counterpart (pI = 5.03) and shares no homology with other known protein motifs in the data base.

*All Three COL6A2 Mutations Cause Nonsense-mediated mRNA Decay in Patients and Their Unaffected Parents*—The effects of these three *COL6A2* gene mutations on the steady state mRNA levels were examined by Northern blot analysis of fibroblasts from the patients and their parents. A representative Northern blot hybridization is shown in Fig. 3*A*. Fibroblasts from the patients in both families expressed little *COL6A2* mRNA, whereas the levels of the *COL6A1* mRNA were similar to those of the normal control (Fig. 3*A, lanes 2* and *5*). The hybridization signals for the *COL6A3* mRNA were rather low in all samples, because the *COL6A3* mRNA is large (9–10 kb) and thus was not efficiently transferred to the nylon membrane. Also, there were some differences in the relative abundance of the alternative spliced *COL6A3* transcripts among samples. This is probably not related to the *COL6A2* mutations, as we have shown previously (23) that the alternative splicing pattern of the *COL6A3* gene varies in tissues and cells. The *COL6A2* gene also transcribes an alternative 6.0-kb mRNA, which retains an intron between two alternatively spliced exons at the 3′-end of the gene (24). The Northern results showed that fibroblasts from both patients contained little 6.0-kb alternative *COL6A2* mRNA, consistent with the absence of the *COL6A2* mRNA. After a prolonged exposure with the PhosphorImager, a low level of the *COL6A2* mRNA could be detected in patient B but not in patient A. Collectively, the data suggested that all three mutations caused nonsense-mediated mRNA decay, and the mutation in patient A resulted in almost complete degradation of the mRNA.

The parents of both families who carried a mutant allele also expressed reduced amounts of the *COL6A2* mRNA, as indi-

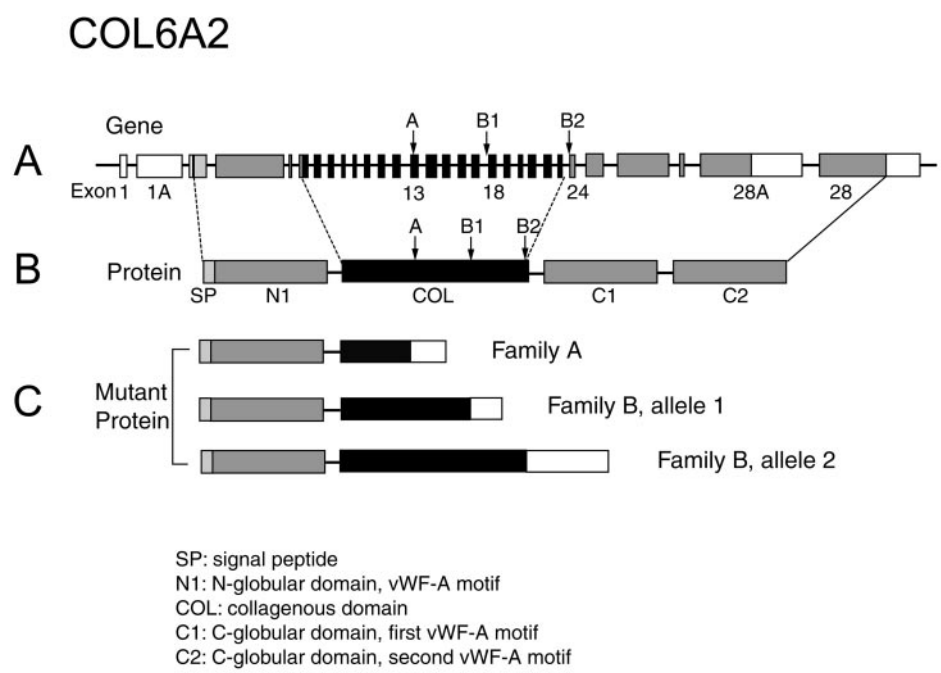


FIG. 1. **Schematic diagram of the *COL6A2* gene structure and the normal and mutant α2(VI) collagen chains.** *A*, the *COL6A2* gene consists of 28 exons plus two alternative exons (*1A* and *28A*) at each end. *Open boxes*, exons encoding untranslated regions; *light gray box*, exon encoding the signal peptide (*SP*); *gray boxes*, exons encoding the N- and C-globular domains; *black boxes*, exons encoding the triple-helical domain. *B*, the α2(VI) collagen chain consists of the signal peptide (*SP*), N-terminal globular domain (*N1*), triple-helical domain (*COL*), and C terminal globular domain (*C1* and *C2*). N1, C1, and C2 are vWF-A motifs, each of which is ~200 amino acids long. The triple-helical domain is 335 amino acids long. Short cysteine-rich connecting peptides (*thin lines*) separate N1, triple-helix, C1, and C2. Locations of the homozygous mutation in family A and heterozygous mutations (*B1* and *B2*) in family B are marked by *arrows. C*, mutant α2(VI) collagen chains are truncated at different positions, which are followed by a missense polypeptide (*open boxes*). Note that the mutant chain encoded by allele 2 in family B contains a complete triple-helical domain but lacks the connecting peptide and thereafter.

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        581                 601                 621
Normal  PGPPGDPGLT ECDVMTYVRE TCGCCDCEKR CGALDVVFVI DSSESIGYTN FTLEKNFVIN
Mutant  PGPPGDPGLT ---------- -----TVRSA VAPWTWSSSS TAPRALGTPT SHWRRTSSST

        641                 661                 681
Normal  VVNRLGAIAK DPKSETGTRV GVVQYSHEGT FEAIQLDDEH IDSLSSFKEA VKNLEWIAGG
Mutant  WSTGWVPSLR TPSPRQGRVW AWCSTATRAP LRPSSWTTNI STPCRASRRL SRTSSGLRAA

        701                 721                 741
Normal  TWTPSALKFA YDRLIKESRR QKTRVFAVVI TDGRHDPRDD DLNLRALCDR DVTVTAIGIG
Mutant  PGHPQPSSLP TTASSRRAGA RRHVCLRWSS RTGATTLGTM TSTCGRCAIA TSQ*

        761                 781                 801
Normal  DMFHEKHESE NLYSIACDKP QQVRNMTLFS DLVAEKFIDD MEDVLCPDPQ IVCPDLPCQT

        821                 841                 861
Normal  ELSVAQCTQR PVDIVFLLDG SERLGEQNFH KARRFVEQVA RRLTLARRDD DPLNARVALL

        881                 901                 921
Normal  QFGGPGEQQV AFPLSHNLTA IHEALETTQY LNSFSHVGAG VVHAINAIVR SPRGGARRHA

        941                 961                 981
Normal  ELSFVFLTDG VTGNDSLHES AHSMRNENVV PTVLALGSDV DMDVLTTLSL GDRAAVFHEK

        1001
Normal  DYDSLAQPGF FDRFIRWIC*
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FIG. 2. **Amino acid sequences of the C-terminal globular domains from the normal (*upper lines*) and mutant (*lower lines*, allele 2 of family B) α2(VI) collagen chains.** Sequence alignment begins at the last 10 amino acids in the triple-helical domain. Note that the mutant C-globular domain (148 amino acids) lacks the cysteine-rich connecting peptide immediately after the triple-helical domain, and the sequence shares no homology with the normal C-globular domain.

cated by quantification of the ratios of the *COL6A2* and *COL6A1* mRNA levels (Fig. 3*B*). For each fibroblast strains, at least two Northern blots were performed and quantified. When the *COL6A2*/*COL6A1* mRNA ratio of fibroblasts from the normal individual was normalized to 1.0, the *COL6A2*/*COL6A1* mRNA ratios for the father and mother of family A and the father and mother of family B were 0.62, 0.73, 0.65, and 0.57, respectively. The analyses also showed that the *COL6A2*/*COL6A1* mRNA ratios of fibroblasts from two Bethlem myopathy patients (0.97 and 1.07) were similar to the control.

Semi-quantitative RT-PCR analysis was performed to examine the levels of two different mutant *COL6A2* mRNAs in family B. With PCR primers flanking the distal mRNA deletion in allele 2, a 581-bp product would be amplified from the normal allele and the mutant allele 1, whereas a shorter 535-bp product would be amplified from the mutant allele 2. The experiment showed that the amount of mutant mRNA from allele 2 was less than that from allele 1 in patient B and that a very small amount of mutant mRNA from allele 2 compared with the normal mRNA was present in the father (Fig. 3*C*). In addition, the results agreed with the Northern analysis in that the amounts of normal *COL6A2* mRNA in both parents were less than that in the normal individual. Similarly, primers flanking the deletion of allele 1 detected a minute amount of mutant transcript in the mother in addition to the normal *COL6A2* mRNA (data not shown).

*Secreted Collagen VI Protein Is Absent in Patient A, Markedly Decreased in Patient B, and Reduced in the Parents*—The synthesis and secretion of collagen VI protein in fibroblasts of the patients were examined by immunoprecipitation after metabolic labeling. The antibody used was specific for the N-terminal globular domain of the α3(VI) collagen chain and therefore would immunoprecipitate α3(VI) chains and also co-immunoprecipitate α1(VI) and α2(VI) chains (migrating at the same position), which are presumably all folded together as triple-helical monomers. A complete absence of α1(VI) and α2(VI) chains was found in the culture medium from fibroblasts of patient A, whereas very low levels of α1(VI) + α2(VI) chains were observed in the medium of fibroblasts from patient B, suggesting that patient B secreted a small amount of mutant collagen VI protein (Fig. 4*A*). There were some α3(VI) chains in the media of both patients, indicating that free α3(VI) chains not associated with α1(VI) and α2(VI) chains could be secreted. Immunoprecipitation was also performed using media of fibroblasts from the parents (Fig. 4*B*). The amounts of α1(VI) and

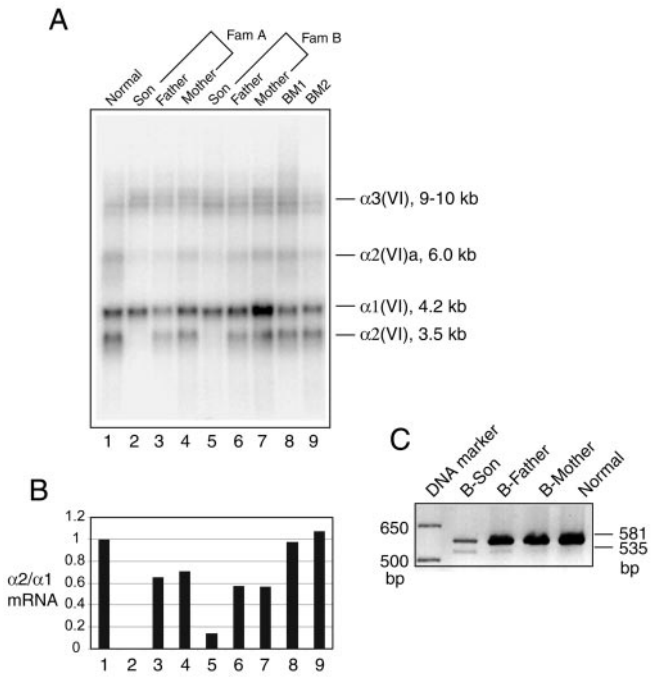


FIG. 3. *A*, Northern blot analysis of the patients and parents in families A and B. Total RNA (3 $\mu$g) was separated on a 0.8% denaturing agarose gel, transferred to a nylon membrane, and hybridized with a mixture of $^{32}$P-labeled cDNA probes for the $\alpha$1(VI), $\alpha$2(VI), and $\alpha$3(VI) collagen chains. Samples are from a normal individual (*lane 1*); the patient, father, and mother from family A (*lanes 2–4*); the patient, father, and mother from family B (*lanes 5–7*); and two patients with Bethlem myopathy (*lanes 8* and *9*). The sizes of the three collagen VI mRNAs and an alternative transcript from the *COL6A2* gene are indicated. *B*, quantification of the Northern blot detected by a PhosphorImager with the ImageQuant software (Amersham Biosciences). The ratio of the $\alpha$2(VI)/$\alpha$1(VI) mRNA for the normal individual was normalized to 1.0. *C*, semiquantitative RT-PCR analysis of the normal and mutant *COL6A2* mRNA in family B. PCR primers were designed to detect mutant mRNA from allele 2 of family B (exon 24 skipping as depicted in Fig. 1). The 535-bp product results from allele 2, whereas the 581-bp product results from allele 1 as well as the normal allele. Patient B and his father contain a low level of the mutant *COL6A2* mRNA from allele 2.

$\alpha$2(VI) chains in the media of all parents appeared to be reduced compared with the control (ranging from 58 to 70% of the control by quantification of two separate experiments).

The amounts of collagen VI protein present within the cells of patients A and B were not drastically reduced compared with their fathers and the normal control (Fig. 5*A*), unlike the results of collagen VI protein secreted into the medium (Fig. 4*A*). However, a doublet was seen at the position of the $\alpha$1(VI)/$\alpha$2(VI) chains in the control and the fathers of both patients, whereas only the lower band of the doublet was observed in both patients. The upper band of the doublet most likely was the $\alpha$2(VI) chain as both patients had little *COL6A2* mRNA. Because the immunoprecipitations were performed with the antibody against the $\alpha$3(VI) chain, the results suggested that the $\alpha$1(VI) chain was able to associate with the $\alpha$3(VI) chain even in the absence the $\alpha$2(VI) chain. However, the associated $\alpha$1(VI) and $\alpha$3(VI) chains were not secreted as evident from the lack of extracellular $\alpha$1(VI) and $\alpha$2(VI) chains in patient A (Fig. 4*A*, *lane 2*).

It is known that secretion of collagen VI occurs after intracellular assembly of the constituent chains into triple-helical monomers, dimers, and tetramers (18). As some $\alpha$1(VI)/$\alpha$2(VI) collagen chains were found in the medium of patient B but not of patient A (Fig. 4*A*), the truncated $\alpha$2(VI) chains of patient B most likely could fold with the other two chains into triple-helical monomers and then assemble into oligomers. This was indeed the case when the intracellular assembly of collagen VI oligomers in patient B was examined by analyzing immunoprecipitated collagen VI within cells under non-reduced conditions on a composite agarose-polyacrylamide gel (Fig. 5*B*). Most of the collagen VI chains in patient B were accumulated as triple-helical monomers, but only low levels of dimers and tetramers were observed. In contrast, almost all of the collagen VI chains were assembled into dimers and tetramers in the normal control. The results suggested that the mutant $\alpha$2(VI) chains in patient B were capable of forming monomers; however, further assembly into dimers and tetramers was severely impaired.

*Long Term Collagen VI Matrix Deposition in the Parents Is Comparable with That in the Normal Control*—Deposition of collagen VI microfibrils in the extracellular matrix was examined by immunofluorescence labeling of fibroblasts with anti-collagen VI antibody. When cultured for 5 days, fibroblasts from the parents of both families revealed a reduction in the amount of microfibrils compared with normal fibroblasts (data not shown), consistent with the biosynthetic results (Fig. 5). However, at day 15 of culture the labeling pattern of the fibroblasts from the parents acquired a normal appearance compared with the control fibroblasts (Fig. 6). Fibroblasts from patient A showed only intracellular staining with a total absence of assembled collagen VI microfibrils in the extracellular matrix, whereas fibroblast cultures from patient B showed some fluorescent spots extracellularly, but there was no extensive microfibrillar network.

*Patient B Deposits Abnormal-shaped Collagen VI Microfibrils in the Extracellular Matrix, Whereas Microfibrils Deposited by the Parents Appear Normal*—The nature of collagen VI deposited in the extracellular matrix was examined by immunoelectron microscopy. Analysis of cultured fibroblasts from the parents of both families (rotary-shadowed replicas) at day 15 revealed that the architecture of the collagen VI filaments appeared unaltered (Fig. 7), in accordance with the immunofluorescence data. In addition, the formation of fibrils and webs appeared normal. Analysis of cultured fibroblasts from patient B revealed altered, coarser microfilaments, with an irregular presence of gold labelings, which correspond to the globular domains (Fig. 7). The microfilaments showed a tendency to form circular arrays, perhaps corresponding to the fluorescent spots seen by fluorescence microscopy, whereas parallel-running fibrils, often present in normal cultures, were absent in the patient. Cultured fibroblasts from patient A showed complete absence of collagen VI assemblies in the extracellular matrix (data not shown).

## DISCUSSION

In this study, we have delineated the consequences of three different *COL6A2* mutations that we have previously identified in two Italian UCMD families in molecular, biochemical, and ultrastructural detail. Our studies show that all three *COL6A2* mutations lead to nonsense-mediated mRNA decay, an RNA surveillance mechanism that detects and degrades mRNAs with PTCs, which encode nonfunctional or deleterious proteins (25–27). The PTC in family A is located 5′ of the two PTCs in family B in the *COL6A2* transcript. It is known that nonsense codons located early in the transcript exert a greater effect on mRNA decay (28, 29). Our Northern blot results are consistent with this contention and show almost complete absence of the *COL6A2* mRNA in patient A, in contrast to a low level of the *COL6A2* mRNA in patient B (Fig. 3). However, this is not the case for the two different *COL6A2* mutations in patient B, as semi-quantitative PCR analysis of the *COL6A2* mRNA from the two mutant alleles suggests that there is more *COL6A2* mRNA from allele 1 than allele 2, even though the PTC in allele

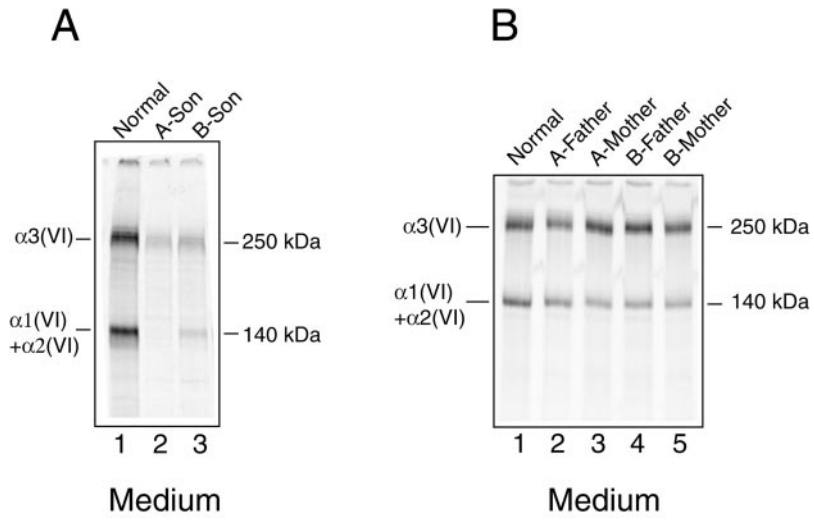


FIG. 4. **Immunoprecipitation of collagen VI in the culture media of fibroblasts from patients and their parents.** Fibroblasts were labeled with [$^{35}$S]cysteine overnight. *A*, culture media from a normal individual (*lane 1*), patient A (*lane 2*), and patient B (*lane 3*) were immunoprecipitated with an antibody specific for the α3(VI) collagen chain. Samples were run on a 6% SDS-polyacrylamide gel and detected by a PhosphorImager. The α1(VI) and α2(VI) collagen chains co-migrate at 140 kDa, whereas several α3(VI) collagen chain variants resulting from alternative splicing migrate around 250 kDa. Note the absence of α1(VI) + α2(VI) chains in patient A, and very low levels of α1(VI) + α2(VI) chains in patient B as compared with the control. *B*, culture media from the parents of families A and B, immunoprecipitated as above. *Lane 1*, normal individual; *lanes 2* and *3*, father and mother of family A; *lanes 4* and *5*, father and mother of family B.

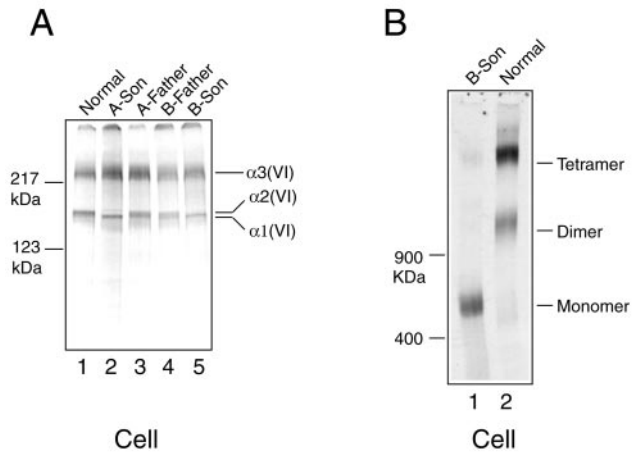


FIG. 5. **Immunoprecipitation of collagen VI in the cell layers of fibroblasts from patients and their fathers.** Fibroblasts were labeled with [$^{35}$S]cysteine overnight. *A*, cell layers from a normal individual (*lane 1*), patient A and his father (*lanes 2* and *3*), and the father and patient of family B (*lanes 4* and *5*) were immunoprecipitated with an antibody specific for the α3(VI) collagen chain. Samples were reduced with 25 mM DTT, run on a 6% SDS-polyacrylamide gel, and detected by a PhosphorImager. Note the presence of two closely migrating bands at the position of the α1(VI) + α2(VI) chains in the normal individual and both fathers, and only a single band at this position in both patients. Because the patients are deficient in the *COL6A2* mRNA, the upper band of the doublet should correspond to the α2(VI) collagen chain. *B*, analysis of collagen dimer and tetramer assembly in patient B. Cell layers from a normal individual (*lane 2*) and patient B (*lane 1*) were immunoprecipitated as above and analyzed without reduction with 25 mM DTT on a composite 0.5% agarose and 3% polyacrylamide gel. The positions of the unreduced (900 kDa) and reduced (400 kDa) forms of laminin are shown on the *left*. Collagen VI tetramer, dimer, and monomer are indicated on the *right*.

1 is located more 5′ than it is in allele 2 (Fig. 3*C*).

Transcripts from the mutant alleles in the parents are also subject to nonsense-mediated mRNA decay as all four parents have lower *COL6A2*/*COL6A1* mRNA ratios compared with the normal control and patients with Bethlem myopathy (Fig. 3*B*). This conclusion is also supported by PCR analysis of the parents in family B, showing a very low level of the mutant mRNA compared with transcripts from the normal allele (Fig. 3*C*). The finding that the *COL6A2*/*COL6A1* mRNA ratios in the parents of both families are greater than 50% suggests that transcription from the normal *COL6A2* allele may be up-regulated. Approximately 50% reduction in the *COL6A1* mRNA was reported for the Bethlem myopathy patient, who carries a frameshift mutation in the *COL6A1* gene resulting in haploinsufficiency (13). The *COL6A2* gene contains two promoters, whereas only one promoter has been found in the *COL6A1* and *COL6A3* genes (30, 31). Whether the alternative *COL6A2* promoter plays a role in up-regulating transcription from the normal allele remains to be determined. An increase in mRNA stability is another possibility. It should be noted that the parents of UCMD patients with a homozygous nonsense mutation in the triple-helical domain of the *COL6A3* gene also do not exhibit clinical symptoms. The *COL6A3* mRNA levels in this family have not been reported (7).

The reduction of the *COL6A2* mRNA in the parents is reflected at the protein level, because the amounts of extracellular α1(VI)/α2(VI) collagen chains in the parents appear somewhat reduced (Fig. 4*B*), as is the case for the short term matrix deposition observed by indirect immunofluorescence of 5-day fibroblast cultures (data not shown). However, the long term accumulation of collagen VI microfibrils in 15-day cultures does not seem significantly different between fibroblasts from the parents and the control (Fig. 6). Although the data from *in vitro* cell culture studies may not necessarily reflect the *in vivo* situation, it is possible that amounts of collagen VI microfibrils accumulated in tissue over time may reach a level similar to normal individuals. Moreover, our results indicated that the mutant transcripts in the parents are mostly degraded. Thus even if a minute amount of truncated protein was produced, it would not be sufficient to exert a dominant negative effect. That collagen VI microfibrils deposited by the parents are structurally normal is also supported by immunoelectron microscopic examination (Fig. 7). We suggest that the apparently normal matrix deposition together with the lack of a significant amount of the mutant protein may explain the absence of clinical symptoms in the parents. It is of interest that a similar

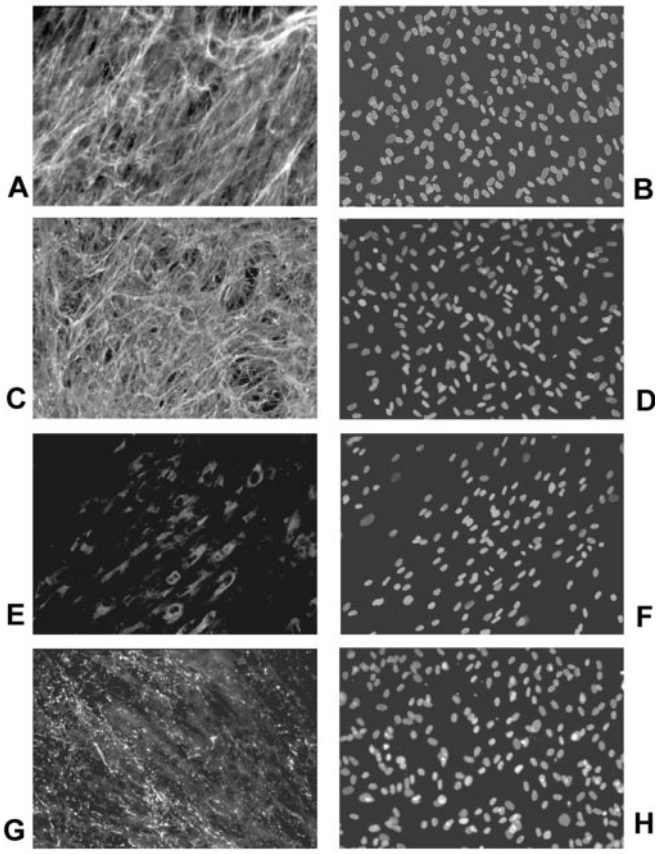


FIG. 6. **Immunofluorescence labeling of fibroblasts with anti-collagen VI antibody at day 15 of culture (*A*, *C*, *E*, and *G*) and 4,6-diamidino-2-phenylindole staining of nuclei, showing analogous cell density (*B*, *D*, *F*, and *H*).** A normal appearance of the collagen VI network was detectable in all four parents from both families (*C* and *D*, representative pictures of the father in family B), and it was comparable with normal fibroblasts at the same day of culture and at the same cell density (*A* and *B*). In fibroblasts from patient A (*E* and *F*), the reaction showed an absence of collagen VI in the extracellular matrix, whereas some fluorescence was retained inside the cytoplasm. In fibroblasts from patient B, only rare fluorescent spots were detectable between the cells, and neither fibrils nor collagen VI networks were present (*G* and *H*). Original magnification, ×40.

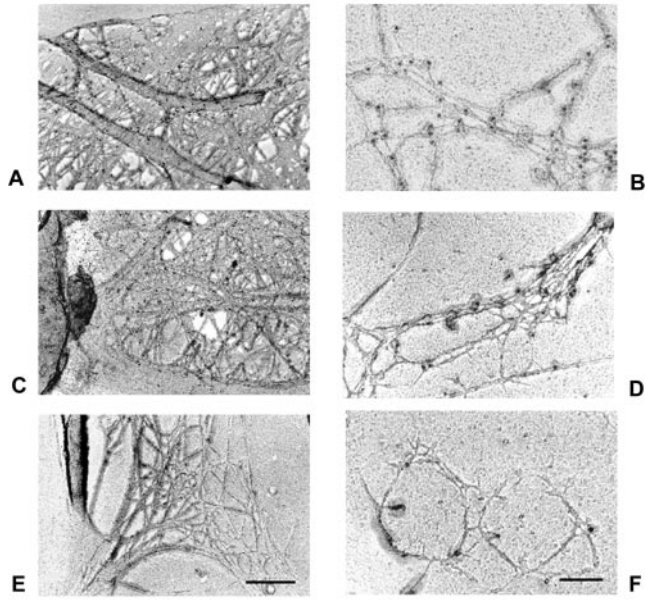


FIG. 7. **Electron microscopy analysis (rotary-shadowed replicas) of cultured fibroblasts immunolabeled with 10 nm colloidal gold-conjugated anti-collagen VI antibody at day 15 of culture.** *A*, *C*, and *E*, lower magnification; *B*, *D*, and *F*, higher magnification. In fibroblast cultures from all parents (*C* and *D*, representative pictures of the father in family B), the architecture of the collagen VI microfibrils appears not to be altered in comparison with the control (*A* and *B*). In cultured fibroblasts from patient B, gold-labeled collagen VI networks in the extracellular matrix were not seen (*E*). However, rare spots with altered collagen VI assemblies were detected, which showed an irregular presence of globular domains (*F*). *Bars*: *A*, *C*, *E* = 5 $\mu$m; *B*, *D*, *F* = 100 nm.

heterozygous mutation in the *COL6A1* gene, leading to nonsense-mediated *COL6A1* mRNA decay, causes Bethlem myopathy (13). In this case, reduced deposition of collagen VI microfibrils was observed in fibroblasts from the patient (13). Whether the presence or absence of the clinical symptoms correlates with possible differences in transcriptional or translational regulation of the *COL6A1* and *COL6A2* genes remains to be determined. On the other hand, the phenotypic variation of similar mutations may also arise from modifying genes yet to be identified.

Immunofluorescence and electron microscopy studies indicate that fibroblasts from patient B synthesize, secrete, and deposit a minute amount of abnormal collagen VI. The mutant protein sheds new light on the biosynthesis and assembly of collagen VI microfibrils. The abnormal collagen VI deposited extracellularly most likely is the product of allele 2, as the predicted protein product of allele 1 is truncated, containing only two-thirds of the triple-helical domain, and therefore unlikely to form a full-length triple-helix with the normal $\alpha$1(VI) and $\alpha$3(VI) chains. The predicted product of allele 2 contains the complete triple-helical domain but an entirely different and shorter C-terminal globular domain than the normal counterpart (Fig. 1). It is known that formation of a collagen triple-helix requires proper chain association/selection, followed by triple-helix nucleation, registration, and propagation (32, 33). The C-terminal globular domains of the fibrillar collagens are important for the chain selection and association, as is the case for the C-terminal NC1 domains of collagens VIII and X (34–36). The protein domain important for chain selection and association in collagen VI is not known. Studies to date indicate that assembly of the collagen VI microfibrillar network requires the presence of all three chains (18). A recent study employing an *in vitro* transcription/translation assay supplemented with semi-permeabilized cells suggests that truncated $\alpha$2(VI) chains with only the C1 domain can associate into monomers made up of three identical chains**;** however, the C2 domain is required for further assembly into dimers and tetramers (37). Our finding that the mutant $\alpha$2(VI) chain without the normal C-globular domain can form triple-helical monomers suggests that the C1 and C2 subdomains of the $\alpha$2(VI) chain are not essential for the chain selection and association. This implies that chain selection and association of collagen VI may begin in the triple-helical domain or the N-globular domain, although it is possible that in the absence of the normal C-terminal globular domain, an alternative recognition site elsewhere is utilized. Also, the possibility that the mutant C-terminal globular domain may contain recognition sequences for chain association cannot be excluded. In this regard, it should be noted that although the C-propeptide of the fibrillar collagen is necessary and sufficient for chain association, it can be replaced with a very short transmembrane domain without affecting triple-helix folding (34).

Our results show that triple-helical monomers containing the mutant C-terminal globular domain can assemble into dimers and tetramers, although with very low efficiency (Fig. 5*B*). In addition, the mutant tetramers are capable of forming

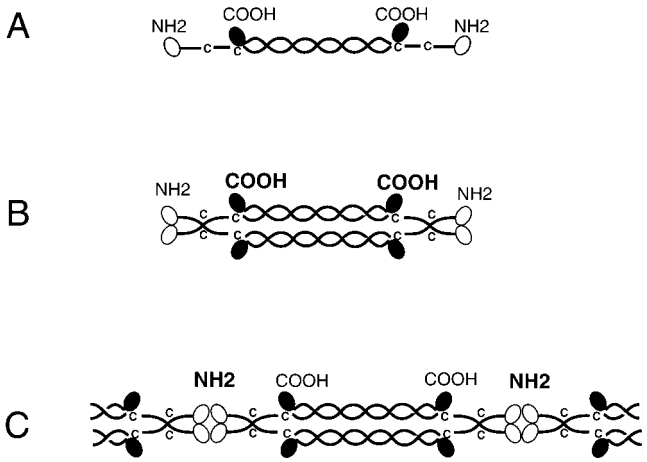


FIG. 8. **Schematic diagram of domains involved in collagen VI oligomerization and microfibril assembly (adapted from Ref. 38).** *A*, two triple-helical collagen VI monomers associate in an antiparallel manner into a dimer through interactions of the C-globular domain of one monomer with the triple-helical domain of the other monomer. Each collagen VI chain has a single cysteine residue in the triple-helical domain (depicted as "*c*"). The cysteine residues in the $\alpha$1(VI) or $\alpha$2(VI) chains are located at the same position and more C-terminal than the cysteine in the $\alpha$3(VI) chain. The dimer is stabilized by disulfide bridges formed presumably between a cysteine residue in the C-globular domain and a cysteine residue in the triple-helical domain of either the $\alpha$1(VI) or $\alpha$2(VI) chain. *B*, two dimers associate in parallel to form a tetramer, which is stabilized by disulfide bridges between the cysteine residues in the triple-helical domain of the $\alpha$3(VI) chain. *C*, the microfibrils are assembled by end-to-end association of the tetramers through the N-terminal globular domains.

larger assemblies, albeit abnormal (Fig. 7). The data are consistent with a previously proposed structural model for collagen VI assembly, which predicts that dimers are formed by interaction of the C-terminal globular domain and the triple-helical domain of two antiparallel monomers and that the association of dimers is stabilized by disulfide bridges (38) (Fig. 8). It is conceivable that the mutant monomers in patient B, which lack the C-terminal globular domain of the $\alpha$2(VI) chain and particularly the short cysteine-rich connecting peptide, would not be able to form dimers efficiently. The model also predicts that end-to-end association of tetramers into microfibrils is primarily mediated through the N-terminal globular domain. In this regard, the mutant tetramers, having normal N-terminal globular domains, would be expected to be able to associate end-to-end into larger assemblies. Our finding by immunoelectron microscopy that these assemblies do not have the expected double-beaded appearance and cannot form extensive networks (Fig. 7) indicates that the C-terminal globular domain is critical for the proper alignment of tetramers and for heterotypic interactions with other matrix molecules. Although the precise protein interactions important for the assembly of collagen VI microfibrils are not known, our results suggest that the C1 and C2 domains of the $\alpha$2(VI) chain contribute to such interactions. It is noteworthy that similar vWF-A motifs in matrilin have been shown to mediate protein-protein interactions involved in the assembly of oligomers and microfilaments (39).

Immunoprecipitation of cell layers from the patients indicate that association of the $\alpha$1(VI) and $\alpha$3(VI) chains can occur without the $\alpha$2(VI) chains (Fig. 5*A*). It is not clear whether these two chains are merely associated together through disulfide bridges or whether the two chains can form a triple-helical monomer of a different chain composition. Secretion of these abnormally associated chains outside cells is apparently prevented, likely by some quality control mechanism.

In conclusion, our studies show that the severe deficiency of collagen VI protein in the two UCMD patients is primarily caused by the near total absence of *COL6A2* mRNA due to nonsense-mediated mRNA decay. The parents of the UCMD patients have reduced amounts of the *COL6A2* mRNA due to degradation of mRNA derived from the mutant allele, but the long term deposition of collagen VI microfibrils is comparable with the normal control. The absence of dominant negative effect from the mutant protein together with the near normal deposition of collagen VI microfibrils may explain the lack of clinical symptoms in the parents. The minute amount of abnormal collagen VI deposited in the extracellular matrix suggests that the C-terminal globular domain is not essential for triple-helix formation but is important for further assembly into higher ordered structures.

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GLYCOBIOLOGY AND EXTRACELLULAR MATRICES:

# Effects on Collagen VI mRNA Stability and Microfibrillar Assembly of Three *COL6A2* Mutations in Two Families with Ullrich Congenital Muscular Dystrophy

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