Am. J. Hum. Genet. 72:998–1004, 2003

Report

Genetic Heterogeneity of Cutis Laxa: A Heterozygous Tandem Duplication within the Fibulin-5 (*FBLN5*) Gene

Dessislava Markova,[1] Yaqun Zou,[1] Franziska Ringpfeil,[1] Takako Sasaki,[3] Günter Kostka,[3] Rupert Timpl,[3] Jouni Uitto,[1,2] and Mon-Li Chu[1,2]

Departments of [1]Dermatology and Cutaneous Biology, and [2]Biochemistry and Molecular Pharmacology, Thomas Jefferson University, Philadelphia; and [3]Max-Planck-Institut für Biochemie, Martinsried, Germany

Inherited cutis laxa is a connective tissue disorder characterized by loose skin and variable internal organ involvement, resulting from paucity of elastic fibers. Elsewhere, frameshift mutations in the elastin gene have been reported in three families with autosomal dominant inheritance, and a family with autosomal recessive cutis laxa was recently reported to have a homozygous missense mutation in the fibulin-5 gene. In the present study, we analyzed the gene expression of elastin and fibulins 1–5 in fibroblasts from five patients with cutis laxa. One patient was found to express both normal (2.2 kb) and mutant (2.7 kb) fibulin-5 mRNA transcripts. The larger transcript contains an internal duplication of 483 nucleotides, which resulted in the synthesis and secretion of a mutant fibulin-5 protein with four additional tandem calcium-binding epidermal growth factor–like motifs. The mutation arose from a 22-kb tandem gene duplication, encompassing the sequence from intron 4 to exon 9. No fibulin-5 or elastin mutations were detected in the other patients. The results demonstrate that a heterozygous mutation in fibulin-5 can cause cutis laxa and also suggest that fibulin-5 and elastin gene mutations are not the exclusive cause of the disease.

Cutis laxa (MIM 123700; MIM 219100) comprises a heterogeneous group of inherited and acquired disorders characterized by loose, sagging, and redundant skin, which often leads to an appearance of premature aging (Uitto and Pulkkinen 2002). A characteristic histopathological abnormality is the fragmentation and paucity of elastic fibers in the skin (Hashimoto and Kanzaki 1975; Kitano et al. 1989). Congenital cutis laxa may be inherited in an autosomal dominant or autosomal recessive pattern (Agha et al. 1978). In the recessive form of the disease, the skin changes are often accompanied by abnormalities in internal organs that are enriched in elastic fibers, such as emphysematous lungs and tortuous arteries. The internal organ involvement is less frequently observed in autosomal dominant cutis laxa.

Reduced elastin mRNA levels have been shown elsewhere in skin fibroblasts from patients with cutis laxa (Olsen et al. 1988), and frameshift mutations in the 3′ end of the elastin gene have subsequently been found in three families with autosomal dominant inheritance (Tassabehji et al. 1998; Zhang et al. 1999). The molecular defects underlying the recessive forms of cutis laxa remained obscure until recently, when two groups of investigators independently showed that mice lacking fibulin-5 developed loose skin, lung emphysema, and vascular abnormalities reminiscent of patients with cutis laxa (Nakamura et al. 2002; Yanagisawa et al. 2002). This led to subsequent identification of a homozygous missense mutation in fibulin-5 in a large consanguineous Turkish family with autosomal recessive cutis laxa and pulmonary emphysema (Loeys et al. 2002).

Fibulin-5, also known as EVEC or DANCE, is a 66-kDa secreted protein identified through its abundant expression in developing and injured arteries (Kowal et al. 1999; Nakamura et al. 1999). The fibulin family members share a common C-terminal globular domain preceded by tandem arrays of calcium-binding epidermal growth factor–like (cbEGF) motifs (Argraves et al. 1990; Pan et al. 1993; Lecka-Czernik et al. 1995; Giltay et al. 1999). Fibulins-1, -2, -4, and -5 are predominantly expressed in tissues rich in elastic fibers, including arteries, cardiac valves, lungs, and skin (Zhang et al. 1995, 1996;

Received October 28, 2002; accepted for publication December 26, 2002; electronically published February 28, 2003.

Address for correspondence and reprints: Dr. Mon-Li Chu, Department of Dermatology and Cutaneous Biology, Thomas Jefferson University, 233 South 10th Street, Philadelphia, PA 19107. E-mail: Mon-Li.Chu@mail.tju.edu

0002-9297/2003/7204-0020$15.00

Giltay et al. 1999; Kowal et al. 1999; Nakamura et al. 1999). In addition, fibulin-1, -2, and -5 have been localized to elastic fibers. However, their precise distribution within the elastic fibers—and, hence, their molecular interactions—are somewhat different. Human disorders previously associated with the fibulin family include two types of retinal dystrophy, resulting from a single mutation in fibulin-3/EFEMP1 (Stone et al. 1999), and a complex synpolydactyly, associated with a chromosomal translocation disrupting the fibulin-1 locus (Debeer et al. 2002).

The similarity in primary structure and expression pattern of the fibulin family members suggest that, in addition to fibulin-5, other fibulins may contribute to the abnormal phenotypes of cutis laxa. We therefore analyzed the gene expression of the fibulin family and elastin in fibroblasts from five patients with sporadic cutis laxa. An affected mother (CL-6) and her son (CL-8), in a family with dominant cutis laxa and a single-nucleotide deletion in exon 30 of the elastin gene (Zhang et al. 1999), were included in the analysis. The patients were all given the diagnosis of cutis laxa on the basis of the characteristic cutaneous findings, including loose and sagging skin with reduced recoil. The diagnosis was confirmed by histopathology with elastin-specific stains (Verhoeff-van Gieson or Orcein), which showed paucity or fragmentation of elastic fibers.

Patient CL-5, an African American female, had extensive folding and redundant skin on the abdominal area and the arms when she was initially seen shortly after birth. No clinical signs of internal organ involvement were noted. However, cardiovascular evaluation by echocardiography and electrocardiography revealed mitral valve regurgitation. Family history was negative for similar clinical conditions. The parents were in good health, and the patient had no siblings. Patient CL-5 was reexamined at age 11 years and was noted to have slightly hyperextensible skin, which demonstrated wrinkling on the backs of hands and wrists, and slightly accentuated excessive skin folds on the abdomen. The patient has recently been diagnosed with scoliosis (12 degrees). Echocardiography, repeated at 2-year intervals, has continued to detect mitral valve regurgitation, whereas the diameter of aorta is normal with no evidence of aneurysms. In summary, the patient has cutis laxa with mild cardiovascular involvement, but the cutaneous manifestations have significantly improved over the 1st decade of life.

Total RNA isolated from fibroblast cultures of patients afflicted with cutis laxa was first examined for the expression of the fibulin-5 mRNA. Northern blot hybridization showed that all patients expressed the 2.2-kb fibulin-5 transcript; however, an additional 2.7-kb mRNA was present in patient CL-5 (fig. 1). The expression of elastin and the other members of the fibulin family was subsequently analyzed by rehybridizing the same nylon membrane consecutively with various probes. Although the transcript levels of fibulin-1 through fibulin-4 and elastin were somewhat variable among fibroblast strains, substantial quantitative differences were noted in several samples. Specifically, the fibulin-2 transcript was not detectable in patient CL-2 fibroblasts derived from lung (fig. 1), yet control lung fibroblasts (not shown) clearly contained this transcript. In addition, both

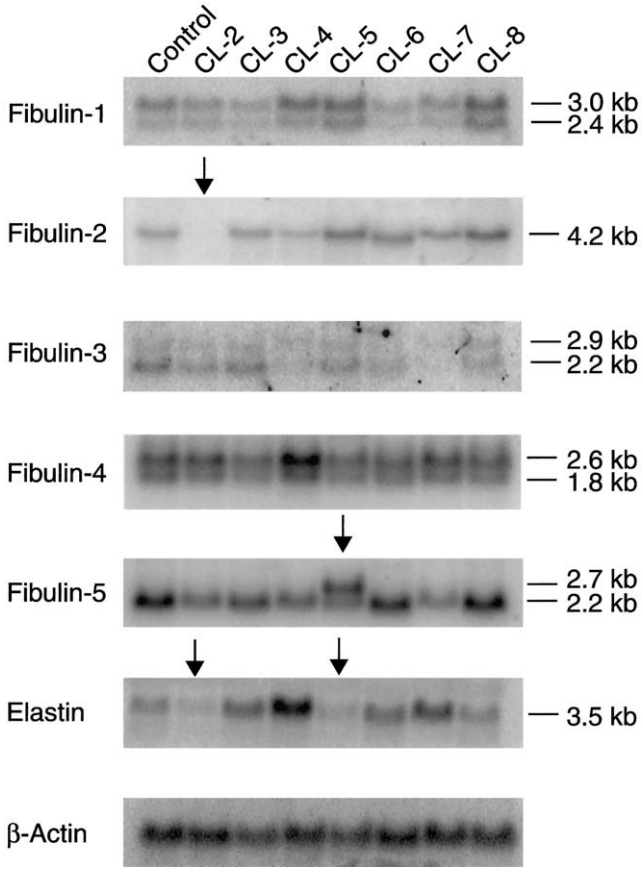

Figure 1 Northern blot analysis of gene expression in fibroblasts from patients with cutis laxa. All fibroblast cultures were derived from skin tissue except for the culture from patient CL-2, which was established from lung tissue. Fibroblasts from patient CL-7 (GM02768) were obtained from Coriell Cell Repositories. Control dermal and lung fibroblasts were cultured from clinically normal individuals. Fibroblasts were grown in Dulbecco's modified Eagle medium with 10% fetal bovine serum at 37 °C in 5% CO_2. The fibroblast cultures were examined at passage 4–7 except for CL-7, which was examined at passage 11–13. Total RNA (10 μg) from fibroblasts of the control subjects and patients CL-2–CL-8 was transferred to Duralon-UV membrane (Stratagene) and hybridized sequentially with α-[^{32}P]dCTP–labeled cDNA probes for fibulin-5, fibulin-4, elastin, fibulin-3, fibulin-2, fibulin-1, and β-actin, in that order. The cDNA probes were generated by RT-PCR. The sizes of the transcripts are indicated on the right. Arrows highlight qualitative and quantitative changes of gene expression (see text).

patient CL-2 and patient CL-5 expressed significantly reduced levels of the elastin mRNA.

Human fibulin-5 consists of 448 amino acids, including a signal peptide of 23 residues at the N-terminus, six tandem cbEGF motifs (IIa–IIf), and a C-terminal globular domain common to all fibulins (fig. 2*A*). The entire fibulin-5 coding region of 1.4 kb in all five patients was amplified by RT-PCR with three primer pairs (fig. 2*A*) and then was subjected to DNA sequencing. The primer pairs were 1F (CAAGCTTTCTTCTCGCCTTC) and 1R (GCCAATATCCGTCGGTGCAG), 2F (TGGACGAGTGTGCAACAGAT) and 2R (TGTGCTCACATTCGTTGATG), and 3F (CGGCACATACTTCTGCTCCT) and 3R (TCAGAATGGGTACTGCGACA). In patient CL-5, the three primer pairs each amplified the expected product and an additional product ~500 bp longer (data not shown). Combination of DNA sequencing and use of allele-specific primers 3F and 660R (GCCTTCCAGAAGCCAATATC) revealed that the larger transcript in CL-5 contained a tandem duplication of 483 bp, corresponding to cbEGF motifs IIb–IIe encoded by exons 5–8 of the fibulin-5 gene (fig. 2*B;* fig. 3).

Genomic DNA from patient CL-5 and the control subjects was digested with *Eco*RI, *Bam*HI, and *Hin*dIII and analyzed by Southern blot hybridization, using a full-length fibulin-5 cDNA as the probe. The hybridization pattern of patient CL-5 was similar to that of the control subjects except for the presence of two additional

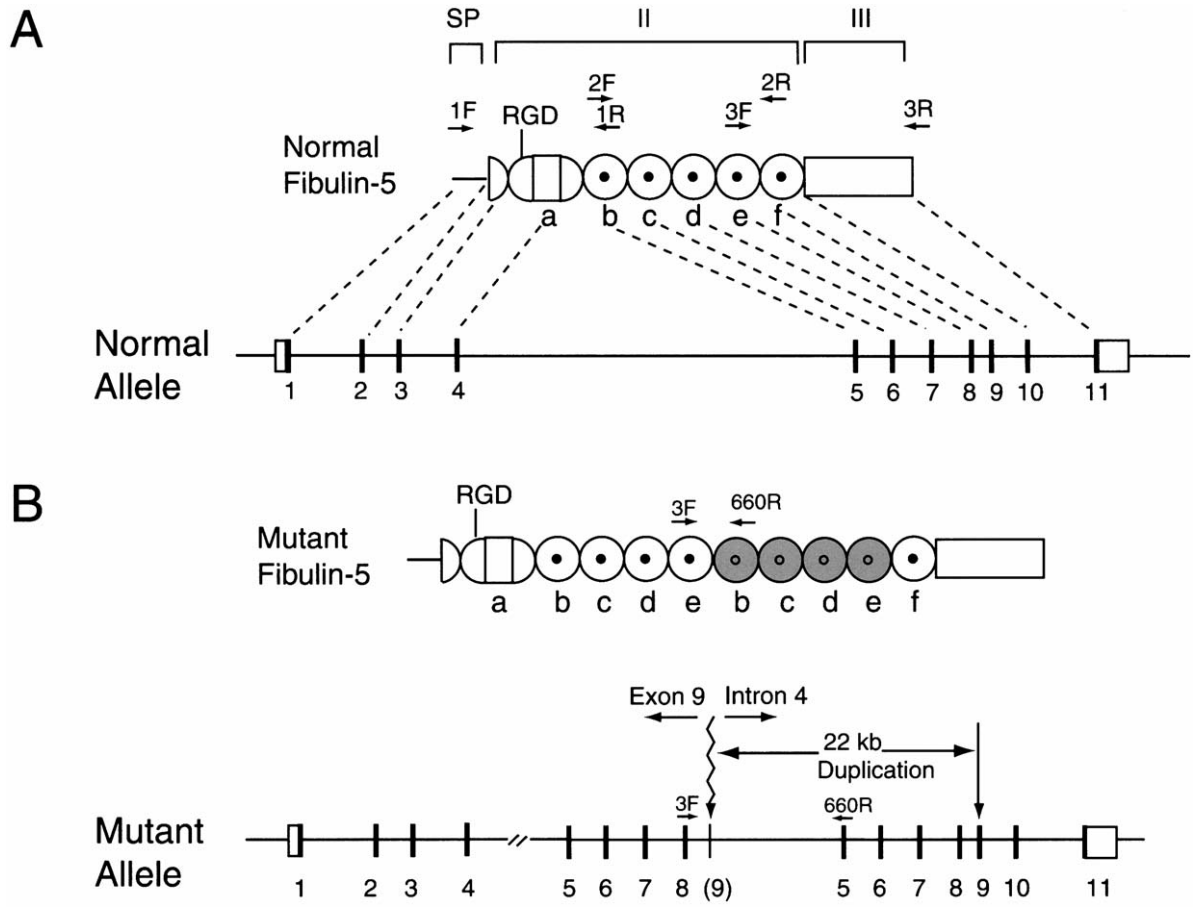

Figure 2 Schematic diagram of normal and mutant fibulin-5 proteins and genes. *A, top,* Human fibulin-5 protein consists of 448 amino acids, including a signal peptide (SP) of 23 residues at the N-terminus, six tandem cbEGF motifs (*domain II, a–f*), and a C-terminal globular domain (*domain III*) of 134 amino acids. The first cbEGF motif (*IIa*) is atypical, as it contains an unusually long linker sequence of 28 amino acids between the 4th and 5th cysteine residues. An RGD sequence is present in the first cbEGF domain. The locations of three primer pairs (1F/1R, 2F/2R, and 3F/3R) used for RT-PCR amplification of the fibulin-5 transcript are indicated. *Bottom,* The fibulin-5 gene, consisting of 11 exons spanning 80 kb of DNA on human chromosome 14. The six cbEGF motifs are each encoded by a single exon (*exons 4–9*). Exons 1 and 2 encode the signal peptide, exon 3 encodes a partial cbEGF motif, and exons 10 and 11 encode the C-terminal globular domain. A large intron of 42 kb separates exons 4 and 5, which encode the first and second cbEGF motifs. *B, top,* The mutant fibulin-5 protein with a tandem duplication of four cbEGF motifs, IIb–IIe (*shaded*). *Bottom,* The mutant allele from patient CL-5 containing a tandem gene duplication of 22 kb, extending from intron 4 to exon 9.

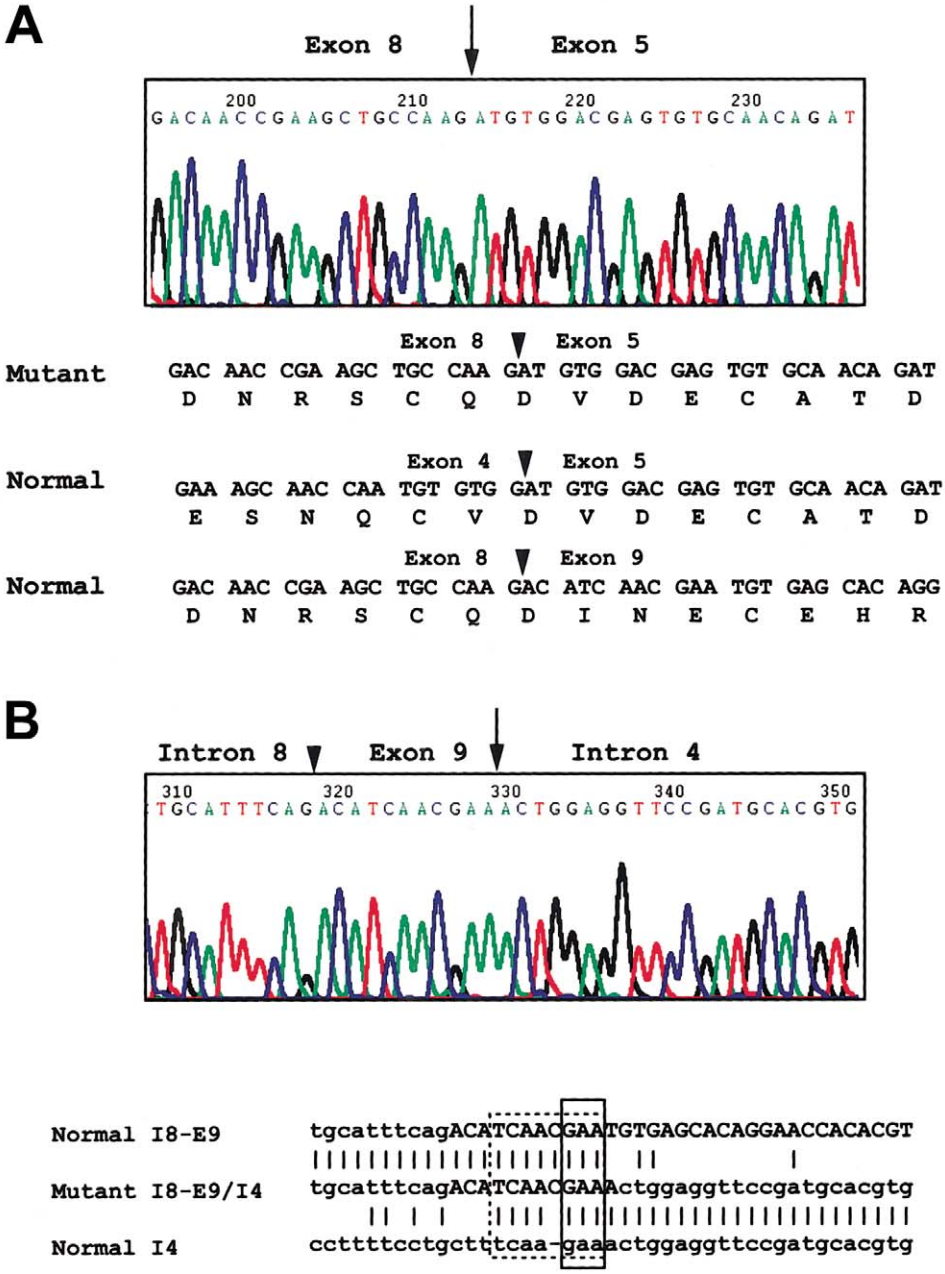

Figure 3 DNA sequence analysis of the fibulin-5 mutation in patient CL-5. *A,* Sequence of the duplication boundary in the fibulin-5 mRNA. RT-PCR amplification of total RNA from patient CL-5, with primers 3F and 660R (see fig. 2*B*), generated a 333-bp mutant-specific product, which was sequenced with the primer 3F. Sequencing showed that nucleotide 852 in the fibulin-5 transcript was followed by nucleotide 369, corresponding to the joining exon 8 and exon 5. DNA and amino acid sequences of the mutant fibulin-5 and the corresponding regions of the normal fibulin-5 are shown. Arrow and arrowheads mark the exon boarders. *B,* Sequence of the duplication boundary in the fibulin-5 gene. PCR amplification of genomic DNA from patient CL-5 with primers 3F and 660R (fig. 2*B*) yielded an 11-kb product, which was cloned and sequenced with primer I8F3 (TCCGTAGTAGTGCCAGGCAA). Sequencing showed that, following nine nucleotides from the beginning of exon 9, there is the sequence of intron 4 at 9,063 nucleotides upstream of exon 5. Sequences of the mutant allele, and the corresponding regions of the normal allele in intron 8/exon 9 and intron 4, are aligned (E9 = exon 9; I4 = intron 4; I8 = intron 8). Exon sequences are shown in uppercase letters, and intron sequences are shown in lowercase letters. The box depicts the 3-bp sequence that is identical between exon 9 and intron 4, where the recombination occurred. The box with dotted lines shows that the nucleotide identity extends an additional 4 bp if a single-nucleotide gap in intron 4 is introduced.

fragments, 7-kb *Hin*dIII and 3-kb *Bam*HI bands (data not shown). This suggested the presence of DNA rearrangement in one of the fibulin-5 alleles of patient CL-5. To delineate the precise breakpoint of the gene duplication, long-range PCR amplification of genomic DNA was performed using primer 3F, located in exon 8, and primer 660R, located in exon 5 (fig. 2*B*). An 11-kb product was obtained, and DNA sequencing of the fragment revealed that the duplication joined sequences of exon 9 (at 10 bp from the 5′ end of the exon) and intron 4 (at 9,063 bp from the 3′ end of intron) (fig. 3*B;* recombination of nucleotides 26060245 and 26037516 in GenBank accession number NT_026437.8). The junction sequence was within a 3-bp sequence that is identical between exon 9 and intron 4 (fig. 3*B*). When a single-nucleotide gap was introduced in intron 4, the sequence identity extended another 4 bp. The result establishes that the mutant allele contains a tandem duplication of 22,729 bp, extending from intron 4 to exon 9 (fig. 2*B*).

Genomic DNA from the mother of patient CL-5 was analyzed by PCR, using primers flanking the boundary of gene duplication. A 295-bp product specific for the mutant allele was amplified from the genomic DNA of patient CL-5, as expected, but not from the genomic DNA of the mother (data not shown), indicating that the mother does not carry the gene duplication. DNA from the father of patient CL-5 was not available for analysis.

Sequence analysis of the fibulin-5 mRNA in the other four patients revealed no deviations from the normal control, except for a silent C/T polymorphism at nucleotide 944. To rule out the possibility that only one allele is expressed, each of the 11 exons and the flanking intron sequences in the fibulin-5 gene in all five patients were amplified by PCR from genomic DNA and analyzed by denaturing high-performance liquid chromatography and DNA sequencing. No mutations in the sequences of the coding regions and splice junctions were detected (results not shown). Furthermore, sequence analyses of the elastin transcripts in all five patients showed no deviations from the normal sequence.

To investigate the molecular mechanism underlying the absence of fibulin-2 expression in patient CL-2, the 17 protein-coding exons, with ~50–150 bp of the flanking intron sequences and 1.0 kb proximal fibulin-2 promoter region, were amplified by PCR from the genomic DNA and then subjected to DNA sequencing. No mutations in the exons and splice junction sequences were detected. Southern blot analysis of genomic DNA from patient CL-2, with the full-length cDNA probe, revealed no DNA rearrangement.

The coding regions of the normal and mutant fibulin-5 transcripts in patient CL-5 were prepared by RT-PCR and cloned into the expression vector pCDNA3 downstream of the T7 promoter. The expression constructs were subjected to coupled in vitro transcription/translation. As predicted by the DNA sequences, the normal transcript yielded a 60-kDa translational product, whereas the mutant transcript resulted in a larger product of 80 kDa (fig. 4*A*).

To examine the synthesis and secretion of the mutant fibulin-5, fibroblasts from patient CL-5 and the control subjects were metabolically labeled overnight and the culture media were immunoprecipitated with an affinity purified polyclonal antibody against fibulin-5. Analysis of the resultant products, by polyacrylamide gel electrophoresis, revealed that patient CL-5 secreted both a larger 85-kDa fibulin-5 protein and the normal 65-kDa fibulin-5 protein (fig. 4*B*). By contrast, only a single 65-

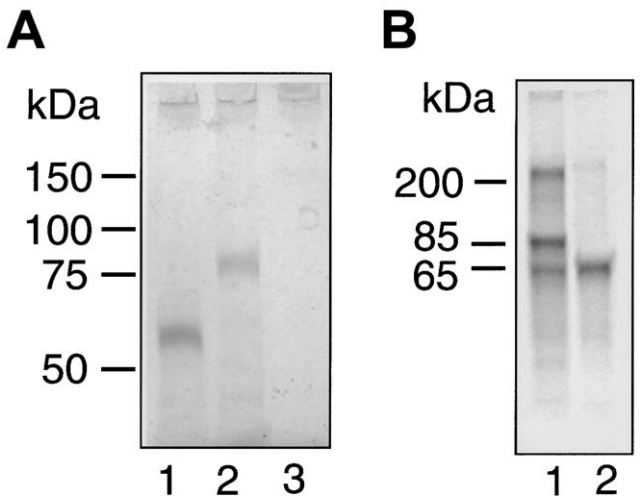

Figure 4 Analysis of the mutant fibulin-5 protein in patient CL-5. *A,* In vitro transcription/translation. The full-length normal and mutant fibulin-5 cDNAs were RT-PCR amplified, using Turbo pfu polymerase (Stratagene), with 1F/3R primers, and cloned into the pcDNA3 expression vector downstream of the T7 promoter. The expression vectors were subjected to coupled in vitro transcription/translation using the TNT Coupled Reticulocyte Lysate System (Promega) in the presence of fluorescent lysyl-tRNA (FluoroTect GreenLys, Promega). The resulting products were separated on a 7.5% polyacrylamide gel and detected by a FluorImager. Translation of the normal (*lane 1*) and mutant (*lane 2*) fibulin-5 constructs resulted in 60- and 80-kDa proteins, respectively, whereas translation of an expression construct of normal fibulin-5 in the opposite orientation yielded no product (*lane 3*). *B,* Immunoprecipitation. Fibroblasts were metabolically labeled with [^{35}S]cysteine (ICN Biochemicals) overnight in serum-free medium, and culture medium (1 ml) from patient CL-5 (*lane 1*) and the control subjects (*lane 2*) was immunoprecipitated with an affinity-purified antibody against fibulin-5, following the procedure described elsewhere (Grässel et al. 1996). The rabbit antiserum was raised from recombinant fibulin-5 protein expressed in 293-EBNA cells, using the pCEP-Pu vector as described elsewhere (Giltay et al. 1999). The antibody was affinity purified on the antigen-coupled Sepharose 4B (Timpl 1982). The immunoprecipitated products were separated on a 7.5% polyacrylamide gel and were detected with a PhosphorImager.

kDa normal fibulin-5 protein was observed in the control subjects. The sizes of the immunoprecipitated products were somewhat larger than those of their in vitro translated counterparts, apparently owing to posttranslational modifications. The antibody also precipitated a 220-kDa protein of unknown identity, which was present in substantially higher amount in the patient, as compared with the control subjects (fig. 4*B*).

Our finding that a heterozygous mutation results in cutis laxa differs from the first fibulin-5 mutation recently reported in the Turkish family (Loeys et al. 2002). Patients who carry the homozygous missense mutation in the Turkish family have severe cutis laxa phenotypes, with internal organ involvement. However, family members heterozygous for the mutation are not affected. The missense mutation is a leucine-to-proline substitution in the fourth cbEGF motif. A similar amino acid substitution in the cbEGF motifs of fibrillin-1 has been shown elsewhere to interfere with protein folding (Wu et al. 1995). This suggests that the missense fibulin-5 protein in the Turkish family may be unstable, and, therefore, the amount of the mutant protein present in the heterozygous carriers might be too low, relative to its normal counterpart, to exert a dominant negative effect. By contrast, the larger fibulin-5 protein in patient CL-5 is stable, as evidenced by the biosynthetic study and, thus, can act in a dominant negative fashion.

The fibulin-5 mutation in patient CL-5 arose from an internal gene duplication of 22 kb, and the chromosomal recombination occurred within a 3-bp sequence that is identical between exon 9 and intron 4. There are several *Alu*-repetitive sequences within the 42-kb intron 4 and one *Alu* sequence in the 2-kb intron 8, but these repetitive sequences do not play a direct role in the gene duplication. The duplicated region consists of four complete exons (exons 5–8), as well as a truncated exon 9 of only 9 bp, which contains an intact splice-acceptor site at the 5′ end but not the splice-donor site at the 3′ end. The truncated exon-9 sequence, although it has an intact splice-acceptor site, is skipped during the pre-mRNA–processing step. The tandem gene duplication appears to have an effect on transcription/processing/stability of the mutant fibulin-5 mRNA, because quantification of the northern blot in figure 1 indicates that the mutant mRNA is present at a level 2.1-fold of the normal fibulin-5 mRNA. Consistent with the increase in the mutant fibulin-5 mRNA, quantification of the immunoprecipitation result (fig. 4*B*) revealed that the mutant fibulin-5 protein is 1.4-fold of the normal counterpart.

In conclusion, our studies provide the first evidence that a heterozygous mutation in the fibulin-5 gene can result in cutis laxa, and they also suggest that mutations in fibulin-5 and elastin are not the exclusive causes of the disease.

Acknowledgments

We are grateful to the patients with cutis laxa and their families for their participation of this study. We thank Dr. Peter Koblenzer, for providing patient samples; Dr. Kouichi Tanaka, for clinical information; Dr. Sergio Jimenez, for the gift of control lung fibroblasts; Dr. Ellen Pfendner, for assistance in dHPLC analysis; and Sarah Fratta, for growing fibroblast cultures. The work was supported by grants from National Institutes of Health (GM55625 to M.L.C. and AR28450 to J.U.).

Electronic-Database Information

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

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for fibulin-5 [accession numbers NM_006329 and NT_026437.8], elastin [accession numbers NM_000501 and NT_007758], and fibulin-2 [accession numbers NM_001998 and NT_005927])
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for cutis laxa and elastin)

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