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Genetics

Two mutations in Marfan syndrome resulting in truncated fibrillin polypeptides

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ABSTRACT **Biochemical and molecular genetic studies have recently suggested that mutations in the gene coding for fibrillin on chromosome 15 result in Marfan syndrome. To our knowledge, only one mutation in the fibrillin gene has been published. Here we report the results of screening 20 unrelated MFS patients for mutations in fibrillin cDNA by the single-strand conformation polymorphism technique. We found two mutations, both of which appear in the heterozygote form and code for a shortened fibrillin polypeptide. The first mutation is a large in-frame deletion of 366 bases of the fibrillin mRNA, shown to result in a truncated but secreted polypeptide found in the fibroblast culture of the patient. The second mutation is a G-to-A transition resulting in the substitution of a stop codon for a tryptophan codon and thus predicting the premature termination of the polypeptide chain. We screened 60 other, unrelated MFS patients for these mutations as well as for the previously reported mutation (arginine-239 to proline) and found none of the three mutations in any of these patients. These data suggest that most MFS families carry their own distinct mutation.**

The Marfan syndrome (MFS) is an autosomal dominant connective-tissue disorder characterized by cardiovascular, ocular, and skeletal manifestations (1). By the random linkage approach, the MFS locus was assigned to the long arm of chromosome 15 in three Finnish families (2). Later, the linkage was confirmed in families from diverse ethnic backgrounds, and the locus was more precisely localized to the immediate vicinity of the polymorphic marker *D15S1* (3–5). To date, linkage analyses of chromosome 15 markers in families from different populations have not revealed any evidence for genetic heterogeneity underlying MFS (3–5).

Independent simultaneous immunohistochemical analyses demonstrated a nearly constant deficiency of fibrillin, an extracellular protein (6), in skin sections and cultured fibroblasts from MFS patients (7). Subsequently, the fibrillin cDNA was cloned (8) and the corresponding locus was mapped by *in situ* hybridization to chromosome 15q21.1 (9, 10), in the vicinity of the marker *D15S1*. The final proof that the fibrillin gene is the MFS gene came from a study by Dietz *et al.* (11): a *de novo* missense mutation at nucleotide 716 substituting proline for arginine (R239P) in the fibrillin gene of two unrelated MFS patients.

The high rate of observed sporadic MFS cases [15–30% of cases (1, 12)] would suggest that numerous different mutations will be found in MFS families. Here we report one deletion and one point mutation in the fibrillin gene of two MFS patients. Both mutations predict a shortened fibrillin polypeptide. A shortened fibrillin chain was detected by protein analysis of fibroblasts from one of the patients.

MATERIALS AND METHODS

Patients. The material consisted of fibroblast lines established from skin biopsies of 20 unrelated MFS patients from Finland and the United Kingdom and blood samples of these and 41 additional MFS patients from Belgium, Finland, the Netherlands, Switzerland, the United Kingdom, and the United States, including patients both with and without family history of MFS. All the samples were taken in accordance with the Helsinki Declaration. The diagnoses were made by using the criteria established by Beighton *et al.* (12).

The patient R.H. was a 48-year-old man with cardiovascular, eye, and skeletal symptoms and signs of MFS. He was a member of a three-generation English pedigree none of whom have ectopia lentis. The propositus' mother had peripheral retinal degeneration and retinal detachment. A brother and sister of the propositus had severe mitral valve prolapse. The brother had a mitral valve replacement at 39 years of age and died suddenly at 44 years after a period of severe congestive heart failure. The sister had associated dysrhythmia requiring β-adrenergic blockade therapy and a moderate aortic root dilation seen on echocardiography.

The patient E.Y. was a 55-year-old Finnish man whose MFS had been diagnosed at early adulthood. He had a characteristic habitus with arachnodactyly, long extremities, and scoliosis. There was downward dislocation and cataract of both ocular lenses, and he was blinded for bilateral retinal ablation. He had surgery for aneurysm of the ascending aorta and aortic valve insufficiency. He had six sisters and two brothers who had no signs of MFS. The parents were deceased, but their medical records did not suggest any MFS features. All the evidence therefore suggests that E.Y. represents a sporadic case of MFS.

Northern and Southern Blot Analyses. Skin fibroblasts were cultured in Dulbecco's modified Eagle's medium containing 10% inactivated fetal bovine serum, penicillin (50 units/ml), streptomycin sulfate (50 μg/ml), and ascorbic acid (50 μg/ml). Total RNA was isolated from confluent cell layers by the guanidinium isothiocyanate method (13). For Northern analyses, 15 μg of total RNA was electrophoresed through a formaldehyde/agarose gel and transferred to a Pall Biodyne nylon membrane. Genomic DNA was extracted from frozen peripheral blood according to the method of Vandenplas *et al.* (14) with minor modifications. For Southern analyses, 5 μg of DNA was digested to completion with 20 units of *Taq* I. The DNA fragments were separated in a 0.9% agarose gel and transferred to Hybond-C-Extra membranes (Amersham) ac-

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Abbreviations: MFS, Marfan syndrome; SSCP, single-strand conformation polymorphism; nt, nucleotide(s).

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cording to the method of Southern (15). Prehybridization and hybridization of the Northern and Southern blot filters with the probe for fibrillin [CLM-5, a probe covering nucleotides (nt) 1628–5802 of the fibrillin cDNA (8)] were performed as described (2, 16).

Primers for PCR, Sequencing, and Solid-Phase Minisequencing. The primers used in this study correspond to the indicated nucleotides according to Maslen *et al.* (8): 1a (nt 1–24), 1b (nt 894–872), 2a (nt 808–828), 2b (nt 1678–1657), 3a (nt 1594–1614), 3b (nt 2421–2400), 4a (nt 2340–2362), 4b (nt 3103–3079), 5a (nt 3018–3039), 5b (nt 3826–3803), 6a (nt 3740–3760), 6b (nt 4656–4535), 7a (nt 4470–4489), 7b (nt 5262–5239), 8a (nt 5180–5199), 8b (nt 5665–5644), 9a (nt 5582–5604), 9b (nt 6033–6009), 10a (nt 5538–5559), 10b (nt 5665–5644), 11a (nt 683–702), 11b (nt 849–831), 12a (nt 4740–4761), 12b (nt 5159–5140), 13b (4783–4762), 14a (nt 5103–5127), 14b (nt 5148–5128), 15a (4800–4821), 15b (5079–5060), seq 1 (nt 5553–5573), and seq 2 (nt 735–717). The oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer. Primers 10b and 11a were biotinylated at their 5′ ends (17).

Single-Strand Conformation Polymorphism (SSCP) Analyses and Characterization of the Mutations. For SSCP analyses (18, 19), first-strand cDNA was synthesized from 1 μg of total RNA by 20 units of avian myeloblastosis virus reverse transcriptase (Promega) using the fibrillin-specific primers 3b, 6b, and 9b (Fig. 1). One-tenth of the cDNA synthesized with primer 3b was then used as a template to amplify overlapping fragments of the fibrillin cDNA by PCR (20) with each of the following primer pairs: 1a/b, 2a/b, and 3a/b. Analogously, cDNA synthesized with primer 6b was used as a template for amplification using the primer pairs 4a/b, 5a/b, and 6a/b, and cDNA synthesized with the primer 9b was used as a template for amplification using the primer pairs 7a/b, 8a/b, and 9a/b (Fig. 1). The PCR products were radiolabeled by adding 1 μCi (37 kBq) of [α-^{32}P]dCTP (Amersham) to the PCR mixture. The PCR was carried out for 30 cycles in a programmable heating block (Techne PHC-1) with denaturation at 95°C for 1 min, annealing at 60°C for 1 min (except for the primer pair 11a/b, 58°C for 1 min), and extension at 72°C for 1–10 min. The conditions for PCR were as previously described (21). Before the SSCP analyses the PCR products larger than 500 bp were cleaved to two fragments by restriction enzyme digestion. Four microliters of the PCR mixture was digested with 30 units of restriction enzyme in a total volume of 60 μl. Two microliters of 0.3% SDS/30 mM EDTA was added to 4 μl of the digestion reaction, and 6 μl of 95% (vol/vol) formamide/20 mM EDTA, 0.05% bromphenol blue/0.05% xylene cyanol was added. The sample was denatured at 80°C for 2 min and applied (3 μl per lane) to a nondenaturing 5% polyacrylamide gel. Electrophoresis was carried out at two conditions: one gel contained 10% (vol/vol) glycerol in 90 mM Tris borate, pH 8.3/4 mM EDTA (1× TBE buffer) and the other gel contained 5% glycerol in 0.5× TBE buffer. In both cases the gels were run at 15 W for 15 hr at room temperature. The gels were dried and the bands were visualized by autoradiography for 5–24 hr with Kodak X-Omat AR film and an intensifying screen.

Sequence Analyses of PCR Products. The amplified DNA fragments were purified by electrophoresis through a 1% agarose gel. The band was excised, crushed into 10 mM Tris, pH 8.0/1 mM EDTA, extracted with phenol, quickly frozen in liquid N_2, and centrifuged. After extraction with chloroform the DNA was precipitated with ethanol and sequenced by the dideoxynucleotide termination method (22) with modifications (23). Sequencing of the individual fragments was carried out with corresponding PCR primers as sequencing primers.

Solid-Phase Minisequencing. Genomic DNA was screened for the G-to-A point mutation at nt 5574 and the R239P mutation by the solid-phase minisequencing method (24) with the conditions previously described (21). The DNA was amplified by using either primer 10a and 5′-biotinylated primer 10b or primers 11a (5′-biotinylated) and 11b. The amplified 5′-biotinylated DNA was then immobilized in a streptavidin-coated microtiter well and denatured at high pH. The mutation was detected by a one-step primer extension reaction directed by primer (seq 1 or seq 2) that anneals to the

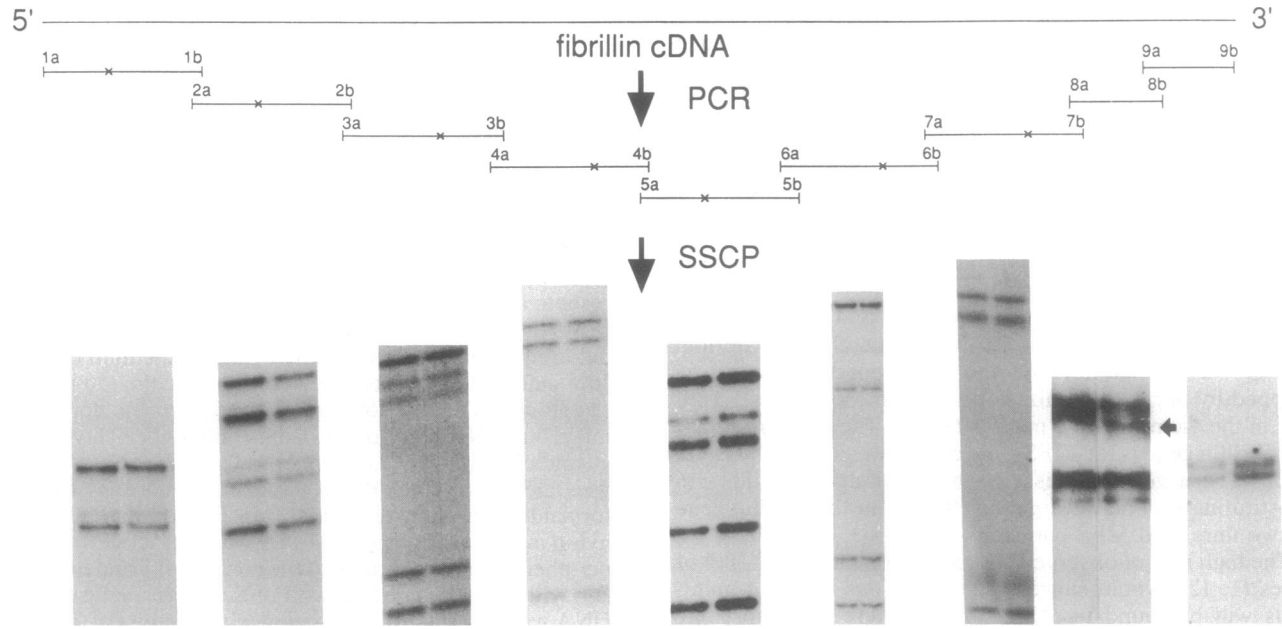

FIG. 1. Strategy for SSCP analysis of fibrillin cDNA. The analyzed 6034 base pairs (bp) of the fibrillin cDNA is presented as a horizontal line and the nine overlapping fragments amplified and analyzed by the SSCP technique are indicated below with the designation of the primers at the ends of each fragment. The cleavage site of the restriction enzyme used is indicated in each PCR fragment (x). Each autoradiograph demonstrates the results of the SSCP analysis of one amplified fragment of one control (left lane) and the Finnish patient E.Y. (right lane). These SSCP analyses were carried out using a 5% polyacrylamide gel containing 10% glycerol in 1× TBE buffer. A shift (indicated with an arrow) was found in the fragment 8a/b of the patient.

DNA directly upstream of the mutation. In this reaction, one labeled dNTP corresponding to the nucleotide at the site of the mutation was incorporated by a DNA polymerase. This method is simple and reliable and the results of the test are obtained as numeric values that unequivocally define the genotype (21).

Analyses of Fibrillin Polypeptides. Confluent flasks of fibroblasts from patients and from normal individuals were incubated with [^{35}S]cysteine (NEN) at 50 μCi/ml in serum-free minimum essential medium without cysteine. After 24 hr, medium was removed and treated with 25 mM diisopropyl fluorophosphate and 3 mM EDTA. The medium was then incubated with gelatin-Sepharose (Pharmacia) on a rotating table for 1 hr. The mixture was poured into a column and the medium that flowed through the gelatin-Sepharose was collected and incubated with fibrillin-specific monoclonal antibody 69 coupled to CNBr-activated Sepharose (Pharmacia) (25). After incubation for 2 hr on a rotating table, the antibody 69-Sepharose and medium were poured into a column and washed extensively with 0.15 M NaCl/50 mM sodium phosphate, pH 7.5/0.05% Tween 20, and bound proteins were eluted with 0.1 M glycine/HCl (pH 2.5), neutralized, applied as a sample to an SDS/4.5% polyacrylamide gel, and electrophoresed. The gel was treated with Amplify (Amersham) and the labeled polypeptides were visualized on Kodak X-AR5 film. Preflashed films were developed after exposure overnight or for 3 days.

RESULTS

SSCP Analyses of MFS Patients. The SSCP method was used to screen amplified cDNA fragments of 20 MFS patients for mutations in the coding region of the fibrillin gene. After detection of an aberrantly moving band in SSCP analysis, the specific nucleotide change was identified by direct sequencing of the corresponding PCR product. To date, 6.9 kilobases (kb) of the ≈10-kb fibrillin cDNA has been cloned, including 5920 bp of coding region followed with 916 bp of the 3′ untranslated region (8). The area of the fibrillin cDNA analyzed covered the known 5920 bp of coding region and first 114 bp of the 3′ noncoding region.

Total mRNA was extracted from cultured fibroblasts of MFS patients. After reverse transcription with fibrillin-specific primers, the fibrillin cDNA was amplified in overlapping fragments between 451 bp and 895 bp in size by using nine sets of primers (Fig. 1). For more sensitive detection of nucleotide changes in the amplified DNA, fragments over 500 bp were cleaved with a suitable restriction enzyme to two fragments of different size. To maximize the sensitivity of the SSCP analyses, each sample was analyzed by nondenaturing electrophoresis under two different conditions.

We found a mobility shift in the DNA fragment of 2 out of 20 analyzed patients, the first in a PCR fragment amplified with primers 7a/b in the sample of patient R.H., a member of a three-generation MFS family, and the second in the fragment amplified with primers 8a/b in a sample of a sporadic Finnish patient, E.Y. (Fig. 1). Both changes were heterozygous; i.e., the fragment of the normal allele was observed in addition to the shifted fragment.

Identification of a 366-bp Deletion of the Fibrillin cDNA. A deletion mutation in the sample of patient R.H. was apparent already prior to SSCP analyses, when amplification of the fibrillin cDNA of R.H. with primers 7a/b revealed, in addition to the normal 791-bp fragment, an ≈425-bp fragment (Fig. 2*A*). A shifted fragment was also observed in SSCP analyses. Sequence analysis of the ≈425-bp fragment showed a deletion of cDNA residues 4762–5127 (Fig. 2*B*). Amplification of the fibrillin cDNA of R.H. and his mother (who is also affected) with an alternative primer pair, 12a/b, located internal to primers 7a/b confirmed the 366-bp deletion and proved the cosegregation of the mutation with the disease in this family (Fig. 2*C*). Next, genomic control DNA was amplified by using two primer pairs immediately flanking the deletion breakpoints: the primer pair 12a/13b, flanking the 5′ breakpoint, and the primer pair 14a/b, flanking the 3′ breakpoint. The results revealed a 3-kb intron between nucleotides 4761 and 4762 and an even larger, unamplifiable intron between nucleotides 5127 and 5128. Amplification of genomic control DNA with primers located inside the deleted region (primers 15a/b) produced a 1200-bp fragment in contrast to the 280-bp fragment predicted from the cDNA sequence. This suggests the existence of at least one intron within the deletion. Thus, the 366-bp deletion in the mRNA is caused by a much larger genomic deletion, which includes at least two exons, one complete intron of ≈1 kb, and portions of two other introns. Next, genomic DNA of controls and the patient R.H. was analyzed by Southern blot hybridization after digestion with different restriction enzymes, using fibrillin cDNA clone CLM-5 as the probe. The hybridization pattern of the DNA samples digested with *Taq* I demonstrated two extra bands in the case of R.H., confirming a deletion at the genomic level. This pattern cosegregated with the disease in the family and was absent from the unaffected members of the family, 40 analyzed control samples, and samples of 61 other, unrelated MFS patients.

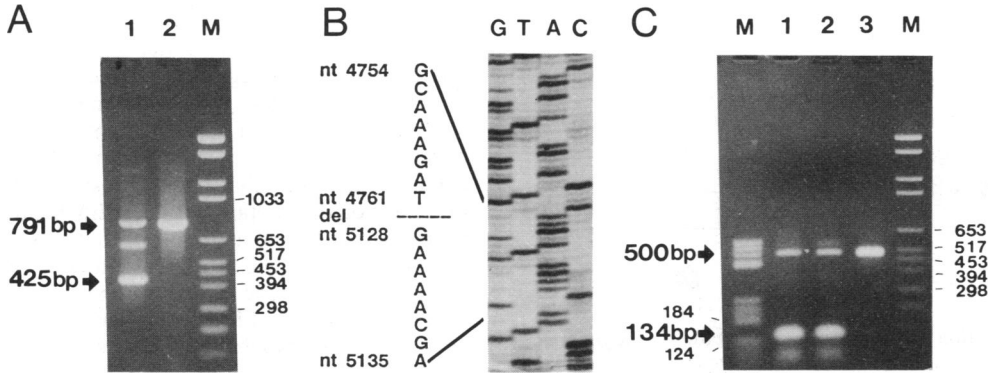

FIG. 2. Detection of a 366-bp deletion of the fibrillin cDNA in patient R.H. (*A*) Agarose gel of fibrillin cDNA of R.H. (lane 1) and a control (lane 2) amplified with the primers 7a/b. In addition to the expected band of 791 bp, R.H.'s sample showed an extra band of 425 bp (the other extra band, of ≈620 bp, turned out to be the amplification product of the deleted allele after the primer 7a had annealed to a highly homologous region 192 bp upstream of its correct annealing site). Lane M, molecular size markers. (*B*) Detection of a 366-bp deletion (del) between nucleotides 4761 and 5128 by sequence analysis of the fibrillin cDNA. (*C*) Agarose gel of cDNA of patient R.H. (lane 1), his mother (lane 2), and a control (lane 3) amplified with a primer set located nested to the primers 7a/b. Lanes M, molecular size markers.

Identification of a Nonsense Mutation. The other detected aberrantly moving SSCP fragment was from a Finnish patient, E.Y. (Fig. 1). Here, a heterozygous G-to-A transition at nucleotide 5574 was detected by sequence analysis of the amplified fragment. As a consequence of this mutation, a stop codon is substituted for tryptophan, predicting premature termination by 116 amino acids. The G-to-A transition was confirmed in genomic DNA by direct sequencing of the PCR product amplified with the primers 8a/b (Fig. 3*A*) and also by the solid-phase minisequencing technique (Fig. 3*B*). None of the siblings had the mutation, when their DNA samples were analyzed by solid-phase minisequencing.

Protein Analyses of Fibrillin Polypeptides. Both the detected mutations predict shortened polypeptides of fibrillin. Immunoprecipitation analyses with fibrillin-specific antibodies of the medium from metabolically labeled fibroblasts of R.H. and subsequent SDS/PAGE analysis demonstrated that the patient's fibroblasts secreted two species of fibrillin molecules, one normal and the other about 15 kDa smaller (Fig. 4). This agrees with the calculated molecular mass of the deleted amino acids, 15.6 kDa. Northern blot analyses of the patient's total RNA with CLM-5 as a probe did not convincingly show a mRNA deletion, possibly due to the low sensitivity and poor size resolution of this method.

In contrast to the analyses of fibrillin polypeptide chains of R.H., immunoprecipitation analyses with fibrillin-specific antibodies failed to detect a shortened fibrillin polypeptide chain in the medium of the fibroblasts of E.Y., the patient with the point mutation producing an early stop.

Screening of Other Patients for Point Mutations. We used solid-phase minisequencing method to screen DNA samples from 61 MFS patients and 40 controls both for the detected point mutation and for the R239P mutation (Fig. 3*B*). Neither mutation was detected in any of the analyzed samples.

DISCUSSION

A mutation causing the Marfan phenotype was recently reported (11) in the gene coding for fibrillin, a 350-kDa

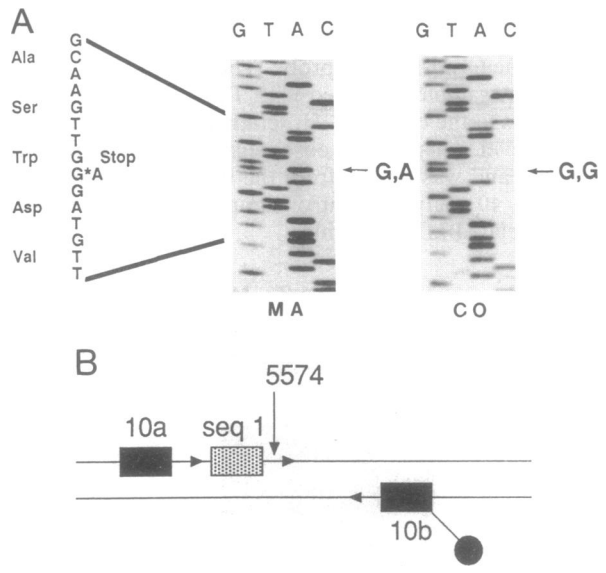

FIG. 3. (*A*) Detection of a nonsense point mutation in the genomic DNA of patient E.Y. Asterisk and arrow indicate the change of G to A at nt 5574 in one fibrillin allele of the patient (MA, mutant allele), resulting in the substitution of a stop codon for a tryptophan codon. CO, control. (*B*) Primers used in minisequencing for detecting the G-to-A point mutation. Genomic DNA was amplified by using 5'-biotinylated primer 10b and the primer 10a. The mutation was detected by a one-step primer extension reaction directed by primer seq 1, which anneals to the DNA directly upstream of the mutation.

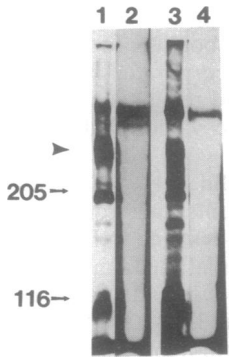

FIG. 4. Detection by immunoprecipitation of two species of fibrillin secreted by the fibroblasts of patient R.H. Lane 1, total labeled medium proteins from the fibroblasts from patient R.H.; lane 2, immunoprecipitated fibrillin from the fibroblasts from patient R.H.; lane 3, total labeled medium proteins from control fibroblasts; lane 4, immunoprecipitated fibrillin from control fibroblasts. All samples were applied with 1% β-mercaptoethanol. Positions of molecular mass standards of 205 and 116 kDa are shown; position of fibronectin is marked with an arrowhead.

glycoprotein that is a constituent of the 10- to 12-nm-diameter microfibrils of extracellular matrix (6).

We have used the published sequence information of fibrillin cDNA to screen for other MFS mutations with the SSCP technique and detected a mutation in 2 of the 20 analyzed patients. The first mutation, which actually did not require SSCP analyses for detection, was a deletion of 122 amino acids shown to segregate in a large three-generation MFS family. The second mutation, found in a sporadic MFS patient, was a nonsense point mutation producing an early stop. As a result, one of the patient's alleles is predicted to code for a truncated fibrillin polypeptide lacking the last 116 amino acids.

In contrast to a previous study (26) in which 9 out of 10 mutations were found by SSCP analysis, the SSCP technique was relatively inefficient here. Possibly this is partially because only two-thirds of the fibrillin cDNA has been cloned, and consequently all the mutations in the 5' third are undetectable. Further, when cDNA copies of mRNA are analyzed, the mutations causing silent alleles or unstable mRNA cannot be detected.

What are the biological consequences of the mutations identified in this study? Like most other MFS patient cell lines (7), immunofluorescence of fibroblasts from both patients displayed reduced amounts of fibrillin fibers (data not shown). Possible explanations include (*i*) a reduced synthesis and impaired fibrillogenesis due to one mutated fibrillin allele, whose product is either poorly expressed or abnormally retained and degraded intracellularly, or (*ii*) an amplified deleterious effect on fibrillogenesis due to the secretion and copolymerization of the mutant gene product with normal fibrillin. The latter mechanism occurs in certain type I collagen mutations that result in osteogenis imperfecta: the presence of the mutant product drastically interferes with normal triple-helical assembly, producing the so-called protein suicide (27). The secretion and copolymerization of the mutant fibrillin gene product with normal fibrillin might equally either slow fibril formation or produce functionally defective structures that are either susceptible to degradation or hamper its interactions with other proteins. These possibilities have been elucidated *in vitro* in a recent study (28) in which different consequences of unknown MFS mutations were dissected at the protein level.

To date, there are no published data on the consequences of the specified fibrillin mutations in MFS at the protein level. Here we have shown that fibroblasts from the patient with the

366-bp deletion in the fibrillin mRNA secrete two species of fibrillin. Such shortened polypeptide chains might disturb the multimer formation of fibrillin in the matrix significantly. The deleted area contains 3 of the 34 six-cysteine repeat motifs identified in the coding region of the fibrillin gene (8). These repeats are thought to participate in the formation of an antiparallel β-sheet conformation of fibrillin polypeptides.

Immunoprecipitation analyses of polypeptides in the medium of fibroblasts from the patient with the G-to-A transition resulting in an early stop did not show shortened fibrillin molecules. Possible explanations include degradation of the mutant polypeptide chain before or after secretion. It is, however, possible that some of the truncated fibrillin molecules are secreted. Although the details of the assembly process and the stabilization of the fibrillin fibers are not known (29), a polypeptide lacking a significant portion of its carboxyl-terminal end could result in defective formation of the fibrillin polymers (25).

Neither of the mutations identified here was observed in the DNA samples of 60 unrelated MFS patients, suggesting that a spectrum of different mutations will be found in the future analyses of MFS families. Definition and detailed characterization of different mutations leading to the MFS phenotype, their characterization at the protein level, and subsequent phenotype–genotype correlations will provide insight into the biological significance of the fibrillin molecule and the specific functions of its various regions.

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