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METHODS

Evaluation and Application of Denaturing HPLC for Mutation Detection in Marfan Syndrome: Identification of 20 Novel Mutations and Two Novel Polymorphisms in the *FBN1* Gene

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Mutations in the human fibrillin 1 gene (*FBN1*) cause the Marfan syndrome (MFS), an autosomal dominant connective tissue disorder. Knowledge about *FBN1* mutations is important for early diagnosis, management, and genetic counseling. However, mutation detection in *FBN1* is a challenge because the gene is very large in size (~200 kb) and the ~350 mutations detected so far are scattered over 65 exons. Conventional methods for large-scale detection of mutations are expensive, technically demanding, or time consuming. Recently, a high-capacity low-cost mutation detection method was introduced based on denaturing high-performance liquid chromatography (DHPLC). To assess the sensitivity and specificity of this method, we blindly screened 64 DNA samples of known *FBN1* genotype exon-by-exon using exon-specific DHPLC conditions. Analysis of 682 PCR amplicons correctly identified 62 out of 64 known sequence variants. In three MFS patients of unknown *FBN1* genotype, we detected two mutations and eight polymorphisms. Overall, 20 mutations and two polymorphisms are described here for the first time. Our results demonstrate 1) that DHPLC is a highly sensitive (89–99%, P = 0.05) method for *FBN1* mutation detection; but 2) that chromatograms with moderate and weak pattern abnormalities also show false positive signals (in all 45–59%, P = 0.05); 3) that the difference in the chromatograms of heterozygous and homozygous amplicons is mostly independent of the type of sequence change; and 4) that DHPLC column conditions, additional base changes, and the amounts of injected PCR products influence significantly the shape of chromatograms. A strategy for *FBN1* mutation screening is discussed. Hum Mutat 19:443–456, 2002. © 2002 Wiley-Liss, Inc.

KEY WORDS: Marfan syndrome; MFS; extracellular matrix; fibrillin 1; *FBN1*; polymorphism; DHPLC; molecular diagnosis; mutation screening; clinical molecular genetics

DATABASES:
FBN1 – OMIM: 134797, 154700 (Marfan syndrome); GDB: 127115; GenBank: NM_000138;
http://insertion.stanford.edu/melt.html (DHPLC MELT Program; Stanford University);
http://www.umd.necker.fr/Site%20Marfan/Marfan_Home_Page.html (Marfan Database)

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INTRODUCTION

Mutations of the human fibrillin 1 gene (*FBN1*, MIM# 134797) cause the Marfan syndrome (MFS, MIM# 154700), one of the most prevalent connective tissue disorders [Dietz et al., 1991]. The autosomal dominant MFS affects ~1:10,000 individuals, without gender or ethnic predisposition [Gray et al., 1994; Pyeritz, 2000]. The disease is characterized by skeletal, ocular, and cardiovascular manifestations and exhibits a broad range of severity [Pyeritz, 2000]. Aortic aneurysms and dissections are the life-threatening events that can be prevented by timely cardiovascular surgery [Finkbohner et al., 1995]. So far, the diagnosis of MFS has been made clinically. Recently, however, the inclusion of *FBN1* mutations as a diagnostic criterion has been proposed [De Paepe et al., 1996; Maron et al., 1998]. Molecular analysis of *FBN1* would be valuable for presymptomatic diagnosis and genetic counseling as well as for a better understanding of the phenotypic variability of MFS and related disorders [Dietz and Pyeritz, 1995].

The *FBN1* gene, located on chromosome 15q21, is about 200 kb in size and contains 65 exons encoding 2,871 amino acids and four additional alternatively spliced exons at the 5′ end of the gene [Kainulainen et al., 1990; Pereira et al., 1993; Biery et al., 1999]. The ~350 *FBN1* mutations reported so far (Marfan Database; personal communication with G. Beroud and C. Boileau) are scattered over all 65 exons and are largely unique to each affected family (http://archive.uwcm.ac.uk/uwcm/mg/search/127115.html) [Dietz and Pyeritz, 1995]. Furthermore, clear genotype–phenotype association has been observed only in the case of neonatal MFS [Putnam et al., 1996]. Consequently, mutation detection should involve the entire coding sequence and all intron-exon junctions of *FBN1*, making the analysis a challenge.

Since the first description of an *FBN1* mutation [Dietz et al., 1991], various methods have been applied to the detection of *FBN1* sequence variations such as DNA sequencing [e.g., El-Aleem et al., 1999], single-strand conformation polymorphism analysis (SSCP) [e.g., Ades et al., 1996], heteroduplex analysis [e.g., Yuan et al., 1999], or long RT-PCR [e.g., Liu et al., 1996]. However, screening for *FBN1* mutations has remained laborious and expensive due to the large number of exons to be analyzed. Recently, the introduction of denaturing high performance liquid chromatography (DHPLC) has allowed the simple, semi-automated, and cost-effective detection of single-base substitutions, small insertions, and deletions [Oefner and Underhill, 1995]. This novel method relies on the differential retention of homo- and heteroduplex DNA on ion-pair reverse-phase chromatography under partially denaturing conditions [Underhill et al., 1997].

DHPLC has been applied to the detection of sequence variation in more than 50 genes [reviewed in Xiao and Oefner, 2001]. It has also been used for the identification of *FBN1* sequence variants in two earlier studies [Liu et al., 1997; Schrijver et al., 1999]. For *FBN1*, however, DHPLC conditions have not been presented and the sensitivity of DHPLC has been tested only with a few known mutations [Liu et al., 1997]. Therefore, we have assessed here the accuracy of DHPLC by blinded screening of a large collection of DNA samples containing previously characterized *FBN1* sequence variants. Furthermore, we have developed a DHPLC-based strategy for *FBN1* mutation screening. The feasibility of this strategy was demonstrated in three individuals with MFS of uncharacterized *FBN1* genotype.

MATERIALS AND METHODS

Samples and Study Design

We analyzed 64 genomic DNA samples containing 60 unique known *FBN1* sequence variants. In addition, we investigated three individuals with Marfan syndrome (BK, PB, and SZ) whose *FBN1* mutations had not been characterized previously. In samples with known *FBN1* genotype, 64 mutations and polymorphisms were encoded and among them four occurred twice (3294C>T, 5305G>A, 5485ins15nt, and 7180C>T; Table 1). Encoded samples were screened blindly in the Zurich laboratory (G.M.), i.e., without prior knowledge of the position, number, and chemical nature of sequence variants, and the code was broken (by B.S.) only after the investigation. To reduce cost and time, in 62 of 64 genomic DNA samples with known *FBN1* genotype (samples encoded 1–65 in Table 1; note that numbers 30, 32, and 49 are not to be found) we screened only 10 exons instead of 65, hereby simulating a smaller *FBN1* gene. For these samples, nine additional exons down- and upstream from the exon car-

TABLE 1. *FBN1* Mutations and Polymorphisms Screened Blindly, DHPLC Profiles, and Detection Temperatures (T)*

Code	Sequence change[a]	Amino acid change[a]	Location	Previous detection method[b]	Reference	DHPLC[c] Profile	DHPLC[c] T(°C)
26	IVS2+1G>A	Splice site	Intron 2	CSGE	Dietz et al. [1993]	B/C	58, 59
48	**306C>T**	–	Exon 3	SSCP/HA	Unpublished	C	60
45	**635delCA**	Frameshift	Exon 6	SSCP/HA	Unpublished	A	64
56	718C>T	R240C	Exon 6	Sequencing	Collod-Beroud et al. [1998]	A	63, 64
40	**IVS6-1G>A** and IVS6-37delT	Splice site –	Intron 6	SSCP/HA	Unpublished Liu et al. [1997]	A	58–60
47	IVS6-37delT	–	Intron 6	SSCP/HA	Liu et al. [1997]	C	60
27	932insT	Frameshift	Exon 8	CSGE	Halliday et al. [1999]	B	57–59
6	960insA	Frameshift	Exon 8	CSGE	Loeys et al. [2001]	B	57
54	**1285C>T** and **1224C>T**	R429X –	Exon 10	SSCP/HA	Unpublished Unpublished	B	60–62
57	**IVS11+2T>C**	Splice site	Intron 11	EMD	Unpublished	OK/B	60–62
58	IVS11+5G>A	Splice site	Intron 11	EMD	Liu et al. [1997]	A	60, 61
31	1960G>A and 1875T>C	D654N –	Exon 15	DHPLC	Halliday et al. [2002] Hayward et al. [1994]	A	60–62
37	IVS17-46A>G	–	Intron 17	SSCP/HA	Nijbroek et al. [1995]	A	56, 57
2	2327G>A	C776Y	Exon 19	CSGE	Loeys et al. [2001]	B/C	55
33	IVS20+1G>A	Splice site	Intron 20	DHPLC	Halliday et al. [2002]	A/B	56–58
43	**2687G> ATCCCATATA**	Frameshift	Exon 22	SSCP/HA	Unpublished	A/B	54–56
53	2954G>A	G985E	Exon 24	SSCP/HA	Collod-Beroud et al. [1999]	C	64
65	3143T>C	I1048T	Exon 25	SSCP/HA	Lönnqvist et al. [1996]	B	61, 62
5	3165T>G	C1055W	Exon 25	CSGE	Loeys et al. [2001]	B/C	60–62
44	**3209A>G**	D1070G	Exon 26	SSCP/HA	Unpublished	B	61, 62
66	3220T>C	C1074R	Exon 26	SSCP	Kainulainen et al. [1994]	B	62
36	**3241T>G**	C1081G	Exon 26	SSCP/HA	Unpublished	B	60–62
50	3294C>T	–	Exon 26	SSCP/HA	Yuan et al. [1999]	A	60–62
61	3294C>T	–	Exon 26	Sequencing	Yuan et al. [1999]	A	60–62
14	3302A>G	Y1101C	Exon 26	CSGE	Loeys et al. [2001]	A/B	60–62
18	3382G>A	V1128I	Exon 27	CSGE	Loeys et al. [2001]	A/B	61, 62
38	**3557A>G**	Y1186C	Exon 28	SSCP/HA	Unpublished	B/C	60–62
39	**3797A>T**	Y1266F	Exon 30	SSCP/HA	Unpublished	B	58, 59
28	3963A>G	–	Exon 31	CSGE	Halliday et al. [1999]	A	58, 59
17	4016G>A	C1339Y	Exon 32	SSCP	Loeys et al. [2001]	C	59–61
29	4365delCT	Frameshift	Exon 35	CSGE	Halliday et al. [1999]	A	61, 62
25	4485delC	Frameshift	Exon 36	CSGE	Halliday et al. [1999]	B/C	59–61
67	4537T>C	C1513R	Exon 36	SSCP	Kainulainen et al. [1994]	B	61
22	4621C>T	R1541X	Exon 37	CSGE	Halliday et al. [1999]	A/B	59–61
13	4721ins23nt	Frameshift	Exon 37	CSGE	Loeys et al. [2001]	A	59, 60
3	4786C>T	R1596X	Exon 38	CSGE	Loeys et al. [2001]	B/C	56
51	**5305G>A**	E1769K	Exon 43	SSCP/HA	Unpublished	B	59, 60
52	**5305G>A**	E1769K	Exon 43	SSCP/HA	Unpublished	B	59, 60
12	5369G>C	R1790P	Exon 43	SSCP	Loeys et al. [2001]	B/C	60
1	5372G>A	C1791Y	Exon 43	CSGE	Loeys et al. [2001]	B/C	59, 60
16	5485ins15nt	1829insCINTA	Exon 44	CSGE	Loeys et al. [2001]	A	63–65
46	5485ins15nt	1829insCINTA	Exon 44	SSCP/HA	Loeys et al. [2001]	A	63–65
15	5500insT	Frameshift	Exon 44	CSGE	Loeys et al. [2001]	B	65
23	5504G>A	C1835Y	Exon 44	CSGE	Halliday et al. [1999]	A	65
8	5726T>C	I1909T	Exon 46	CSGE	Loeys et al. [2001]	B	60, 61
19	6160C>T	Q2054X	Exon 49	CSGE	Loeys et al. [2001]	B/C	59, 60
42	**6302C>A**	T2101K	Exon 50	SSCP/HA	Unpublished	B/C	61–63
9	6461C>G	P2154R	Exon 52	CSGE	Loeys et al. [2001]	A	58–60
7	6583G>T	G2195X	Exon 53	CSGE	Loeys et al. [2001]	B/C	59, 60
21	6773G>A	C2258Y	Exon 55	CSGE	Halliday et al. [1999]	A	59–61
55	**6884G>A**	C2295Y	Exon 56	Sequencing	Unpublished	B	61, 62
41	**6919T>C** and IVS56+17G>C	C2307R –	Exon 56 Intron 56	SSCP/HA	Unpublished Nijbroek et al. [1995]	OK/C	59–61
60	**6995/6delT**	Frameshift	Exon 56	Sequencing	Unpublished	B	61, 62
24	7180C>T	R2394X	Exon 57	CSGE	Halliday et al. [1999]	B	62, 63
62	7180C>T	R2394X	Exon 57	Sequencing	Halliday et al. [1999]	B	62, 63
35	7398C>A	Y2466X	Exon 59	DHPLC	Halliday et al. [2002]	B/C	59–61

(*Continued*)

TABLE 1. Continued.

Code	Sequence change[a]	Amino acid change[a]	Location	Previous detection method[b]	Reference	DHPLC[c] Profile	DHPLC[c] T(°C)
34	7399C>T	Q2467X	Exon 59	DHPLC	Halliday et al. [2002]	B	59–61
20	7754T>C	I2585T	Exon 62	SSCP	Loeys et al. [2001]	B	62–64
11	**7852G>A**	G2618R	Exon 63	CSGE	Unpublished	A/B	64, 65
10	7872C>G	N2624K	Exon 63	CSGE	Loeys et al. [2001]	A/B	63–65
4	**7915T>A**	Y2639N	Exon 63	SSCP	Unpublished	B	64, 65
64	**7916A>G**	Y2639C	Exon 63	Sequencing	Unpublished	B	64
63	**8003G>A**	G2668D	Exon 63	Sequencing	Unpublished	B	63, 64
59	**8437insC**	Frameshift	Exon 65	Sequencing	Unpublished	B/C	58–60

*Novel sequence variants are indicated with boldface type.
[a]Positions are given according to Pereira et al. [1993].
[b]Mutations were previously detected in the laboratories of A.D.P (code 1–20), D.H. (code 21–35), C.B. (code 36–54), G.P. (code 55–65), and B.S. (code 66, 67). CSGE, conformation-sensitive gel electrophoresis; EMD, enzyme mismatch detection; HA, heteroduplex analysis; SSCP, single-strand conformation polymorphism analysis.
[c]A, B, C, and OK denote high, medium, low, and no deviation, respectively, from the normal DHPLC profile. Regarding DHPLC oven temperatures used see Table 3.

rying the known sequence variation were randomly chosen (by B.S.) by means of a computer program. In each of the remaining two DNA samples (encoded 66 and 67 in Table 1), 31 exons were analyzed because after having identified the mutations, screening was stopped. For each of the three MFS patients (BK, PB, and SZ), we analyzed all 65 coding exons.

Polymerase Chain Reaction (PCR)

DNAs were amplified exon-by-exon using previously reported intron-specific primers [Nijbroek et al., 1995; Liu et al., 1997], except for amplification of exons 1, 17, 18, 22, 35, 47, 56, 61, and 65 (Table 2), for which at least one primer was redesigned using the Oligo 4.1 software (National Bioscience, Plymouth, MN). PCR-amplifications (6–20 amplicons per exon; Table 3) were performed in a final volume of 75µl containing 100–140ng of genomic DNA, 1× PCR buffer (Promega, Madison,WI), 1.6mM $MgCl_2$ (Promega), 0.1mM of each dTTP, dATP, dCTP, dGTP (Promega), 0.2µM of each primer (Microsynth, Balgach, Switzerland), and 3.75U

TABLE 2. Novel Forward (pcrF) and Reverse (pcrR) PCR Primers for the Amplification of Exons and Flanking Intron Regions of the *FBN1* Gene as Well as Internal Primers Used for Cycle Sequencing of the PCR Products (seqF and seqR)

Exon	Primer sequence (5′ →3′)	PCR product length
1	(pcrF) TGG CGG CTC GGC ATC AT	211 bp
	(pcrR) CCA CAC CAA AGG AGG GAA CC	
7	(seqF) CCC CGG GCT CTG TCA G	
	(seqR) CCA ACT GCA AAG GCA TAA GA	
17[a]	(pcrF) TGC AAA CAA GGG AAT CAT	211 bp
	(pcrR) GCC AGA GAG GGA GTC AG	
	(seqF) TCT GCA AAC AAG GGA ATC ATT	
	(seqR) GTA AAT TTT GAA AGG AAT CCT TA	
18	(pcrF) ATC GAT TTT TTT CCT CCT GTA G	288 bp
	(pcrR) TAT GCA GGC AAT GTT TCA GA	
22	(pcrF) TGC TCC AGG TCA TCT TTC	303 bp
35	(pcrF) CTC CCT CCC CCC AAG ATA T	181 bp
	(pcrR) CGG GAC ACC AGG GAG CT	
42	(seqF) GTC CCA CCT TTG TTT AAT CA	
	(seqR) AAT GCC GTC ATG ACT CAC	
47	(pcrF) TGG GGA CTT TTC TGC AGA TG	183 bp
	(pcrR) AGC ATT CTT TCC AGG TCT TTC TAA	
56	(pcrF) TTT GGT CCT TCA ATA AAA TCA	196 bp
	(pcrR) TGT GGA GGC TGA GGT TAG	
61	(pcrF) TTG GCC TTT TCC GAG TTA TC	254 bp
	(pcrR) TGG ATC GCA GCT GAA GTC T	
65	(pcrF) AGT GGC ATA TGT ACA TTG T	507 bp
	(pcrR) TTG GGG GAA AAT ATA GTT	

[a]Reverse internal primer (seqR) used for cycle sequencing of exon 17 was as previously described for PCR amplification [Nijbroek et al., 1995].

TABLE 3. DHPLC Conditions for the Analysis of the 65 *FBN1* Exons*

Exon	PCR product length (bp)[a]	DHPLC analysis conditions		No. of amplicons	No. of polymorphic amplicons (unique/total)	No. of false positive		
		Gradient start-stop (% B)	Oven T (°C)			B	B/C	C
1	211	47-61	62, 63, 64	10	0/0	1	0	1
2	248	47-61	57, **58**, **59**	12	1/1	0	0	2
3	196	47-61	55, **60**, **61**	14	1/2	0	0	1
4	271	47-61	57, 58, 59	15	0/0	0	0	2
5	182	47-61	59, 60, 61	15	0/0	0	0	3
6	307	47-61	62, **63**, **64**	16	2/2	0	0	0
7	222	47-61	**58**, **59**, **60**	16	2/3	0	0	0
8	224	47-61	**57**, **58**, **59**	16	2/2	0	0	1
9	256	47-61	62, 63, 64	14	0/0	0	0	1
10	268	47-61	**60**, **61**, **62**	14	1//1	0	0	1
11	249	47-61	**60**, **61**, **62**	9	2/2	0	0	0
12	223	47-61	60, 62, 63	7	0/0	0	1	1
13	218	47-61	58, 59, 60	9	0/0	0	0	0
14	210	47-61	57, 58, 59	9	0/0	0	0	0
15	212	47-61	**60**, **61**, **62**	9	3/5	0	0	0
16	251	47-61	60, 61, 62	8	0/0	0	0	3
17	211	47-61	60, 61, 62	10	0/0	0	0	3
18	288	47-61	**56**, **57**, **58**	12	2/7	0	0	0
19	211	47-61	**55**, 57	13	1/1	0	0	0
20	220	47-61	**56**, **57**, **58**	13	1/1	0	1	2
21	288	47-61	62, 63, 64	14	0/0	1	0	1
22	303	51-65	**54**, **55**, **56**	16	1/1	0	0	1
23	332	47-61	54, 57, 59	16	0/0	1	2	0
24	418	47-61	59, 62, 63, **64**	16	1/1	0	0	0
25	271	47-61	**60**, **61**, **62**	17	2/2	0	0	1
26	227	47-61	**60**, **61**, **62**	18	5/6	1	0	2
27	249	47-61	**61**, **62**, 63	17	1/1	0	0	0
28	245	47-61	**60**, **61**, **62**	15	2/4	0	0	2
29	214	47-61	59, 60, 61	15	0/0	0	0	2
30	292	47-61	57, **58**, **59**	16	1/1	0	0	2
31	230	47-61	57, **58**, **59**	16	1/1	0	0	1
32	226	47-61	**59**, **60**, **61**	14	1/1	0	1	1
33	224	47-61	59, 60, 61	10	0/0	0	0	0
34	203	47-61	62, 63, 64	11	0/0	0	0	1
35	181	45-59	**61**, **62**, **63**	12	2/2	0	0	0
36	201	47-61	**59**, **60**, **61**	12	2/2	0	0	1
37	260	47-61	**59**, **60**, **61**	11	2/3	0	0	0
38	155	42-56	**56**, 57, 58	13	1/1	0	0	0
39	217	45-59	59, 60, 61	12	0/0	1	1	1
40	201	45-59	58, 59, 60	12	0/0	0	0	0
41	332	47-61	**59**, **60**, **61**	12	1/3	0	2	0
42	142	42-56	59, 60, 61	15	0/0	1	0	1
43	200	45-59	**59**, **60**	16	4/5	0	0	1
44	193	45-59	**63**, **64**, **65**	16	3/4	0	1	0
45	362	47-61	56, 57, 58	15	0/0	0	0	3
46	317	47-61	58, 59, **60**, **61**	14	1/1	0	0	4
47	183	47-61	59, 60, 61	13	0/0	0	0	2
48	219	47-61	58, 59, 60	12	0/0	0	0	5
49	232	47-61	**59**, **60**, 61	13	1/1	0	0	1
50	249	47-61	**61**, **62**, **63**	13	1/1	1	0	0
51	171	42-56	59, 60, 61	13	0/0	0	0	2
52	415	47-61	**58**, **59**, **60**	12	2/4	1	0	1
53	287	47-61	**59**, **60**	11	1/1	0	0	1
54	222	47-61	59, 60, 61	14	0/0	0	2	2
55	236	47-61	**59**, **60**, **61**	17	1/1	0	0	1
56	196	47-61	**59**, **60**, **61**, **62**	20	4/10	1	0	0
57	295	47-61	**62**, **63**, 64	18	1/2	0	1	1
58	243	47-61	56, 57, 58	18	0/0	0	1	0
59	222	47-61	**59**, **60**, **61**	17	2/2	2	0	0
60	209	47-61	59, 60	16	0/0	0	0	3
61	254	47-61	60, 61, 62	15	0/0	0	0	2
62	211	47-61	**62**, **63**, **64**	13	1/1	0	0	4
63	407	47-61	62, **63**, **64**, **65**	13	5/5	0	1	2
64	344	51-65	60, 61, 62	10	0/0	3	0	0
65	507	51-65	**58**, **59**, **60**	6	1/1	0	1	0

*The number of analyzed and polymorphic amplicons, and false positive signals are given for each exon (B, B/C, and C denote DHPLC profiles; see Fig. 1). Oven temperatures (T) at which sequence variations were detectable are indicated by bold numbers (cf. Tables 1 and 4).

[a]PCR product lengths are based on our data resulting from bi-directional sequencing.

of *Taq* DNA polymerase (Promega) with the following cycling profile: 3 min denaturation at 94°C and 35 cycles of denaturation at 94°C for 40 sec, annealing at 59.5°C (for exons 1, 35, and 64) or 54.5°C (for the other exons) for 40 sec, extension at 72°C for 40 sec, followed by a 10 min final extension step at 72°C. All thermal cycles were run on a Hybaid PCR Express thermocycler (Hybaid, Teddington, Middlesex, UK). The quality and quantity of PCR products were determined on 1.5% agarose gels by standard procedures.

Denaturing HPLC Analysis

DHPLC analysis was performed either on a WAVE DNA fragment analysis system (Transgenomic, Santa Clara, CA) (for samples with codes 66 and 67 in Table 1, and for patient PB in Table 4) or on a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) (for the remaining samples) both containing a DNASep column (Transgenomic) and a 80 cm coil of PEEK tubing before the column in the oven (L-7300 from Transgenomic or CTO-10AVP from Shimadzu, Kyoto, Japan). Prior to DHPLC, aliquots of untreated PCR products (~50μl) were denatured at 95°C for 3 min followed by gradual re-annealing to 35°C over 45 min, allowing the formation of heteroduplexes in the PCR products.

Re-annealed PCR products (5–15μl) were automatically loaded on a DNASep column kept at an oven temperature as given in Table 3. The optimal column temperatures were selected based on our test runs with amplicons from each exon using small temperature gradients, previously used conditions [Liu et al., 1997], and recommendations of the WAVEMAKER software (Transgenomic) and the DHPLC Melt program

TABLE 4. **Mutations Identified in MFS Patients With Unknown *FBN1* Genotype (BK, PB, and SZ) as well as Polymorphisms Detected in This Study***

No.[a]	Sequence and amino acid change	Location	Reference	Sample (code)	Allele frequency	DHPLC[b] Profile	DHPLC[b] T (°C)
Mutations							
1	4409G>A, C1470Y	Exon 35	Unpublished	BK	1/4	B	62, 63
2	4621C>T, R1541X	Exon 37	Halliday et al. [1999]	PB, 22	2/5	A/B	59–61
Polymorphisms							
3	**306C>T**	Exon 3	Unpublished	48, 57	2/10 (0.06–0.51)	C	60
4	IVS6-37delT	Intron 6	Liu et al. [1997]	40, 47, 48, SZ	4/4 (0.51–1.00)	C	60
5	**1224C>T**	Exon 10	Unpublished	54	1/11 (0.02–0.38)	B	60–62
6	1875T>C	Exon 15	Hayward et al. [1994]	2, 31, 43, 67, SZ	5/10 (0.24–0.76)	B/C	60–62
7	IVS15+30G>A	Intron 15	Loeys et al. [2001]	2	1/10 (0.02–0.40)	–	–
8	IVS17-46A>G	Intron 17	Nijbroek et al. [1995]	2, 5, 31, 33, 37 43, 67, SZ	8/10 (0.49–0.94)	A	56, 57
9	3294C>T	Exon 26	Yuan et al. [1999]	50, 61	2/15 (0.04–0.38)	A	60–62
10	IVS27-5G>A	Intron 27	Hewett et al. [1991]	25, 67, SZ	3/8 (0.14–0.69)	A	60, 61
11	IVS28+ 15delTTTTA	Intron 28	Nijbroek et al. [1995]	25, 67, SZ	3/8 (0.14–0.69)		
12	3963A>G	Exon 31	Halliday et al. [1999]	28	1/7 (0.03–0.51)	A	58, 59
13	IVS40-35C>T	Intron 40	Nijbroek et al. [1995]	3, 8, BK	3/7 (0.16–0.75)	A	59–61
14	5343G>A	Exon 43	Loeys et al. [2001]	15	1/11 (0.02–0.38)	B/C	59, 60
15	IVS52+128A>G	Intron 52	Yuan et al. [1999]	21, 24, BK	3/14 (0.08–0.48)	A	58
16	IVS56+17G>C	Intron 56	Nijbroek et al. [1995]	10, 20, 21, 24 41, 59, 62, SZ	8/13 (0.36–0.82)	B	61, 62

*Notations are according to Table 1. Lower and upper confidence limits of observed relative allele frequencies of polymorphisms are given in parentheses (P = 0.05). Novel sequence variants are indicated with boldface type.

[a]Polymorphisms no. 10 and 11 occurred simultaneously, whereas polymorphisms no. 4, 5, 6, and 16 were found in amplicons also carrying an encoded *FBN1* mutation (code 40, 54, 31, and 41 in Table 1). Polymorphism no. 7 occurred in one amplicon homozygous for polymorphism no. 6 (1875T>C). Two amplicons (samples with code 2 and 33) were homozygous for polymorphism no. 8 (IVS17-46A>G).

[b]DHPLC profiles refer only and soley to amplicons heterozygous for the given polymorphism.

[Jones et al., 1999] (http://insertion.stanford.edu/melt.html). PCR products were eluted from the column using a 7-min linear acetonitrile gradient created by mixing 0.1M triethylamine acetate buffer (TEAA, Fluka, Milwaukee, WI), pH 7.0, without (eluent A) or with 25% (v/v) acetonitrile (eluent B) (Labscan, Dublin, Ireland) at a constant flow rate of 0.9ml per min. The start and end points of the gradients were empirically adjusted to each PCR product (Table 3). After each gradient, the column was washed by 100% eluent B for 0.5 min, and equilibrated for 3.2 min with a mixture of eluents A and B containing 5% less eluent B than used at the start of the gradient (Table 3). After about 100 injections, the column was cleaned by 75% (v/v) acetonitrile at 60°C for 30 min, and equilibrated for 30 min with a 1:1 mixture of eluent A and B.

The elution of PCR products was monitored with an UV detector at 260nm in millivolts and analyzed using either the D-7000 HSM program version 3.0 (Transgenomic) or the Class-VP software version 5.032 (Shimadzu). To infer an effect of different sequence variants on DHPLC elution profiles, chromatograms differing from the elution profile of control samples were empirically classified by the principal investigator (G.M.) into five categories (A, A/B, B, B/C, and C) indicated by the letters A, B, and C according to high (A), medium (B), and low (C) pattern abnormality, based on differences in peak number, width, and relative height (Fig. 1).

DNA Sequencing

PCR products with pattern abnormalities as well as all 65 coding exons of one of the three individuals with unknown *FBN1* genotype (patient SZ) were directly sequenced in both directions by means of ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) using PCR primers except for exons 7, 17, and 42 for which (semi-)nested internal primers were used (Table 2). Prior to sequencing, PCR products with low DNA concentration (approx. <30ng/μl) were purified by the QIAquick PCR Purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In DNA samples with previously uncharacterized *FBN1* genotype, the detected mutations were verified by restriction enzyme digestion and repeated sequencing on newly amplified PCR products.

Data Analysis

For observed proportions (p), upper and lower confidence limits ($p_{U,L}$) were calculated using the Wilson score method [Wilson, 1927]:

$$p_{U,L} = \frac{p + \frac{z^2}{2n} \pm z\sqrt{\frac{p(1-p)}{n} + \frac{z^2}{4n^2}}}{1 + \frac{z^2}{n}},$$

where n is the sample size and z is chosen according to the desired level of confidence ($z = 1.960$ for 95% confidence limits) [Sokal and Rohlf, 1995]. This equation is based on the binomial distribution and has the advantage of a simple closed form, equally applicable to very small ($n \approx 5$) and large sample sizes [Newcombe, 1998].

RESULTS

The DHPLC profile of amplicons containing sequence variations showed different aberrations from the corresponding normal elution profile, ranging from high to low pattern abnormality (Fig. 1). The DHPLC profile of each sequence variant detected is indicated in Tables 1 and 4. By repeated analyses, DHPLC produced highly reproducible chromatograms, allowing the identification of sequence variants based on chromatogram shape differences between heterozygous and homozygous samples. However, we found that not only sequence alterations but also the amounts of injected PCR

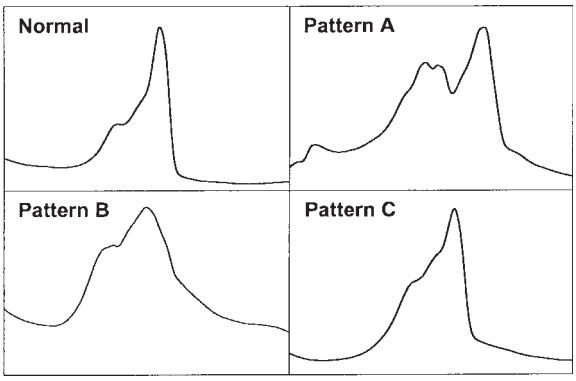

FIGURE 1. **Representative chromatograms for high (pattern A), medium (pattern B), and low (pattern C) deviations from the corresponding normal DHPLC elution profile (normal), exemplified by amplicons from exon 26 at 61°C.**

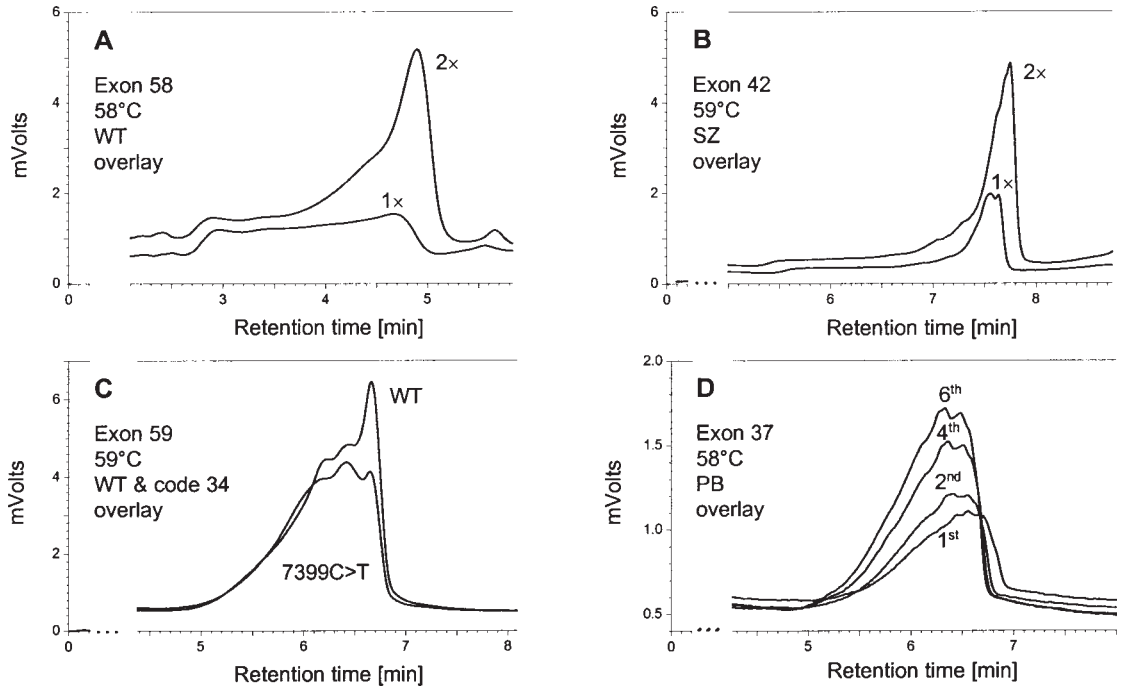

FIGURE 2. **DHPLC profiles demonstrating the influences of the amount (1×, 2×) of amplicons injected (A and B) and a mutation (C) on the chromatogram. Increased signal intensity and slight elution profile changes were also observed in the first ~6 DHPLC runs after the column was cleaned with 75% (v/v) acetonitrile at 60°C for 30 min and equilibrated for 30 min (D). Corresponding chromatograms are overlaid.**

products and DHPLC column conditions (e.g., after column cleaning) influenced the shape of chromatograms (Fig. 2).

Blind DHPLC analysis of 682 PCR products varying in size from 142 to 507 base pairs and subsequent sequencing of 177 amplicons revealed 62 of the 64 known *FBN1* sequence variants, resulting in a detection rate of 97% (89–99%, P = 0.05). In amplicons of exon 11 (249 bp) and exon 56 (196 bp), respectively, blinded DHPLC analysis failed to detect the encoded mutations (codes 57 and 41 in Table 1). The amplicon of exon 11 displayed only DHPLC signals lower than 2mV. The amplicon of exon 56 contained a polymorphism (IVS56+17G>C; Table 4) in addition to a mutation (6919T>C, code 41; Table 1). However, after repetition of PCR and DHPLC both amplicons showed an abnormal DHPLC profile (Fig. 3), which is predicted to harbor a sequence variation. Interestingly, the amplicon of exon 56 displayed a less abnormal DHPLC pattern (profile C) than those amplicons that contained only the polymorphism IVS56+17G>C (profile B; Fig. 3B and C).

In the three MFS patients (BK, PB, and SZ) of uncharacterized genotype, DHPLC analysis of all 65 exons resulted in 20 abnormal elution profiles. Of these amplicons, only two carried a mutation (4409G>A in exon 35 and 4621C>T in exon 37) and seven harbored a polymorphism (Table 4). In the third patient (SZ), six polymorphisms were found by DHPLC (Table 4), but no mutation was detected. Even bi-directional sequencing of the entire coding region of the *FBN1* gene failed to detect any pathogenic mutation as well as any additional sequence changes in this patient.

Taking together all DHPLC analyses, a total of 95 sequence variants were detected (Tables 3 and 5), 69 of which were unique (Tables 1, 3, and 4). Sequence analysis of 197 amplicons with aberrant DHPLC patterns revealed that the elution profiles of 102 fragments showed false positive signals (Table 5), resulting in an overall specificity of 48% (41–55%, P = 0.05). However, false-positive signals were produced only by samples with DHPLC profiles B (14/102; 8–22%, P = 0.05), B/C (15/102; 9–23%, P = 0.05) and, most frequently, with profile C (73/102; 62–79%, P = 0.05); high pattern abnormalities (A and A/B) revealed no false-positive signals. In addition, amplicons with sequence variation

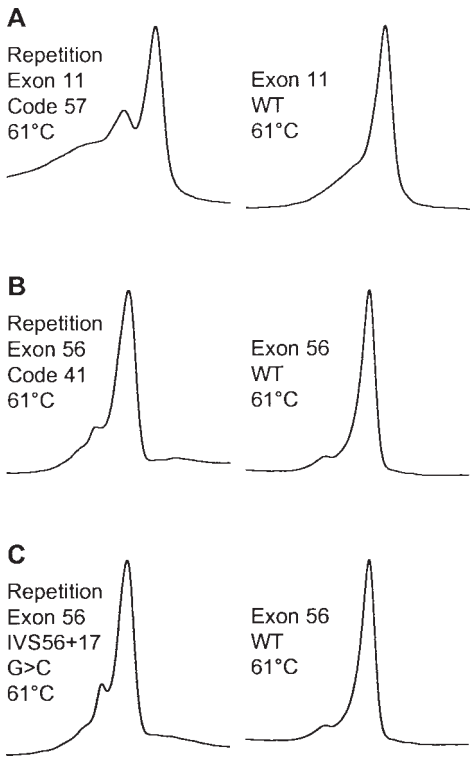

FIGURE 3. **Chromatograms after repeated DHPLC analysis of PCR products in which blind DHPLC analysis failed to detect an encoded *FBN1* mutation; this was probably due to the low quality of amplicon (A) or the simultaneous occurrences of two single-base changes (B: 6919T>C and IVS56+17G>C). DHPLC profiles of corresponding wild-type (WT) samples and amplicon carrying only one of the two single-base changes (IVS56+17A>G) are also shown (C).**

(true positives) showed significantly less often (9/82; 6–20%, P = 0.05) a low pattern abnormality (C) compared to false positives (73/82; 80–94%, P = 0.05).

Based on the 197 sequenced PCR products with pattern abnormalities (Table 5), we did not find a clear relationship between DHPLC profile and the type of sequence change because the 95% confidence limits of their observed proportions overlapped each other (data not shown). Exceptions are 1) high pattern abnormalities (A and A/B), which were caused most frequently (19/30; 46–78% and 6/8; 41–93%; P = 0.05) by A-C and G-T mismatches resulting from A>G, G>A, C>T, and T>C transitions; and 2) amplicons with base changes A>G (mismatches A–C, G-T) and G>C (mismatches C–C, G–G), which most frequently produced DHPLC profile A (10/15; 42–85%, P = 0.05) and B (7/8; 53–98%, P = 0.05), respectively (cf. Table 5). However, different sequence variants are represented unequally,

TABLE 5. **Number of PCR Products Sequenced in This Study for Different Types of *FBN1* Sequence Changes and DHPLC Profiles***

		Degree of DHPLC pattern abnormality					
Sequence variation		A	A/B	B	B/C	C	Total
Base change							
A>T (A-A, T-T)		0	0	1	0	0	1
T>A (A-A, T-T)		0	0	1	0	0	1
C>G (C-C, G-G)		1	1	0	0	0	2
G>C (C-C, G-G)		0	0	7	1	0	8
A>C (A-G, T-C)		0	0	0	0	0	0
C>A (A-G, T-C)		0	0	0	2	0	2
T>G (A-G, T-C)		0	0	1	1	0	2
G>T (A-G, T-C)		0	0	0	1	0	1
A>G (A-C, G-T)		10	1	2	1	1	15
G>A (A-C, G-T)		3	3	5	4	2	17
C>T (A-C, G-T)		6	2	3	2	2	15
T>C (A-C, G-T)		0	0	5	3	1	9
double/complex		5	0	1	1	1	8
	Σ	25[a]	7[b]	26[c]	16[d]	7[e]	81
Small insertion	Σ	3[f]	1[g]	3[h]	1[h]	0	8
Small deletion	Σ	2[i]	0	1[j]	1[j]	2[j]	6
No sequence variation	Σ	0	0	14	15	73	102
Total		30	8	44	33	82	197

*A, B, and C denote high, medium, and low pattern abnormality, respectively (cf. Fig. 1). Possible heteroduplex mismatches are given in parentheses for each single-base change.
[a]6 mutations, 14 polymorphisms, 1 amplicon with both of them, 3 amplicons with two polymorphisms, and 1 amplicon with one mutation and 1 nt deletion.
[b]7 mutations.
[c]18 mutations, 7 polymorphisms, and 1 amplicon with both of them.
[d]11 mutations, 4 polymorphisms, and 1 amplicon with two polymorphisms.
[e]3 mutations, 3 polymorphisms, and 1 amplicon with both of them.
[f]One 23 nt and two 15 nt insertions.
[g]10 nt insertion.
[h]1 nt insertion(s).
[i]2 nt deletions.
[j]1 nt deletion(s).

ranging from 0 to 17 in frequency, and most of them also by a total number of lower than eight, rendering the identification of significant proportion differences more difficult and a priori impossible, respectively (Table 5).

DISCUSSION

In this study, we have evaluated the utility of DHPLC for the detection of *FBN1* mutations. Our study was based on a large collection of DNA samples containing both new and previously identified *FBN1* sequence variants and on a total of 876 derived PCR products (217 kb) representing all 65 coding exons of the *FBN1* gene. DHPLC followed by direct sequencing of a total of 197 PCR products allowed us to draw conclusions on the sensitivity and specificity of

DHPLC as well as on the specific analytical conditions used.

Sensitivity of DHPLC

Blinded analysis of *FBN1* revealed a sensitivity of DHPLC as high as 97% (89–99%, P = 0.05). This value is similar to that found in the study of many other genes (>96%) [reviewed in Xiao and Oefner, 2001], and more exact than that described in a DHPLC study of *FBN1* on only 22 DNA samples (85–100%, P = 0.05) [Liu et al., 1997]. The true sensitivity of DHPLC, however, may be lower than has been generally observed because the tested sequence variants may not have been representative of all sequence changes of *FBN1* due to the likewise limited detection rate of previously used mutation screening methods [reviewed in Nollau and Wagener, 1997].

Out of the 64 known *FBN1* sequence variants, only two were not detected by blinded DHPLC analysis: mutations in amplicons of exon 11 and 56 (Table 1; Fig. 3). The failure to detect the mutation in exon 11 most likely was due to a low chromatogram signal (A_{260}<2mV) since this mutation was clearly detected after increasing the amount of DNA in the analysis (Fig. 3A). The detection of the mutation in exon 56 (6919T>C) was rendered more difficult by the presence of an additional polymorphism (IVS56+17G>C; Fig. 3B and C). In this sample, even the substitution of dGTP by 7-deaza-2′-dGTP in the PCR did not result in a higher DHPLC pattern abnormality, in spite of the fact that both the mutation and the polymorphism were located in the high-melting domain (data not shown). In contrast, three other amplicons (codes 40, 54, and 31 in Table 1), each of which contained both a mutation (IVS6-1G>A, 1285C>T, and 1960G>A) and a polymorphism (IVS6-37delT, 1224C>T, and 1875T>C), did not show lower DHPLC profile abnormalities than corresponding amplicons that carried only the polymorphism (Tables 1 and 4).

From these observations we conclude that the signals of DHPLC profiles should have intensities higher than 2mV at A_{260} (corresponds to ~50–200ng of PCR product) in order to achieve higher sensitivity (Fig. 2); and that amplicons of exon 56 should always be sequenced even if DHPLC does not indicate any sequence variation. In addition, GC-clamped primers in PCR and 3M betaine in hybridization procedure can be used to improve the efficiency of mutation detection by DHPLC [Narayanaswami and Taylor, 2001]. In the case where the sample contained both a polymorphism and a mutation in exon 56 (code 41), however, the use of betaine did not lead to any improvements (data not shown). Nevertheless, amplicons potentially harboring polymorphisms, e.g., those given in Table 4, should be sequenced first if DHPLC fails to detect a pathogenic mutation (cf. Fig. 4).

Specificity of DHPLC

The frequency of amplicons with DHPLC profile abnormality but without any sequence variation was significantly (P = 0.05) higher in our study (102/197) than in previous studies (1/137 and 4/166, respectively) [Gross et al., 2000; Spiegelman et al., 2000]. In our study, however, false-positive signals were only found in amplicons with medium or low DHPLC profile abnormalities that may have been ignored in previous studies. We can only speculate about the causes of the false-positive results, but most likely they are due to the different quality (i.e., presence of unspecific PCR fragments) and quantity of the PCR products compared (cf. Fig. 2), even if this is not always suggested by agarose gel analysis. Furthermore, false-positive results were found significantly more frequently in two DNA samples (code 59, 62; data not shown), suggesting that differences in the quality of genomic DNA samples after extraction can also lead to false-positive DHPLC signals. Nevertheless, it cannot be ruled out that some of the false positives in reality harbor a mutation at low frequency (<10%) that is not detectable by sequencing [Jones et al., 2001], e.g., due to asymmetric PCR or somatic mosaicism. In order to reduce false positives, quality and quantity (A_{260}≥2mV) of PCR products, as well as column, oven, and buffer conditions, should be kept as identical as possible for each chromatogram to be compared. To achieve this, it is necessary 1) to quantify PCR products prior to or by means of DHPLC, 2) to analyze corresponding samples by DHPLC successively, and maybe 3) to optimize PCR conditions by designing further novel PCR primers (cf. Table 2).

The number of false-positive signals may also be influenced by the subjective classification of low-pattern abnormalities. For instance, if

amplicons with DHPLC profile C (Table 5) had been classified as normal, the specificity of DHPLC would have increased from 48% (41–55%, P = 0.05) to 75% (66–82%, P = 0.05). In this case, however, six additional polymorphic fragments would have remained undetected, decreasing the sensitivity to 88% (77–94%, P = 0.05). If high-throughput mutation detection is needed and sensitivity lower than 95% can be accepted (e.g., for non-diagnostic purposes), the threshold for pattern abnormality can be defined between categories B and C (Fig. 1).

Correlation Between DHPLC Profile and Mutation Type

We did not observe any clear correlation between DHPLC profile type and underlying mutation (Table 5). We also did not find evidence for a correlation between amplicon length and profile type (R^2 = 0.009 based on Table 3; data not shown). These results suggest that the degree of DHPLC pattern abnormality is mainly influenced by the sequences flanking the mismatch rather than by the nature of the mismatch itself. The reason for this phenomenon may lie in neighboring stacking interactions [Ke and Wartell, 1993] and hydrogen bonding between non-Watson-Crick base oppositions such as G-T and G-A [Aboul-ela et al., 1985]. In our study, two pairs of mutations (encoded 35, 34; and 4, 64 in Table 1) provide evidence for a mismatch stabilization effect of flanking bases, both representing neighboring mutations (Δ1 bp) which result in different single-base mismatches but in

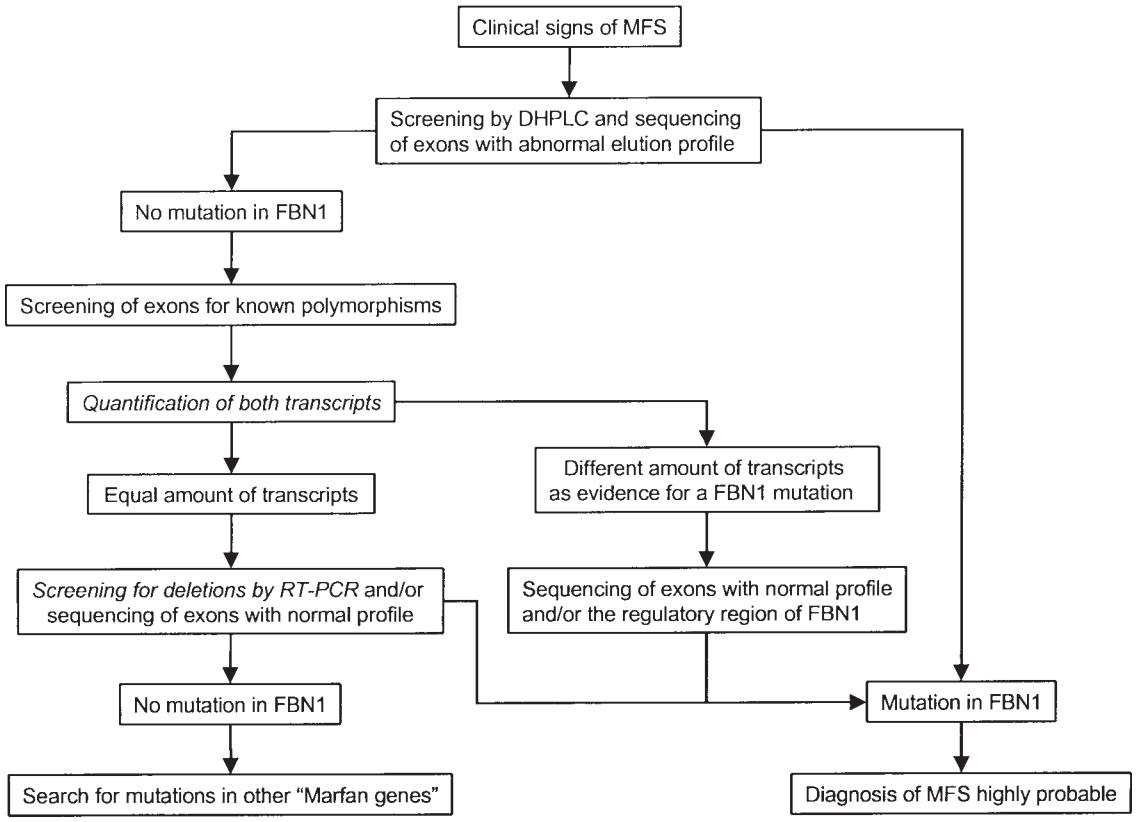

FIGURE 4. **Flowchart for a possible mutation screening strategy in the *FBN1* gene. Analyses to be performed at RNA (extracted preferably from fibroblasts) level are indicated by italics. It should be noted that the relative quantification of allele-specific transcripts can provide evidence for the presence of mutations that alter pre-mRNA splicing, generate premature termination codon, or affect the gene regulatory region because such mutations can influence mRNA abundance [e.g., Nagy and Maquat, 1998; Valentine, 1998]. However, transcript quantification requires both a known sequence variation in the *FBN1* coding region and an accurate method [e.g., Karttunen et al., 1996; Mátyás et al., 2001]. Furthermore, large deletions are maybe not detectable by using this strategy and unexpected homozygous mutations can be reliably identified only by adding wild-type DNA to all PCR samples prior to DHPLC [Liu et al., 1998]. In addition, DHPLC prescreening can also be performed at the cDNA level [Schrijver et al., 1999] but, in this case, mutation detection rate may be lower due to the reduced abundance of mutated mRNA [reviewed in Maquat, 1995]. Since DHPLC is at least 10 times less expensive than sequencing [Wagner et al., 1999], the costs for mutation detection by our strategy are ~50% lower than for that by sequencing alone.**

identical (B, B) or very similar (B, B/C) pattern abnormalities (Tables 1 and 5).

DHPLC Analysis Conditions

We have presented here for the first time specific DHPLC conditions for each *FBN1* exon. Performing the analysis of all selected temperatures, a total of 195 injections are required for each DNA sample. Considering the fact that the discrepancy between actual and displayed temperature in the column oven can increase over a period of months [Xiao and Oefner, 2001], the use of more than one temperature per exon provides a potentially higher detection rate, especially for those mutations that are detectable at a single temperature only (Tables 1 and 4). Furthermore, the use of only one oven temperature per exon (e.g., 64°C for exon 24; Table 3) harbors the risk that some mutations in the low-melting domains may be missed. However, if high-throughput is preferred rather than high sensitivity, DHPLC could be carried out only at a single temperature for exons in which sequence variations were detectable at several temperatures (Table 3). Our DHPLC conditions were evaluated for 39 exons by analyzing heterozygous samples (Table 3); for the remaining 26 exons, DHPLC conditions could be examined only by means of homozygous PCR fragments.

Screening Strategy for *FBN1* Mutations

While direct sequencing may be the most efficient way to identify a mutation in small genes, screening methods appear to be advisable for larger genes such as *FBN1*. We have outlined a possible strategy for mutation detection in *FBN1* based on DHPLC as depicted in Figure 4. We have applied this strategy to screen DNA from three MFS patients (BK, PB, and SZ). Indeed, in two patients (BK and PB) we identified two *FBN1* mutations, one of which is described here for the first time (4409G>A, C1470Y); whereas we could rule out the presence of *FBN1* mutation in the third patient (SZ) by quantification of both transcripts (1.0:1.0), RT-PCR, and bi-directional sequencing of all 65 exons (data not shown). In this latter case, a second Marfan locus (MFS2) [Collod et al., 1994] may be mutated.

In order to apply our strategy to large-scale screening, further improvements in sample throughput will be necessary. Using robotic PCR and capillary array HPLC systems interfaced with multi-color fluorescence detection [Huber et al., 2001; Xiao et al., 2001] and labeling different fragments that share a common melting temperature will increase throughput of DHPLC analysis.

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