

## Calpainopathy—A Survey of Mutations and Polymorphisms

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### Summary

Limb-girdle muscular dystrophy type 2A (LGMD2A) is an autosomal recessive disorder characterized mainly by symmetrical and selective atrophy of the proximal limb muscles. It derives from defects in the human CAPN3 gene, which encodes the skeletal muscle-specific member of the calpain family. This report represents a compilation of the mutations and variants identified so far in this gene. To date, 97 distinct pathogenic calpain 3 mutations have been identified (4 nonsense mutations, 32 deletions/insertions, 8 splice-site mutations, and 53 missense mutations), 56 of which have not been described previously, together with 12 polymorphisms and 5 non-classified variants. The mutations are distributed along the entire length of the CAPN3 gene. Thus far, most mutations identified represent private variants, although particular mutations have been found more frequently. Knowledge of the mutation spectrum occurring in the CAPN3 gene may contribute significantly to structure/function and pathogenesis studies. It may also help in the design of efficient mutation-screening strategies for calpainopathies.

### Introduction

Limb-girdle muscular dystrophy type 2A (LGMD2A [MIM 253600]) is an autosomal recessive disorder characterized by symmetrical and selective atrophy of the pelvic, scapular, and trunk muscles; elevated serum cre-

atine kinase; and a necrotic regeneration pattern on muscular biopsies. Classically, calf hypertrophy is rare and always discrete, and there is no mental, cardiac, or facial disturbance. In the vast majority of cases, the symptoms arise in childhood, progress gradually, and, 10–20 years after onset, patients are frequently unable to walk and are confined to a wheelchair (Beckmann and Fardeau 1998). A limb-girdle muscle involvement can also be recognized in a number of muscular disorders—in particular, in the other progressive muscular dystrophies that also have been regrouped under the term “LGMD2”; the latter comprise the four sarcoglycanopathies (LGMD2C–LGMD2F; (Roberds et al. 1994; Bönnemann et al. 1995; Lim et al. 1995; Noguchi et al. 1995; Nigro et al. 1996), which are characterized by a disruption of the sarcoglycan complex (Campbell 1995), and three additional LGMD2 entities (LGMD2B, LGMD2G, and LGMD2H [Bashir et al. 1994; Moreira et al. 1997; Weiler et al. 1998]), which present no alterations in the sarcoglycan complex. The causative gene for LGMD2B recently has been identified as coding for dysferlin (Bashir et al. 1998; Liu et al. 1998).

The gene responsible for LGMD2A had been localized, by linkage and linkage-disequilibrium analyses, to chromosome 15q15.1–15.3, within a 1-cM region almost entirely covered by a 1.6-Mb YAC (Beckmann et al. 1991; Fougousse et al. 1994; Allamand et al. 1995). Characterization of genes from this region led eventually to the identification of the LGMD2A gene that encodes calpain 3, a member of the calpain family (Chiannilkulchai et al. 1995; Richard et al. 1995). The latter regroups nonlysosomal calcium-dependent cysteine proteases, whose precise functional roles remain elusive.

The human calpain 3 gene (CAPN3) covers a genomic region of ~40 kb (Richard et al. 1995). It is expressed predominantly in the skeletal muscle tissue, as a 3.5-kb transcript, driving the translation of a 94-kD protein containing a proteolytic domain, a Ca<sup>++</sup>-activated domain, and two other domains with no known homology

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**Table 1****Primer Pair Used for Detection of Mutations, by Allele-Specific PCR**

MUTATION	PRIMER(S) <sup>a</sup> (5'→3')		TOUCHDOWN PCR	
	Upper <sup>a</sup>	Lower	Temperature Range (°C)	Formamide Concentration (%)
550ΔA	ATAAGATGACTGCCTGCCAAC (1) TAGATGACTGCCTGCCAC (2)	TTCCTGTGAGTGAGGTCTCG	65–60	
G222R	CTACGAAGCTCTGAAAGGTG (1) CTACGAAGCTCTGAAAGGTA (2)	GGCTTTCTTCATGATCTTGT	60–55	2
IVS6–1G→A	CTCTGGTTACTGCTCTACAA (1) CTCTGGTTACTGCTCTACAG (2)	AGCACGAAAAGCAAAGATAAA	63–58	
R489W	GCCCTGATGCAGAAGAACC (1) GCCCTGATGCAGAAGAACT (2)	CCAGGAGCTCTGTGGGTCA	65–60	4
R489Q	GCCCTGATGCAGAAGAACC (1) GCCCTGATGCAGAAGAACCA (2)	CCAGGAGCTCTGTGGGTCA	65–60	4
R572W	AGGGGGAATTCATCCTCCG (1) AGGGGGAATTCATCCTCTG (2)	TTCAACCTCTGGGAGTGGGCC	60–55	2
R572Q	AGGGGGAATTCATCCTCCG (1) AGGGGGAATTCATCCTCCA (2)	TTCAACCTCTGGGAGTGGGCC	60–55	2
S744G	AAGAATGGGGTTGATTTGGAG	GCATTTTCGCATCTCGTAGCT (1) GCATTTTCGCATCTCGTAGCC (2)	65–60	2
R748Q	AAGAATGGGGTTGATTTGGAGA	TGCGTCGTTGACTGCATTTT (1) TGCGTCGTTGACTGCATTTT (2)	60–55	
R769Q	AAGAATGGGGTTGATTTGGAGA	ATGTGTTTGTCTGCGTACC (1) ATGTGTTTGTCTGCGTACT (2)	65–60	
1872C→T	CACCAGAGCAAACCGTCCAC	CAGGGCTTGTTTTGCCTTTG (1) CAGGGCTTGTTTTGCCTTTA (2)	63–58	...
2069ΔCA	CAAGGACCTGAAGACACACG (1) CACAAGGACCTGAAGACACG (2)	ACCCTGTATGTTGCCTTGG	65–60	2
2362AG→TCATCT	CATCTGCTGCTTCGTTAG (1) TGCTGCTTCGTTTCATCT (2)	GGAGATTATCAGGTGAGATGCC	60–55	

<sup>a</sup> For each mutation, two sequences are used—one corresponding to the normal allele (1) and the other corresponding to the mutated allele (2).

(Sorimachi et al. 1989). In addition, CAPN3 has three unique sequences—NS, IS1, and IS2. Additional transcripts resulting from alternative splicing and alternative promoter use have been characterized (Ma et al. 1998a, 1998b; Herasse et al., in press; F. Fougerousse, personal communication).

Since the discovery of the molecular defect in LGMD2A, we have collectively undertaken studies to determine the nature, number, and distribution of CAPN3 mutations and to assess potential genotype-phenotype correlations. To date, 97 distinct mutations have been identified, 56 of which have not been described previously.

## Subjects and Methods

### *Description of Families and Preparation of Samples from Patients*

This report summarizes studies, performed in five laboratories (Généthon [France], Hospital Ntra. Sra. Aranzazu [Spain], Hospital Sant Pau [Spain], the DNA diagnostic laboratory [Netherlands], and University of

Newcastle upon Tyne [United Kingdom]), of families from diverse ethnic or geographic origins (including Brazil, Bulgaria, Canada, France, Greece, Israel, Italy, Japan, Lebanon, the Netherlands, Poland, Portugal, Russia, Spain, Switzerland, Turkey, United Kingdom, United States, and Vietnam). Families with LGMD that were from of the U.S. Amish community, from Réunion Island, and Basque country were distinguished from, respectively, families in the remaining United States, Metropolitan French (i.e., continental France), and Spanish families, since each of them represents a particular genetic isolate with a predominant founder effect. All patients presented in this report fulfill the clinical criteria of a classic LGMD phenotype—namely, autosomal recessive inheritance, progression of muscle weakness in a limb-girdle distribution (with sparing of facial muscles), and a clearly dystrophic muscle biopsy (Fardeau et al. 1996a, 1996b). Calpain 3–deficient patients were also ascertained by the Newcastle group, by demonstration of both reduced 94-kD-band labeling and preserved sarcoglycans and dystrophin on western blots of skeletal-muscle biopsies (Anderson et al. 1998).

**Table 2****Mutations in the CAPN3 Gene**

Exon/Intron and Origin	No. of Families (No. of Homozygous Patients)	Position <sup>a</sup> (bp)	Mutation <sup>b</sup>	Position in Amino Acid <sup>c</sup>	Mutation	Reference
1:						
Spain	1	10	GTC→ATC <sup>d</sup>	4	V4I	Hospital Sant Pau
Turkey	1 (1)	19–23	ΔGCATC	6–7	19–23ΔGCATC	Dinçer et al. (1997), Richard et al. (1997)
Réunion Island	1	43	ΔG	15	43ΔG	Généthon
Spain	1	60	ΔA	20	60ΔA	Hospital Sant Pau
Italy	1	77	CCG→CTG <sup>d</sup>	26	P26L	Généthon
France	1	224–225	insT	76	224–225insT	Généthon
France	1	229	GAC→AAC	77	D77N	Généthon
Lebanon	1 (1)	257	TCT→TTT	86	S86F	Richard et al. (1997)
France	1 (1)	277–300	24-bp change	93–100	ΔFPIQFVWK	Généthon
2:						
Brazil	1 (1)	328	CGA→TGA <sup>d</sup>	110	R110X	Richard et al. (1995)
Bulgaria	1	352	AGA→GGA	118	R118G	Généthon
3:						
United States	1	402	ΔC	134	402ΔC	Richard et al. (1997)
France	1 (1)	409	TGC→CGC	137	C137R	Généthon
Polish	1	418–419	insC	140	418–419insC	MGC Leiden
Vietnam	1	424	CAG→TAG	142	Q142X	Hospital Ntra. Sra. Aranzazu
France	1	484	ATC→CTC	162	I162L	Généthon
4:						
France	1	545	CTG→CAG	182	L182Q	Richard et al. (1995)
France	1	548	CCA→CTA	183	P183L	Généthon
France	5	550	ΔA	184	550ΔA	Richard et al. (1995, 1997)
Greece	1					University of Newcastle
Italy	1 (1)					Richard et al. (1997)
Netherlands	2					MGC Leiden
Russia	1 (1)					Généthon
Turkey	5 (5)					Dinçer et al. (1997)
UK	1					University of Newcastle
Réunion Island	1	551	ACG→ATG <sup>d</sup>	184	T184M	Généthon
United Kingdom	1	566	CTG→CCG	<189	L189P	University of Newcastle
Bulgaria	1	598–612	15-bp change	200–204	ΔFWSAL	Généthon
Germany	1					Häffner et al. (1998)

IVS4-7:		5,328-bp change		IVS4+404Δ5328		Généthon	
Basque	1						
Netherlands	1	640	GGT→AGT	G214S	214	MGC Leiden	
France	1	643	TCC→CCC	S215P	215	Richard et al. (1997)	
United States	1	643-663	21-bp change	ΔSYEALKG	215-221	Richard et al. (1997)	
United Kingdom	1	649	GAA→AAA	E217K	217	University of Newcastle	
Basque	4	664	GGG→AGG	G222R	222	Urtasun et al. (1998)	
Spain	1 (1)					Hospital Sant Pau	
Spain	1	676	GAG→AAG	E226K	226	Hospital Natr. Sra. Aranzazu	
France	1	695	ACA→ATA	T232I	232	Généthon	
France	2	701	GGG→GAG	G234E	234	Richard et al. (1995)	
Lebanon	1 (1)	717	ΔT	717ΔT	239	Richard et al. (1997)	
France	2	759-761	ΔGAA	ΔK254	253-254	Généthon	
IVS5:							
Germany	1	801+1	GT→AT	IVS5+1G→A	...	Häffner et al. (1998)	
6:							
France	2	887	ΔA	886ΔA	296	Généthon	
France	1	945	ΔG	945ΔG	315	Richard et al. (1995)	
IVS6:							
Réunion Island	16 (9)	946-1	AG→AA	IVS6-1G→A	...	Richard et al. (1995), Penisson-Besnier et al. (1998)	
7:							
Lebanon	1	956	CCG→CTG <sup>d</sup>	P319L	319	Richard et al. (1997)	
France	1	1002	CAC→CAG	H334Q	334	Richard et al. (1997)	
Turkey	1 (1)	1006	TAC→AAC	Y336N	336	Diğer et al. (1997)	
8:							
France	1 (1)	1061	GTG→GGG	V354G	354	Richard et al. (1995)	
France	1	1079	TGG→TAG	W360X	360	Richard et al. (1995)	
Japan	2 (2)	1080	TGG→TGC	W360C	360	Kawai et al. (1998)	
IVS9:							
France	1	1194-9	A→G	IVS9-9A→G	...	Généthon	
10:							
Spain	1	1292-1293	insT	1292-1293insT	431	Hospital Natr. Sra. Aranzazu	
France	1	1309	CGC→TGC <sup>d</sup>	R437C	437	Hospital Natr. Sra. Aranzazu	
Israel	1 (1)	1318	CGG→TGG <sup>d</sup>	R440W	440	Richard et al. (1997)	
Spain	1	1319-1322	ΔGGGG	1319ΔGGGG	440-441	Hospital Natr. Sra. Aranzazu	
France	1	1322	GGT→GAT	G441D	441	Généthon	
Netherlands	1	1333	GGG→AGA <sup>d</sup>	G445R	445	MGC Leiden	
Netherlands	1	1342	CGC→TGC <sup>d</sup>	R448C	448	MGC Leiden	
Spain	1					Hospital Natr. Sra. Aranzazu	
France	2 (1)	1342	CGC→GGC	R488G	448	Généthon	
France	1	1343	CGC→CAC <sup>d</sup>	R448H	448	Généthon	
United States	1					Généthon	

(continued)

**Table 2 (continued)**

Exon/Intron and Origin	No. of Families (No. of Homozygous Patients)	Position <sup>a</sup> (bp)	Mutation <sup>b</sup>	Position in Amino Acid <sup>c</sup>	Mutation	Reference
11:						
United Kingdom	1	1373	ΔC	458	1373ΔC	University of Newcastle
Spain	1	1435	AGC→GGC	479	S479G	Hospital Natr. Sra. Aranzazu
United Kingdom	1					University of Newcastle
Basque	1	1456	CAG→GAG	486	Q486E	Urtasun et al. (1998)
Basque	2	1465	CGG→TGG <sup>d</sup>	489	R489W	Urtasun et al. (1998)
Réunion Island	2	1466	CGG→CAG <sup>d</sup>	489	R489Q	Généthon
United States	1	1468	CGG→TGG <sup>d</sup>	490	R490W	Richard et al. (1997)
France	1					Richard et al. (1995)
France	5 (2)	1469	CGG→CAG <sup>d</sup>	490	R490Q	Généthon
Turkey	1					Dinçer et al. (1997)
France	1	1477	CGG→TGG <sup>d</sup>	493	R493W	Généthon
Spain	1					Hospital Sant Pau
Italy	1	1486	GGG→AGG	496	G496R	Richard et al. (1997)
Netherlands	1	1505	ATT→ACT	502	I502T	MGC Leiden
13:						
France	1	1611	TAC→TAA	537	Y537X	Généthon
Turkey	1 (1)					Dinçer et al. (1997), Richard et al. (1997)
France	1	1622	CGG→CAG <sup>d</sup>	541	R541Q	Généthon
Switzerland	1 (1)	1699	GGG→TGG	567	G567W	Richard et al. (1997)
France	2 (1)	1714	CGG→TGG <sup>d</sup>	572	R572W	Richard et al. (1997)
Réunion Island	1 (1)	1715	CGG→CAG <sup>d</sup>	572	R572Q	Richard et al. (1995)
France	1					Généthon
Europe	1	1743/1744	ΔTG	581–582	1743ΔTG	Hospital Natr. Sra. Aranzazu
15:						
Spain	1	1785/1788	ΔAAAAG	595–596	1785ΔAAAAG	Hospital Sant Pau
Japan	1 (1)	1795/1796	insA	599	1795–1796insA	Kawai et al. (1998)
16:						
Italy	1	1817	TCC→TTC <sup>d</sup>	606	S606L	Richard et al. (1997)
Europe	1					Hospital Natr. Sra. Aranzazu
France	2	1838	ΔA	613	1838ΔA	Généthon
France	1	1865	ΔAG	622	1865ΔAG	Généthon
Réunion Island	1 (1)	1872	GGC→GGT <sup>d</sup>	624	1872 C→T	Richard and Beckmann (1995)
Netherlands	1	1913	CAG→CCG	638	Q638P	MGC Leiden
17:						
Bulgaria	1	1981/1984	ΔATAG	661–662	1981ΔATAG	Généthon
France	1	1981	ΔA	661	1981ΔA	Généthon
United Kingdom	1	1983	ΔA	661	1983ΔA	University of Newcastle

IVS17:													
Spain	2	1992+1	GT→AT	...	IVS17+1G→T	Hospital Nattr. Sra. Aranzazu							
United Kingdom	1	1993-1	AG→AT	...	IVS17-1G→T	University of Newcastle							
19:													
Réunion Island	1	2069/2070	ΔAC	690	2069ΔCA	Richard et al. (1995)							
United Kingdom	1	2093	CGT→CCT	698	R698P	University of Newcastle							
Turkey	1 (1)	2105	<u>GCG</u> → <u>G<sub>T</sub>G</u> <sup>d</sup>	702	A702V	Dinçer et al. (1997), Richard et al. (1997)							
France	1 (1)					Généthon							
France	1	2113	GAT→CAT	705	D705H	Hospital Nattr. Sra. Aranzazu							
Greece	1	2114	GAT→GCT	705	D705G	University of Newcastle							
IVS20-exon21:													
Spain	1	(2185-12)/2194	22-bp change	...	V(IVS20-12)/2194Δ	Hospital Nattr. Sra. Aranzazu							
IVS20:													
France	1	2185-2	AG→GG	...	IVS20-2AG→GG	Généthon							
Canada	1 (1)					Généthon							
21:													
France	1	2192	TTC→TCC	731	F731S	Généthon							
Réunion Island	3	2230	<u>AGC</u> → <u>GGC</u>	744	S744G	Richard et al. (1995), Penisson-Besnier et al. (1998)							
Basque	3	2243	<u>CGA</u> → <u>CAA</u> <sup>d</sup>	748	R748Q	Urtasun et al. (1998)							
Spain	4 (2)					Hospital Sant Pau, Hospital Ntra. Sra. Aranzazu							
Turkey	1 (1)					Dinçer et al. (1997), Richard et al. (1997)							
IVS21:													
Spain	1	2263+2	GT→GA	...	IVS21+2T→A	Hospital Nattr. Sra. Aranzazu							
22:													
United States (Amish)	19 (19)	2306	<u>CGG</u> → <u>CAG</u> <sup>d</sup>	769	R769Q	Richard et al. (1995)							
Brazil	1 (1)					Richard et al. (1995)							
France	2					Richard et al. (1995)							
Brazil	1	2313-2316	ΔAGAC	771-772	2313ΔAGAC	Richard et al. (1995)							
France	1	2317-2321	AAACA→T	773-774	2317AAACA→T	Richard et al. (1995)							
Netherlands	1	2319-2320	insA	774	2319-2320insA	Généthon							
Brazil	1	2362-2363	AG→TCATCT	788	2362AG→TCATCT	MGC Leiden							
Réunion Island	3					Richard et al. (1997)							
Basque	39 (31)					Richard et al. (1997)							
Spain	8 (3)					Urtasun et al. (1997)							
United States	2					Richard et al. (1998)							
						Hospital Sant Pau, Hospital Nattr. Sra. Aranzazu							
						Richard et al. (1997)							

<sup>a</sup> Numbered on the basis of the cDNA (EMBL accession number 85030), starting from ATG.

<sup>b</sup> Mutated nucleotides are underlined.

<sup>c</sup> Numbered on the basis of the protein sequences, starting from the first methionine residue.

<sup>d</sup> Modified CpG site.

**Table 3****Nature and Frequency of the Polymorphisms Identified in the CAPN3 Gene**

Location	Name	Position (bp)	Event	CpG?	Frequency <sup>a</sup>
Promoter	-408T→C	-408	T→C	No	ND
Exon 1	T3T	9	ACC→ACT	No	<.01
Exon 1	T32T	96	ACT→ACC	No	.12
Exon 2	C106C	318	TGC→TGT	Yes	.04
Exon 2	E107K	319	GAG→AAG	Yes	.05
Exon 3	F165F	495	TTC→TTT	No	.01
Exon 3	Q166Q	498	CAG→CAA	No	<.01
Exon 5	A236T	708	GCA→ACA	No	.10
intron 9	IVS9-26C→G	1194-26	C→G	No	.07
Exon 13	I556I	1668	ATC→ATT	Yes	<.01
Exon 18	L673L	2019	CTC→GTC	No	<.01
Intron 22	2380+12ΔA	2380+12	ΔA	No	.10

<sup>a</sup> Based on study of >100 independent chromosomes; ND = not determined.

Altogether, 180 families were selected on the basis of identification of variation in the CAPN3 gene. Several series of control chromosomes were examined, including those of the parents of patients, those of CEPH individuals, and/or those of control individuals from matched geographic origins. Genomic DNA was prepared from either peripheral-blood lymphocytes or cultures of lymphoblastoid cell lines.

#### Identification of DNA Sequence Variants

There was no uniform strategy for variant identification, since different protocols were used in each of the centers involved. These protocols included the following:

1. Illegitimate transcription analyses of cDNA—These were performed as in the study by Richard et al. (1995), by use of total cellular RNA extracted from lymphoblastoid cell lines, followed by a final characterization by DNA sequencing.

2. Heteroduplex analysis—Ten microliters of the PCR product were denatured at 94 °C for 5 min and were allowed to renature at room temperature for ≥1 h. The product was electrophoresed on Hydrolink gel prepared according to the manufacturer's recommendations. For PCR products of ~150 bp, the conditions of migration are an overnight (~16 h) run at 400 V. After the gel was stained by ethidium bromide, the products are visualized under UV light. Identification of the variant sequences was performed by DNA sequencing.

3. SSCP—Five microliters of the PCR products were denatured at 95°C for 5 min and cooled on ice, with 10 μl of loading buffer (95% formamide and 0.1% bromophenol blue). The samples were separated on 0.4-mm-thick 6% acrylamide 5% glycerol gels in 0.5 × Tris-borate EDTA. Gels were run at room temperature, at 8 W for 16 h. The samples were transferred to nylon filters and hybridized with the primers used in the PCR,

according to the ECL protocol (Amersham), as described by Vignal et al. (1993). Identification of the variant sequences was performed by DNA sequencing.

4. Direct sequencing—One hundred nanograms of DNA were amplified under buffer and cycle conditions described by Richard et al. (1995). Each exon was amplified by specific primers, chosen in introns. The products of PCR were directly sequenced, with the same primers, by dye-dideoxy sequencing, after purification through either Microcon devices (Amicon) or polyacrylamide gel Biogel P-25 (Bio-Rad).

5. Allele-specific PCR—Oligonucleotides for mutations 551ΔA, G222R, IVS6-1G→A, R489W, R489Q, R572W, R572Q, S744G, R748Q, R769Q, 1872C→T, 2069ΔCA, and 2362AG→TCATCT were designed such that their 3' bases were at the point of the mutation (table 1). One hundred nanograms of DNA were amplified by touchdown PCR in a 50-μl volume containing 1 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.1% Triton X-100, 200 μM of each dNTP, 100 ng of each primer, and 2 units of *Taq* polymerase (Perkin-Elmer). It was necessary to add various amounts of formamide, to increase the specificity of some PCRs. After 5-min denaturation at 96°C, thermocycling was performed as follows: 94°C for 40 s and then 30-s annealing steps starting 5°C higher than the annealing temperature and decreasing 1°C every two cycles until the annealing temperature was reached. These steps were followed by 20 cycles at 94°C for 40 s and 30 s at the annealing temperature. After PCR, the products were electrophoresed on a 2% horizontal agarose gel stained with ethidium bromide.

#### Haplotype Analysis

Short tandem repeats covering a region of 1 cM at the LGMD2A locus at 15q15.2 (markers D15S514, D15S779, D15S782, D15S780, and D15S778) were

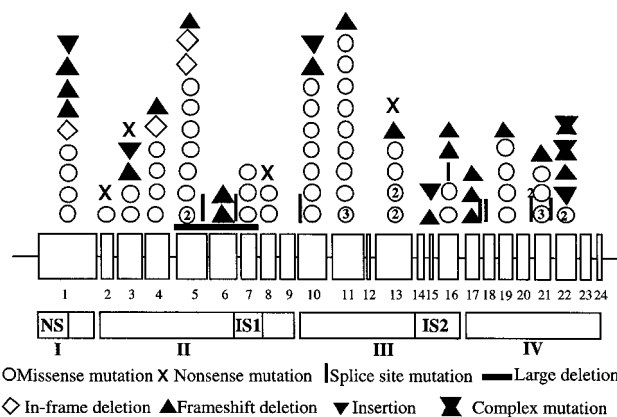
used to assign haplotypes, as described in a previous publication (Richard et al. 1997).

## Results

Altogether, 114 variants have been detected in the CAPN3 gene (table 2). Ninety-seven mutations were identified after screening of the CAPN3 gene in a total of 180 families from 22 different ethnic or geographic origins. Ninety-six mutations were characterized in our laboratories, and the additional one (IVS5+1G→A) was previously reported as having occurred in a German family (Häffner et al. 1998). In most families, both mutations were identified, with the exception of 24 families for which only one allele each has been uncovered. Twelve polymorphisms were uncovered in the course of these studies (table 3). Five additional variants (table 4), found only in patients, were not demonstrated to be causative mutations and therefore could not be classified as belonging to either of the two formerly described groups.

### Mutations Identified

A summary of all the CAPN3 mutations is presented in table 2 and figure 1. The names of the mutations follow published guidelines (Antonarakis 1998). The mutations include 65 single-base-pair substitutions—which resulted in a change of amino acid in 53 cases, in premature stop codons in 4 cases, and in splicing defects in 8 cases. Twenty-three (35%) of these 65 point mutations affect a CpG dinucleotide. An additional 32 mutations were small insertions and/or deletions. Therefore, ~45% (44/97) of the CAPN3 mutations are likely to result in a truncated protein and, therefore, are likely to be inactivating. For the missense mutations, the following arguments are used to infer their pathogenic nature: (i) the fact that they were not encountered in control populations (in general, ≥100 independent chromosomes were examined) and (ii) the observation that all but one of these mutations involve changes of amino acids that are strictly conserved among the human, mouse, rat, bovine, and porcine CAPN3 sequences, if



**Figure 1** Distribution of mutations along the CAPN3 transcript and protein. The mutations are represented, according to their type, along the schematic representation of the CAPN3 gene and protein. The number of independent events is given either inside or above the symbol of the mutation. The 24 exons are indicated by unblackened boxes (with the exon numbers below them). The four domains (I-IV) and the muscle-specific sequences (NS, IS1, and IS2) of calpain 3 also are shown.

not among all the calpains (table 5). Furthermore, some mutations were demonstrated to induce consequences with regard to three biochemical characteristics of CAPN3—namely, titin-binding ability, autolysis capacity, and fodrinolysis capacity (Ono et al. 1998).

Interestingly, one of the splicing mutation (1872C→T) changes the third base of a codon to a synonymous codon yet leads to both the creation of a novel internal splice site and the subsequent loss of the end of the exon and, eventually, to a premature in-phase stop codon (Richard and Beckmann 1995). Demonstration of the pathogenic nature of this sequence variant required the examination of the transcription product. The seven remaining splice mutations are located within 5' or 3' splice sites. IVS5+1G→A was reported to induce skipping of exon 5 (Häffner et al. 1998), and IVS6-1AG→AA leads to the use of a cryptic splice site upstream in the intron. The consequences, at the RNA level, of the other splice-site mutations were not ex-

**Table 4**

### Unclassified Variants

Exon/Intron	Origin	No. of Families	Position (bp)	Event	Amino Acid Position	Putative Effect
7	Spain, Netherlands	2	984	TGC→TGT <sup>a</sup>	C328	Splicing?
10	Switzerland	1 (1)	1263	CTG→CTA <sup>a</sup>	L421	Splicing?
IVS12	Japan	1 (1)	1525-1534	ΔC	...	Splicing?
22	Turkey	1	2320	CAC→GAC	H774	H→D
23	United Kingdom	1	2393	GCA→GAA	A798	A→E

NOTE.—Data are as defined in the footnotes to table 2.

<sup>a</sup> Modified CpG site.



Table 5

## Conservation of Amino Acids at Locations of Missense Mutations and Nonclassified Variants of the CAPN3 Protein

MUTATION	AMINO ACIDS IN								
	CAPN3 of <sup>aa</sup>						Other Calpains <sup>b</sup>		Calpain Homologue(s) <sup>b</sup>
	Human	Mouse	Rat	Porcine	Bovine	Chicken	Mammalian	Nonmammalian	
V4I	V	V	V	V	...	V	Not relevant <sup>c</sup>	Not relevant <sup>c</sup>	Not relevant <sup>c</sup>
P26L	P	P	P	A	...	S	Not relevant <sup>c</sup>	Not relevant <sup>c</sup>	Not relevant <sup>c</sup>
D77N	D	D	D	D	...	D	D	D	D
S86F	S	S	S	S	...	S	S, A	A, S	S, A
R118G	R	R	R	R	...	R	R	R	N, R, S
C137R	C	C	C	C	...	C	S, C	S, N, C	C, S, N
I162L	I	I	I	I	...	I	I	I, V	I
L182Q	L	L	L	L	...	L	L	L	L
P183L	P	P	P	P	...	P	P	P	P
T184M	T	T	T	T	...	T	T, I	T, V	T
L189P	L	L	L	L	...	L	L	L	L
G214S	G	G	G	G	...	G	G	G	G
S215P	S	S	S	S	...	S	S, C	C, S	C, S
E217K	E	E	E	E	...	E	E	E, A	E, Q
G222R	G	G	G	G	...	G	G	G	G, L
E226K	E	E	E	E	...	E	E	E	D, E
T232I	T	T	T	T	...	T	T	T	T
G234E	G	G	G	G	...	G	G	G	G, T
P319L	P	P	P	P	P	P	Not relevant <sup>c</sup>	Not relevant <sup>c</sup>	Not relevant <sup>c</sup>
H334Q	H	H	H	H	H	H	H	H	H, Y
Y336N	Y	Y	Y	Y	Y	Y	Y	Y	Y
V354G	V	V	V	V	V	V	I	I, M	I, V
W360C	W	W	W	W	W	W	W	W	W, L
R437C	R	R	R	R	...	R	N, T, R, S	E, Q, N, S, T	E, D, N
R440W	R	R	R	R	...	R	R	R, P	L, R, P, Q
G441D	G	G	G	G	...	G	G	G, N	M, G, Q
G445R	G	G	G	G	...	G	G	G	G
R448H or G or C	R	R	R	R	...	R	R, L	R, I	Y, I, R
S479G	S	S	S	S	...	S	S, T	S, T	T
Q486E	Q	Q	Q	Q	...	Q	Q	Q	Q
R489W or Q	R	R	R	R	...	R	R	R, I, T	L, K, R
R490Q or W	R	R	R	R	...	R	R, Q	R	R
R493W	R	R	R	R	...	R	R, K	R, K	R
G496R	G	G	G	G	...	G	G	G	G
I502T	I	I	I	I	...	I	I	I	I
R541Q	R	R	R	R	...	R	R	R	R
G567W	G	G	G	G	...	G	G, A	G, A, V	G, S
R572Q or W	R	R	R	R	...	R	R	R	R
S606L	S	S	S	S	...	S	Not relevant <sup>c</sup>	Not relevant <sup>c</sup>	Not relevant <sup>c</sup>
Q638P	Q	Q	Q	K	...	...	Not relevant <sup>c</sup>	Not relevant <sup>c</sup>	Not relevant <sup>c</sup>
R698P	R	R	R	R	...	R	R, K	R, K, N	K
A702V	A	A	A	A	...	A	D, N, S, H	N, A, D, H	D
D705H or G	D	D	D	D	...	D	D, E	D, Q, T	D
F731S	F	F	F	F	...	F	F, Y	F, Y, I	Y
S744G	S	S	S	S	...	S	S, A, T	F, S, G, A	S
R748Q	R	R	R	R	...	R	R	R, D	R
R769Q	R	R	R	R	...	R	R	R	R
H774D	H	H	H	Y	...	N	D, E, N, Q, K	D, E	Q
A798E	A	A	A	A	...	A	Q, A, C, S, L, I	E, A, K, S	Q

<sup>a</sup> Indicative of conservation among all known calpain 3 proteins; an ellipsis (...) denotes that no information is available.

<sup>b</sup> In each cell, amino acids are listed according to frequency in published sequences.

<sup>c</sup> With regard to amino acids of the three specific parts of calpain 3: NS, IS1 or IS2.

aminated, because cells were not available from these patients. Nevertheless, present knowledge of splicing mechanisms could be used to infer putative consequences, in accordance with the exon-definition model (Robberson

et al. 1990). IVS9-9A→G generates a new dinucleotide, AG, that could potentially be used as a splicing anchor (Smith et al. 1989); use of the latter would result in a frameshift mutation, by addition of 8 bases

**Table 6**  
**Haplotype Analysis of Recurrent Mutations**

MUTATION (CpG?) AND ORIGIN	HAPLOTYPE <sup>a</sup>				
	D15S514	D15S779	D15S782	D15S780	D15S778
G234E:					
France ( <i>n</i> = 1)	204	96	128	2	179
France ( <i>n</i> = 1)	200	96	130	5	177
R490Q (yes):					
France ( <i>n</i> = 2)	202/206	96	130/128	3	181/187
France ( <i>n</i> = 3)	202	82/94	122	4/5	183/179
Turkey ( <i>n</i> = 1)	210	86	128	5	181
R572W (yes):					
France ( <i>n</i> = 1)	202	96	120	3/5	177
France ( <i>n</i> = 1)	210	96	132	5	179
R572Q (yes):					
Réunion Island ( <i>n</i> = 1)	198	92	130	5	181
France ( <i>n</i> = 1)	210	84	130	3	181
IVS20–2AG→GG:					
France ( <i>n</i> = 1)	196/210	96/98	128/132	2	181
Canada ( <i>n</i> = 1)	200	86	116	3	177
R748Q (yes):					
Spain ( <i>n</i> = 2)	202	90	128	3	183
Spain ( <i>n</i> = 2)	210	84/96	130/128	3	181
Turkey ( <i>n</i> = 1)	210	82	126	5	181
R769Q (yes):					
Amish ( <i>n</i> = 19), France ( <i>n</i> = 2)	210/202	96	122	4	181
Brazil ( <i>n</i> = 1)	202/210	84	126	4	181

<sup>a</sup> Only haplotypes corresponding to recurrent mutations are presented. Markers are listed according to their linear map order. The disease locus is located between markers D15S779 and D15S782. In situations in which the haplotype could not be reconstructed, the two alleles have been given, separated by a virgule (/). For all markers except D15S780, the numbers represent allele size (in bp); in the case of marker D15S780, the numbers represent allele designations as given in the CEPH database.

in the mRNA. IVS17+1G→T, IVS17–1G→T, and IVS20–1AG→GG may lead to either single-exon skipping or cryptic-splice-site utilization.

Most of the deletion/insertion mutations caused a frameshift, leading to a secondary premature stop codon. Four of the mutations ( $\Delta$ FPIQFVW,  $\Delta$ FWSAL,  $\Delta$ SYEALKG, and  $\Delta$ K254), however, were in-frame deletions that would theoretically result only in limited amino acid deletions. Two mutations were slightly more complex and resulted in the combination of small insertions and deletions. 2317AAACA→T consists of a 5-bp deletion of nucleotides 2317–2321 of the coding region, coupled with a 1-bp insertion, and 2362AG→TCATCT consists of a 2-bp deletion coupled with a 6-bp insertion. The net result in both cases is a shift in the reading frame, followed by a premature stop codon.

The first major genomic deletion in the calpain 3 gene was identified in a Basque patient. Reverse transcription-PCR of the illegitimate transcription products showed that exons 5–7 were missing. Since no sequence alteration was identified in the splice sites of exons 4–8, a genomic deletion was suspected. Two PCR primers—one located in exon 4 and the other located in exon 8—that, in normal genomic DNA, were separated by

~7 kb, led to the amplification of a fragment of ~1.5 kb in DNA from this patient, whereas no amplification was obtained in DNA from control individuals. Sequencing of the corresponding PCR product enabled us to identify the mutation event as a 5,328-bp deletion spanning exons 5–7. The 5' breakpoint is in the right arm of the 3'→5'-oriented *Alu*-Jb repeat in intron 4, and the 3' breakpoint maps to the left arm of the *Alu*-Sg repeat in intron 7, which is oriented in the same direction. The deletion therefore seems to result from an unequal recombination event between two adjacent *Alu* elements.

### Polymorphisms

To date, 12 polymorphisms have been characterized in the CAPN3 gene (table 3). They were excluded from being LGMD2A mutations, because of their occurrence in normal healthy populations and/or occurrence along the same chromosome that has a mutation. The range of frequencies of these polymorphisms varies between <0.01% and 12% (table 3). None of these polymorphisms is in linkage disequilibrium with a particular mutation. Three of the variants are due to C→T transitions. Three polymorphisms map in noncoding regions. Seven

**Table 7**  
**Detection of Variants by Modification of Restriction Enzymes**

MUTATION	ENZYMATIC SITE(S) <sup>a</sup>	
	Normal Allele	Variant Allele
19ΔGCATC	<i>HbaI, HinPI, SfaNI</i>	<i>AluI</i>
43ΔG	<i>AluNI, NspBII</i>	<i>HaeII, Eco47III</i>
60ΔA		<i>Ava I</i>
P26L	<i>BcnI, NciI</i>	
T32T	<i>DdeI</i>	<i>SecI</i>
D77N	<i>AvaII</i>	
S86F	<i>MnII</i>	
I162L	<i>AluI, BstYI</i>	<i>BanII, Bsp1286I</i>
T184M	<i>MaeII</i>	
S215P		<i>AvaII, Sau96I</i>
ΔK254	<i>MboI</i>	
945ΔG	<i>NciI, BcnI</i>	<i>AvaI<sup>b</sup></i>
P319L	<i>MspI, HpaII</i>	
H334Q		<i>StuI</i>
A236T		<i>HpbI</i>
W360X	<i>NlaIV, SecI</i>	
W360C	<i>NlaIV, SecI</i>	<i>EaeI, GdiII</i>
IVS9-26C→G		<i>Tth111II</i>
L421L	<i>PstI</i>	
1292insT	<i>Tth111II</i>	
R437C	<i>NspII, HaeIII</i>	
R440W	<i>RsaI</i>	
Q486E	<i>SfaNI</i>	
R489W	<i>Cfr10I</i>	
R489Q	<i>Cfr10I</i>	<i>NspBII</i>
R493W	<i>AvaII</i>	
G496R	<i>MaeI</i>	<i>DdeI</i>
R572W	<i>BcnI, NciI</i>	
R572Q	<i>BcnI, NciI</i>	
1838ΔA		<i>AluNI</i>
1981ΔATAG		<i>BbvI, Fnu4HI</i>
2000ΔA	<i>BglII</i>	
A702V	<i>HinPI, HbaI</i>	<i>HgiAI</i>
F731S	<i>XmnI</i>	
S744G	<i>AluI</i>	
2317AAACA→T	<i>Tth111II</i>	<i>BspHI, P1eI</i>
A798E	<i>NsiI</i>	<i>BspHI</i>

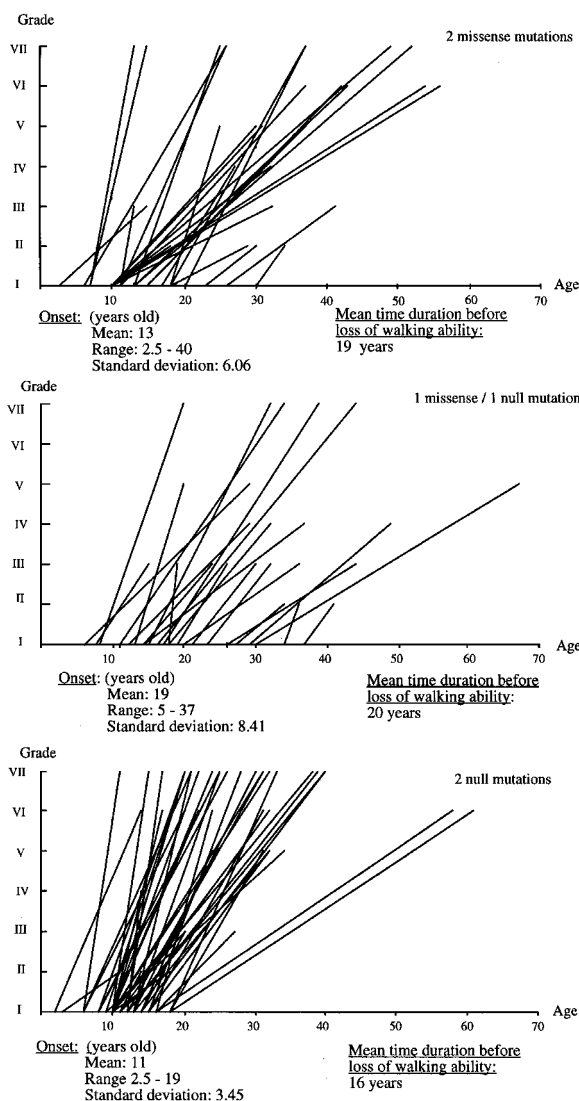
<sup>a</sup> Enzymatic sites corresponding to enzymes that cut too frequently to be used in an analysis are not given.

<sup>b</sup> Present only on genomic DNA, not on cDNA.

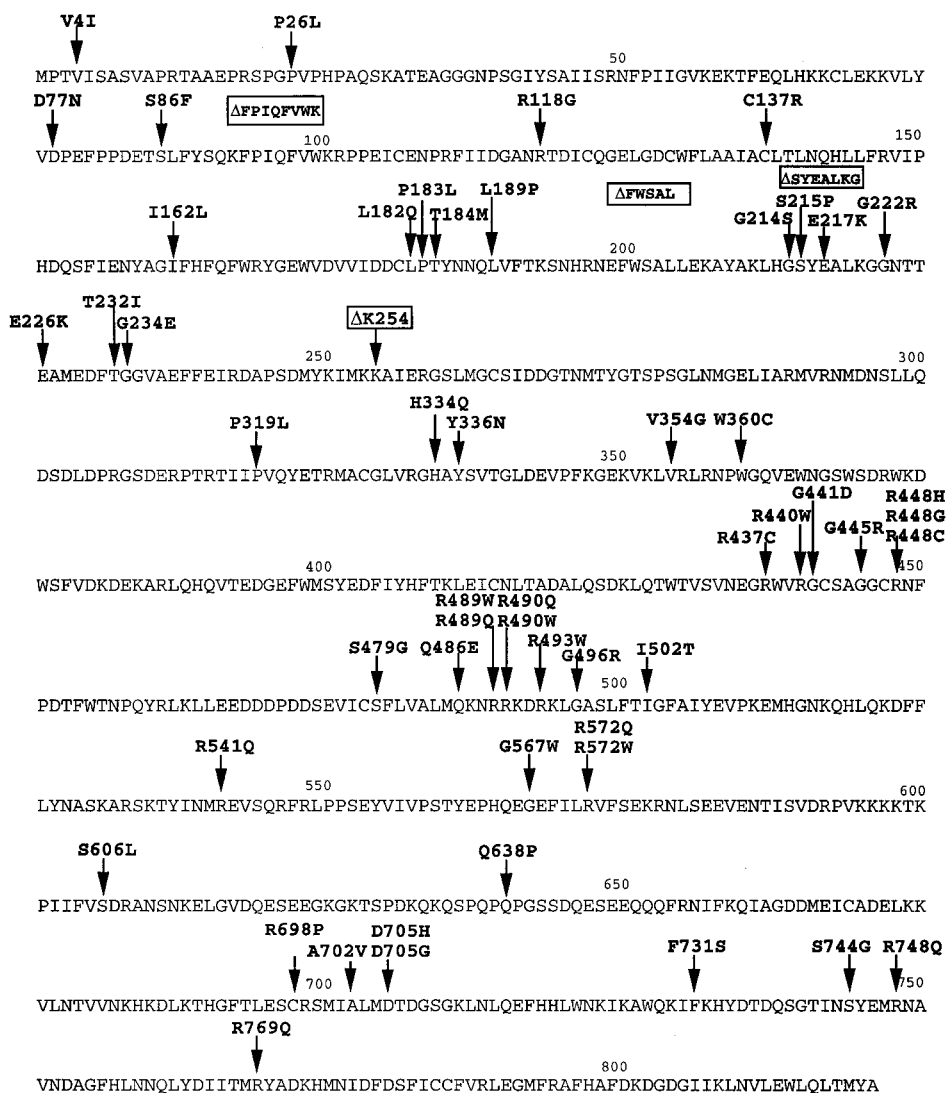
exonic polymorphisms do not lead to an alteration of the encoded amino acid, and the majority of them involve the third base of a codon. The last two polymorphisms, E107K and A236T, which are present, respectively, in exons 2 and 5, lead to an amino acid substitution. It should be noted, however, that the minor variants correspond to an amino acid that can also be found at the equivalent positions in other members of the calpain family: for E107, a lysine is present at the equivalent position in the human nCL4 protein, and, for A236, a threonine is present at the corresponding position in rat, mouse, and porcine calpain 3, as well as in the human, mouse, rat, and chicken calpain 1 proteins. This suggests that these changes may not have major deleterious effects on the protein's function(s).

*Unclassified Variants*

Also detected were five changes that could not be classified as either a polymorphism or a deleterious mutation, even though they were observed only in patients (table 4). One intronic change, leading to a deletion of a C in intron 11 at position -34 of exon 12, was observed in a homozygous state in a Japanese patient who was homozygous for a chromosome 15 haplotype. Two additional changes are single-base substitutions involving the third base of a codon: a TGC→TGT transversion at position 984 in exon 7 (C984→T [C328C]) and a CTG→CTA transversion at position 1263 in exon 10



**Figure 2** Evolution curves for LGMD2A patients. Functional stages are graded as I-VII, according to the Gardner-Medwin and Walton (1974) scale. Only the curves for patients for whom two points of the disease evolution are known are represented, whereas the calculation of means and duration takes into account all available data.



**Figure 3** Location of missense mutations and in-frame deletions along the CAPN3 protein

(G1263→A [L421L]). C984→T (C328) was observed in three unrelated LGMD2 families of different geographic origins but not in >100 control individuals. We are confident that two of these families are authentic LGMD2A families, since one mutant allele already has been characterized. No additional changes were identified in the corresponding patients' DNA, despite exhaustive screening. G1263→A (L421) was encountered only in a Swiss family in which the segregation is consistent with the chromosome 15 location of the disease locus.

All these changes may represent neutral polymorphisms; however, it is also possible that they represent authentic LGMD2A mutations that affect the structure of the transcript. Unfortunately, no biopsies or cell lines were available for the corresponding patients. Hence, we could not examine whether these changes have consequences at the RNA level. We can, however, attempt

to predict their putative manifestations on the transcription products. IVS12–34ΔC may affect the branch site, since it is located at the corresponding consensus position. C984→T and G1263→A create potential splicing sites, with scores of 59% and 80%, respectively (Shapiro and Senapathy 1987). The last two unclassified variants, H774D and A798E, lead to a change in the amino acid composition; but the presence, at the corresponding position, of the variant amino acid in other members of the calpain family is a strong argument in favor of their representing rare innocuous polymorphisms (table 5).

#### Recurrent Mutations or Founder Effect

Twenty-nine mutations were each found in more than one family; however, it is necessary to include a correction for founder effect, since several mutations

have been amplified in specific populations. This is particularly true for the Amish community, in which the R769Q mutation is encountered in a homozygous state in all 19 families with LGMD2A that were tested. A predominant mutation is also observed in two other populations with high consanguinity. IVS6-1G→A and 2362AG→TCATCT are present on >60% (25/39) and 76% (42/55) of the carrier chromosomes in families from Réunion Island and in the Basque isolate, respectively. Haplotype analyses by microsatellite markers that flank the LGMD2A locus confirmed that the mutations derived from the same ancestral mutational event (Allamand et al. 1995; Richard et al. 1995; Urtasun et al. 1998). The same analysis allowed us also to infer a relationship between families, even in the absence of any known genealogical or geographic link. In particular, the 550ΔA mutation observed in patients from eight different countries was found, each time the information was available, to be associated with the same LGMD2A marker haplotype. This is also the case for (i) Y537X and A702V, which are found in Turkish and metropolitan French families; (ii) R769Q, which is present in both Amish and metropolitan French families; and (iii) 2362AG→TCATCT, which is present in 2 North American, 1 Brazilian, 3 Réunion Island, and 49 Spanish families. In other cases, haplotype data allowed us to rule out the possibility of a common founder effect and suggested, instead, that the mutations represent events that have occurred several times independently. Overall, if only the clearly independent events are considered, the number of recurrent mutations collapses to seven, representing 16 independent events (table 6); among these mutations, five correspond to C→T transitions involving CpG sites. Thus, altogether, 105 independent mutations were observed.

#### *Distribution of CAPN3 Mutations*

Mutations are relatively evenly distributed over all exons of the calpain 3 gene, with the exception of exons 9, 12, 14, 23, and 24, all of which are among the smallest exons and in which no mutations have been found so far (fig. 1). These exons are to be contrasted with exon 21, which carries an excess of mutations ( $P < .01$ ). When the nature of the mutations is considered, exons 5, 11, and 21 show an excess of missense mutations ( $P < .05$ , .001, and .05, respectively), and, when allowance is made for the small numbers, exons 15, 17, and 22 may show an excess of deletions or insertions. Interestingly, a mutation cluster spanning a region of 11 amino acids in exon 11 accounts for 18% (11/61) of the independent missense events. This may be due to the elevated number of CpG sites at this location. No differences in the dis-

tribution of mutations were observed between the four calpain 3 domains.

#### *Rapid Screening for Particular Mutations*

In some cases, rapid testing for the presence of a particular mutation in a patient's DNA may be important, especially in the case of recurrent or population-specific mutations. Direct sequencing may be the method of choice, but alternatives allowing the analysis of a great number of individuals exist. Some CAPN3 mutations can be tested rapidly because they cause a change in a restriction-enzyme site (table 7). Short deletions could be tested directly by electrophoresis of the corresponding PCR products. In addition, we have developed allele-specific PCR assays (table 1) for the mutations present in families from the Amish community, from Réunion Island, and from the Basque country. This has allowed us to test rapidly the presence of the corresponding mutations in new patients with these origins. We also have done this for the frequently observed mutation 550ΔA, which is always associated with the same haplotype, allowing to test candidate carriers presenting either this particular haplotype or a related haplotype.

#### *Phenotypic Characteristics of Patients with LGMD2A*

Clinical information was available for 163 of the patients presented here. For many of them, clinical characteristics have been reported elsewhere, and a typical formula has been defined, first in patients from Réunion Island (Fardeau et al. 1996b). This formula is characterized mainly by a symmetrical, very selective atrophic involvement of limb-girdle and trunk muscles, with the gluteus maximus and thigh adductors being most affected (Fardeau et al. 1996a, 1996b). The same pattern of muscle involvement was also reported for 3/4 of the examined metropolitan French patients, with occasionally minor variations around this pattern. The same holds for the majority of LGMD2A patients of Turkish or Basque origin (Dinçer et al. 1997; Topaloglu et al. 1997; Beckmann and Fardeau 1998; Urtasun et al. 1998). Overall, there was a marked heterogeneity of severity. The mean age at onset is 13.7 years (range 2-40 years old; fig. 2), and the mean age at loss of walking ability is 17.3 years (range 5-39 years; fig. 2). No sex difference was evident in either age at onset or evolution.

In an attempt to draw correlations between the clinical severity and the nature of gene mutations, evolution curves associating age at onset and functional stages were separated and compared according to the nature of the mutations (null/null, null/missense, and missense/missense) (fig. 3). The age at onset in patients carrying two null mutations is quite homogeneous and occurs generally at age 15 years. In contrast, age at onset in patients who either had two missense mutations or were

compound heterozygotes for one missense mutation and one null mutation is much more variable and can occur as early as age 2.5 years or as late as age 40 years. Furthermore, patients carrying two null mutations lost walking ability at age <40 years, whereas patients with either one missense and one null mutation or two missense mutations had broader variability in the evolution curves. These data further confirm and extend prior observations regarding the relative severity of null mutations versus missense mutations.

## Discussion

In this report, we have presented all the mutations and polymorphisms identified so far in CAPN3, the gene involved in LGMD2A. A total of 114 variants have been identified in the calpain 3 gene: 97 mutations, 12 polymorphisms, and 5 variants that could not be classified as either mutation or polymorphism. Until one assesses the impact of the latter on mRNA processing and stability, it may be difficult to ascertain the neutrality or nonpathogenicity of these variants. The precedent demonstration of the pathogenic character of the 1872C→T mutation, which does not change the nature of the encoded amino acid (Richard and Beckmann 1995), confirms that one needs to interpret with caution the apparently “neutral” mutations.

The 97 mutations reported thus far, 56 of which are newly described, include 4 nonsense, 32 deletion/insertion, 8 splice-site, and 53 missense mutations. If we consider the additional, unclassified IVS12–34ΔC, C495→T, and G1263→A variants as causative mutations, then the number of mutations characterized thus far would total 100. The mutations are distributed along the length of the CAPN3 gene, with a slight mutational “hot spot” in exon 21. If we consider missense mutations alone, exon 11 shows a notable excess of mutations. This exon constitutes a good primary target for screening of mild calpainopathies, in light of the degree of disease severity of carriers of either two missense mutations or a missense mutation associated with a null mutation.

Most mutations (68/97 [70%]) represent private variants, although particular mutations were found more frequently. Among these particular mutations, 11 (38%) are associated with a founder effect, 7 (24%) are really recurrent, and no information was obtained for the remaining mutations. CAPN3 mutations were identified in 181 families originating from 19 countries, further demonstrating the worldwide distribution of the disease. For 24 families, only one mutant allele was identified, despite the fact that all coding regions in these patients have been examined. These observations therefore suggest that mutations may occasionally lie in an essential noncoding regions, such as the promoter region or an intron. We also have described the first large genomic

deletion of the calpain 3 gene, which is due to an unequal recombination between two intragenic *Alu* elements. The methodologies usually used for mutation screening are not oriented toward identification of such alterations. It would be interesting, given the relatively high *Alu*-sequences content of the calpain 3 region, to verify what fraction of the 23 unidentified mutations can be explained by such events. For this purpose, it may be useful to resort to use of additional procedures, such as Southern blot.

## Diagnostic Applications

After the initial confirmation of CAPN3 as the LGMD2A gene, the primary clinical motivation for performing a systematic mutation analysis was to provide accurate and unambiguous LGMD2A diagnosis for patients with progressive muscular dystrophies. In particular, the identification of mutations in a family allowed the latter to be classified as belonging to the LGMD2A group. This is of importance in light of (i) the genetic heterogeneity of LGMD2 (which has at least eight causative genes, LGMD2A–LGMD2H) and (ii) the difficulty in clinically distinguishing one entity from another. Even if it is now possible, with the help of antisarcoglycans antibodies, to test for defects in the corresponding proteins and to distinguish sarcoglycanopathies from the other progressive muscular dystrophies, unambiguous diagnosis still rests on the identification of the underlying mutations. Hence, genetic and immunochemical analyses provide important means by which to refine and simplify diagnosis.

The wide spectrum of CAPN3 mutations, the relative large size of the CAPN3 gene, and, finally, the fact that most mutations seem to be private and relatively evenly distributed over most calpain 3 exons create significant practical problems for diagnosis of calpainopathies. Because of all these characteristics, no single mutation-detection method appears to be ideal, and the challenge of mutation identification remains important. The assessment of the calpain 3 protein in muscle biopsies, by either western blot or immunohistochemistry, may become an additional tool with which to investigate calpainopathies, although it should be remembered that the autoproteolytic nature of calpain 3 could render this diagnostic test difficult to interpret (Anderson et al. 1998; Beckmann and Fardeau 1998). Nevertheless, the potential of this diagnostic strategy, which has been followed by the Newcastle team, is clearly demonstrated in the present study.

Since the realization of an objective molecular-genetic diagnosis still presents a formidable challenge, it could be helpful, whenever possible, before a direct mutation search is performed, to further classify the family—or, at least, to exclude particular loci—by linkage analysis.

To streamline the genetic analyses, we have developed a fluorescent-marker kit, using a set of markers bracketing the LGMD2A–LGMD2F disease loci (Richard et al., in press). In an additional step, haplotype analyses can be used to infer a common ancestral origin and thus to point to a specific mutation(s). The geographic origins of the patients is another element that can direct the mutation screening.

We also have reported herein the polymorphisms identified in the study of our patient's cohort, since it is essential, in a diagnostic test, to distinguish between them and the morbid DNA-sequence anomalies and, thereby, to prevent erroneous diagnosis. This is of particular importance in the detection of mutations in exons 1 and 22 and in intron 22, which contain polymorphisms that can reach 10%–12% heterozygosity in the population examined. The inclusion of appropriate controls in the mutation analysis of this exon will help us to distinguish between these neutral polymorphisms and causative mutations.

#### *LGMD2 Phenotype*

The availability of molecular diagnosis has enabled precise symptomatology, by identification of clues allowing the recognition of specific clinical features of calpainopathies, compared with other progressive muscular dystrophies caused by defects in structural proteins. Molecular classification of patients as belonging within the different LGMD2 forms helps us to validate the specific topography and characteristics of muscle involvement (for a precise description of LGMD2A patients, see (Fardeau et al. 1996a, 1996b; Dinçer et al. 1997; Topaloglu et al. 1997; Beckmann and Fardeau 1998; Urtasun et al. 1998). To sum up, the typical formula with regard to calpain 3-deficient patients includes, in the early stage of the disease, a predominant wasting of muscles from the posterior compartments of the limbs, clearly visible by both clinical examination and computed-tomography (CT) scan, whereas in sarcoglycanopathies there is a marked quadriceps femoris involvement (Eymard et al. 1997). The differences, in the distribution of muscle wasting, between the different forms renders CT-scan analysis an important element in a clinical diagnosis. An additional sign that may help us to distinguish calpainopathies from sarcoglycanopathies is the fact that, in the former, calf hypertrophy is less frequent, whereas macroglossia is never seen.

Both the characterization of mutations and the study of phenotype/genotype correlations should allow us to elucidate the molecular basis for the expression of disparate phenotypes among calpain 3-deficient patients. No evident correlations between the mutations and the clinical manifestations of LGMD2A has been established, either between the families or even within the families, although, in general, null mutations result in

clinical consequences that are more severe than those that result from missense mutations. Furthermore, the study of this large cohort of calpain 3-deficient patients has failed to show that the patient's gender has any influence on either age at onset or disease evolution.

#### *Locus-Specific Database*

Given both the widespread geographic distribution of this disease and the growing number of reported cases, it seems important, in order to help investigators and clinicians in their diagnostic process, to maintain and update a CAPN3-mutation database. With this goal in mind, we are in the process of constructing a locus-specific database Website in accordance with HUGO guidelines for content and structure of mutations databases, and we encourage others to join us and to participate in a collective effort to regroup all CAPN3 mutations in a publicly available database. Meanwhile, some of these data are already accessible in the Leiden Muscular Dystrophy pages.

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### **Electronic-Database Information**

Online Mendelian inheritance in Man (OMIM), <http://www3.ncbi.nlm.nih.gov:80> (for LGMD2A [MIM 253600])  
Leiden Muscular Dystrophy pages, <http://www.dmd.nl>

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