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Missense mutations in the β-myosin heavy-chain gene cause central core disease in hypertrophic cardiomyopathy

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ABSTRACT **Hypertrophic cardiomyopathy (HCM) is an important cause of sudden death in apparently healthy young individuals. In less than half of kindreds with HCM, the disease is linked to the β-myosin heavy-chain gene locus (*MYH7*). We have recently described two missense *MYH7* gene mutations [Arg-403 to Gln (R403Q) and Leu-908 to Val (L908V)] and found that the mutant message is present in skeletal muscle (soleus) and that the mutant β-myosin obtained from soleus muscle has abnormal *in vitro* motility activity. Having identified a second kindred with the R403Q mutation, and 3 other kindreds with two additional mutations (G741R and G256E), we performed histochemical analysis of soleus muscle biopsies from 25 HCM patients with one of these four mutations. Light microscopic examination of the NADH-stained biopsies revealed the presence of central core disease (CCD) of skeletal muscle, a rare autosomal dominant nonprogressive myopathy characterized by a predominance of type I "slow" fibers and an absence of mitochondria in the center of many type I fibers. CCD was present in 10 of 13 patients with the L908V mutation, 5 of 8 patients with the R403Q mutation, 1 of 3 patients with the G741R mutation, and 1 patient with the G256E mutation. Mild-to-moderate myopathic changes with muscle fiber hypertrophy were present in 16 patients. Notably, CCD was present in 2 adults and 3 children with the L908V mutation who did not have cardiac hypertrophy. In contrast, soleus muscle samples from 5 patients from 4 kindreds in which HCM was not linked to the *MYH7* locus showed no myopathy or CCD. Soleus muscle biopsies from 5 control subjects also showed normal histology. This work demonstrates that (*i*) *MYH7*-associated HCM is often a disease of striated muscle but with predominant cardiac involvement and (*ii*) a subset of HCM patients with *MYH7* gene missense mutations have CCD.**

Hypertrophic cardiomyopathy (HCM) is an important cause of arrhythmias, syncope, and sudden death (1). Most cases are familial and are inherited in an autosomal dominant pattern. HCM is considered a primary cardiac disease with variable clinical and morphologic expression, evident within as well as between kindreds (2–4). This pleiotropy is demonstrated in kindreds with severely affected members in which obligate carriers of the disease gene have normal hearts (2, 4).

The disease gene for some HCM kindreds has been identified by linkage analysis as localizing to the β-myosin heavy-chain gene (*MYH7*) locus on the long arm of chromosome 14 (3–5). Subsequently, distinct missense mutations in the *MYH7* gene resulting in substitution of conserved amino acids have been described (4, 6, 7). In addition to this allelic genetic heterogeneity, nonallelic heterogeneity of HCM has also been established (3, 8).

The mechanisms by which point mutations in the *MYH7* gene lead to development of HCM are unknown. Although MYH7 protein is called ventricular heavy-chain myosin, it is also expressed in skeletal muscle of animals (9–11). We have also shown that normal and mutant β-myosin message and mutant β-myosin protein are also expressed in the slow fibers of skeletal muscle in humans (12). As the soleus muscle contains 75–90% slow (type I) fibers (13), we biopsied this muscle from patients with distinct point mutations in the *MYH7* gene. Findings were compared to biopsies obtained from HCM patients from kindreds in which the disease was shown not to be linked to the *MYH7* gene locus as well as from normal controls.

METHODS

Patients. Thirty patients from 10 HCM kindreds participated in the study. In 6 of the kindreds, HCM was associated with missense mutations in the *MYH7* gene (Table 1). In the remaining 4 HCM kindreds, the *MYH7* gene locus was excluded. Informed consent was obtained in accordance with study protocols (87-H-57 and 91-H-50) approved by the Institute Review Board of the National Heart, Lung, and Blood Institute. HCM was defined as increased cardiac wall thickness in the absence of another cause for the cardiac hypertrophy.

PCR and Southern Blot Analysis. Each family member was phlebotomized, and DNA was extracted from isolated nuclei of peripheral leukocytes as described (14). A set of intronic primers was designed, each of which encompassed 1 of the 40 *MYH7* gene exons and yielded a single unique fragment of the expected size in a PCR amplification. Primer pairs used to generate an exon-containing fragment of the *MYH7* gene with two mutations were (*i*) exon 9 (amino acid residue 256): 5′ primer, 5′-GACTTGGACTGGTGGAGGAATGG-3′; 3′ primer, 5′-TAGACCTGAAGACAGAGACACC-3′; (*ii*) exon 20 (amino acid residue 741): 5′ primer, 5′-GGATCTGCAGGTGACCCTGAAT-3′; 3′ primer, 5′-ACAACAGGAAAAGCATCAGAGG-3′. PCR was performed in a 100-μl vol using the Amplitaq enzyme (Perkins–Elmer/Cetus) according to the manufacturer's recommendations except that a thermocycler oven (Bios, New Haven, CT) was used. Each of the denaturation, annealing, and extension segments was for 40 sec, and 35 cycles were performed. Annealing temperatures of ≥61°C were used. Radioactive labeling of the amplified fragment was accomplished through the addition of 0.1 μCi of [α-^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq) (Amersham) to the 100-μl reaction vol.

Single-Strand Conformation Polymorphism (SSCP) Detection. Polymorphisms were detected by SSCP analysis of PCR-amplified fragments containing each of the 40 *MYH7* gene exons by a modification of the procedure described by Orita *et al.* (15). Briefly, 1 μl of a 100-μl reaction mixture was

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Abbreviations: HCM, hypertrophic cardiomyopathy; SSCP, single-strand conformation polymorphism; CCD, central core disease.

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Table 1. Histochemical analysis of soleus muscle

Mutation	Kindred/ patient	Age, yr/sex	ECG	LVWT, mm	CCD*
	HCM linked to *MYH7* gene				
V908L	2755/I-10	64/F	+	15	+++
	2755/II-1	53/F	+	26	+++
	2755/II-7	33/F	+	27	−
	2755/II-12	55/M	+	15	+
	2755/III-29	24/M	+	22	+
	2755/III-32	32/F	+	11	++
	2755/III-33	33/F	−	10	−
	2755/III-34	27/M	+	11	+
	2755/III-35	20/F	+	32	+
	2755/III-65	26/M	−	14	−
	2755/IV-32	5/F	−	5	+
	2755/IV-33	4/F	−	4	+
	2755/IV-34	2/M	−	4	+
R403Q	2002/II-3	39/F	+	16	+++
	2002/II-4	35/F	+	22	+
	2002/III-1	25/F	+	19	+++
	2002/III-9	18/M	+	8	−
	2002/IV-3	2/F	+	6	−
	2258/III-2	32/M	+	16	+
	2258/IV-3	3/M	+	11	−
	2258/IV-4	2/M	+	8	+
G741R	2191/III-1	45/F	+	16	−
	2191/IV-2	20/M	+	19	+
	2251/II-1	29/F	+	14	−
G256E	2280/II-1	30/M	+	17	+++
	HCM not linked to *MYH7* gene				
	2000/JK-618	35/M	+	24	−
	2001/JG-126	27/M	+	40	−
	2179/MP-120	28/F	+	16	−
	2179/MS-108	43/F	+	20	−
	2125/MC-04	33/M	+	22	−

ECG, abnormal (+) or normal (−) 12-lead electrocardiogram; LVWT, maximal left ventricular wall thickness; F, female; M, male. *+, 1–5 fibers with cores per magnification field; ++, 6–20 affected fibers per field; +++, >20 fibers per field; −, CCD absent.

diluted with 9 μl of a denaturing solution (95% formamide/20 mM EDTA/0.05% bromphenol blue/0.05% xylene cyanol), heated to 80°C, plunged into an ice bath, and resolved on a 5% polyacrylamide/10% (vol/vol) glycerol gel run at 30 W at room temperature and also on a 5% polyacrylamide gel run at 4°C.

Sequencing. The PCR fragments showing polymorphisms by SSCP analysis were sequenced without subcloning through the chain-termination technique (16) by a modification of a described procedure (4) and a Sequenase kit (United States Biochemical). Briefly, a biotin phosphoramidite (Midland Certified Reagent, Midland, TX) was used to add a biotin molecule to the 5′ end of an oligonucleotide primer in the last step in its synthesis on the nucleic acid synthesizer (model 380B; Applied Biosystems). This primer, together with its mate, which was not biotinylated, was used to generate the PCR-amplified fragment to be sequenced. Twenty microliters of this product was then incubated at room temperature, with 20 μl of magnetic beads bound to streptavidin (Dynal, Oslo) for 15 min. The product, now bound to beads, was then denatured with 0.2 M NaOH and washed with 1× TE (10 mmol Tris, pH 7.5/1 mmol EDTA) and then water. After the beads were collected with a magnet, they were resuspended in 7 μl of water and the bound template was sequenced by using the complementary primer according to the manufacturer's recommendations.

Muscle Biopsy. Using local anesthesia, biopsy specimens were obtained from the soleus muscle through a longitudinal incision on the medial aspect of the leg, below the belly of the gastrocnemius. A sample was snap-frozen in isopentane cooled in liquid nitrogen and processed for a battery of muscle enzyme histochemistry (17). A second sample was fixed in glutaraldehyde and processed for EM.

RESULTS

Identification of Distinct Mutations of the *MYH7* Gene. Sequencing of PCR fragments showing polymorphism by SSCP gel analysis identified five distinct missense mutations in the *MYH7* gene in 6 kindreds. We have previously described two of these mutations—Arg-403 to Gln (R403Q) and Leu-908 to Val (L908V) (4). The remaining three mutations are shown in Fig. 1.

Exclusion of the *MYH7* Gene Locus. Our technique of scanning the 40 exons of the *MYH7* gene identified polymorphisms in the flanking intronic regions. These were used in linkage analysis of the disease against the *MYH7* gene locus in kindreds lacking identified exon mutations. Two such kindreds have been described (3, 4). Recombinations of intronic polymorphisms against the disease phenotype allowed the exclusion of the *MYH7* locus in two other kindreds—2179 and 2125 (data not shown).

Histochemical Analyses and EM Examination of Soleus Muscle Biopsies. The effect of abnormal *MYH7* on skeletal muscle of HCM patients was studied by analyzing soleus muscle biopsies of 25 patients from 6 kindreds with four distinct *MYH7* gene mutations. The findings were compared to histochemical analyses of biopsies from 5 HCM patients in 4 kindreds in which the disease was not linked to the *MYH7* gene as well as in 5 normal individuals.

Enzyme histochemical studies on frozen sections (10 μm thick) using ATPase (pH 9.4) and NADH histochemical reactions showed the type I predominance expected for the soleus muscle (Fig. 2 *B, D, F, H,* and *J*). Normal muscle and muscles from HCM patients in the non-myosin-linked kindreds showed normal muscle fiber cytoarchitecture with hematoxylin and eosin, trichrome, and NADH reactions (Fig. 2 *A* and *B*). In contrast, muscle biopsies from 16 patients with distinct *MYH7* gene mutations revealed various degrees of myopathic changes (mild in 12 and moderate in 4 patients) with the modified Gomori trichrome, characterized by variations in fiber size, hypertrophic fibers with internal nuclei, increased connective tissue, splitting of muscle fibers, and occasional necrotic fibers (Fig. 2 *C, E, G,* and *I*). The most significant histochemical abnormality was the presence of cores, easily identified with the oxidative enzymatic stains in the center of many muscle fibers, related to the absence of mitochondria (Fig. 2 *D, F, H,* and *J*). Central core disease (CCD) of skeletal muscle was present in 10 of 13 patients with the L908V mutation, in association with myopathic features (Fig. 2 *C* and *D*) or without myopathy in asymptomatic children (Fig. 2 *E* and *F*). CCD was also present in 5 of 8 patients with the R403Q mutation with or without associated myopathy (Fig. 2 *G* and *H*), in 1 of 3 patients with the G741R mutation, and in 1 patient with the G256E mutation associated with myopathy (Fig. 2 *I* and *J*). The cosegregation of CCD and myopathy with HCM, in adults and children, in kindreds 2755 and 2002, with the L908V and R403Q mutations, respectively, is shown in Fig. 3 and Table 1. Notably, CCD was present in 2 adults and 3 children with the L908V mutant allele in the absence of demonstrable cardiac hypertrophy. The enzyme histochemical demonstration of CCD was confirmed (Fig. 4). In two patients, the myopathic changes were not accompanied by evidence of CCD. In contrast, skeletal muscle biopsy from patients with HCM not linked to the *MYH7* gene locus showed no myopathy or CCD (Table 1). Soleus muscle biopsies from 5 control subjects also showed normal histology.

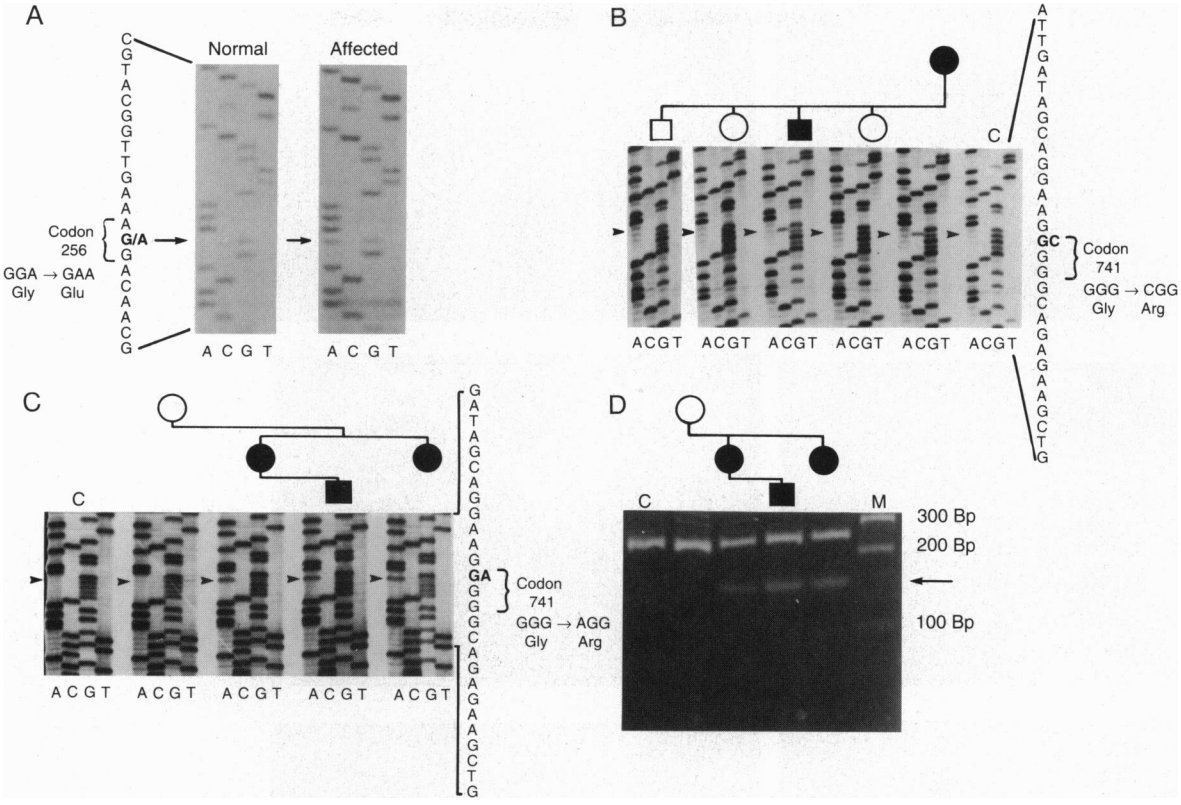

FIG. 1. Identification of three *MYH7* gene mutations. (*A*) Portion of sequence of total PCR product encompassing exon 9 of two individuals from kindred 2280. Sequence on the left from an affected individual shows a G → A transition resulting in a Gly → Glu substitution at amino acid residue 256. The affected individual, a heterozygote, has both a G and the mutant A (arrow). (*B*) Subset of kindred 2191 showing a portion of sequence of total PCR product encompassing exon 20. Sequence from the two affected individuals (solid symbols) shows a G → C transversion resulting in Gly → Arg substitution at amino acid residue 741. (*C*) Subset of kindred 2251 showing a portion of sequence of total PCR product encompassing exon 20. Sequence from the 3 affected individuals (solid symbols) shows a G → A transition also resulting in Gly → Arg substitution at amino acid residue 741. Sequence from unaffected family member (open symbol) and a normal control (C) show the absence of the mutant A (arrowhead). (*D*) *Mbo* II digest of fragments sequenced in *C*. The G → A transition creates an *Mbo* II site, yielding two fragments of 132 and 151 bp, which comigrate on a 10% acrylamide gel marking the presence of the mutant allele in the affected individuals. The uncut fragment is 283 bp and both mutant and normal fragments are present in the affected individuals. M, molecular size markers.

DISCUSSION

We have recently demonstrated that both mutant and normal cardiac *MYH7* gene message is expressed in skeletal muscles and that mutant cardiac myosin purified from skeletal muscle of patients with the R403Q and L908V mutations has an abnormal function in an *in vitro* motility assay (18, 19). In this report, we show that the mutant β-myosin present in skeletal muscle has a demonstrable effect on the structure, and, in particular, the mitochondria, of myofibers. This effect appears to be absent in skeletal muscle of HCM patients in whom the disease is not a consequence of a *MYH7* gene mutation.

In more than two-thirds of HCM patients with one of four distinct *MYH7* gene mutations, light microscopic examinations of NADH-stained fresh-frozen skeletal muscle sections revealed the absence of mitochondria in the center of many type I fibers—the hallmark of CCD (20–26). These observations were confirmed by EM, which showed the ultrastructural appearances associated with cores. Associated myopathic changes were mild and in all but two patients (2755/I-10 and 2002/III-9) CCD was present in the absence of significant muscle weakness, indicating that the cores were not secondary to severe myopathy.

A striking finding was the dissociation of the extent of CCD and severity of cardiac disease. For example, in kindred 2755, patient III-33 had extensive CCD of skeletal muscle despite the absence of cardiac hypertrophy. Similarly, 3 children (IV-32, IV-33, and IV-34) with the same L908V mutation, shown to have CCD, had normal echocardiograms and electrocardiograms. Thus, the disease processes in skeletal muscle and cardiac muscle do not always parallel each other. The skeletal muscle disease in children may precede cardiac hypertrophy and exist in adults in the absence of cardiac disease determined by echocardiography. A concomitant of CCD has not been observed in the heart. This may be due to the fact that missense mutations of the *MYH7* gene are uncommon—of 80 unrelated HCM kindreds in which we scanned the entire *MYH7* gene coding sequence, only 8 have been shown to have missense mutations (N.D.E. and L.F., unpublished observations). Thus, appropriate studies may not have been performed on cardiac tissue from HCM patients with *MYH7* gene mutations. Another explanation may be that distribution of mitochondria in myocardial cells is different from that in skeletal myofibers.

Cores and myopathy were not apparent in soleus muscle biopsy samples of 5 patients from 4 unrelated HCM kindreds unlinked to the *MYH7* gene. The cardiac manifestations of the non-β-myosin HCM genes were indistinguishable from the HCM associated with the mutant alleles of the *MYH7* gene. Thus, the cardiac hypertrophy in β-myosin- and non-β-myosin-associated HCM may be secondary to a compensatory final common pathway. In contrast, the effects of β-myosin and non-β-myosin genes on skeletal muscle are different. CCD is a rare nonprogressive autosomal dominant myopathy, and in some kindreds it cosegregates with malignant hyperthermia (20–26). Both CCD and malignant hyperthermia have been linked to the skeletal ryanodine receptor,

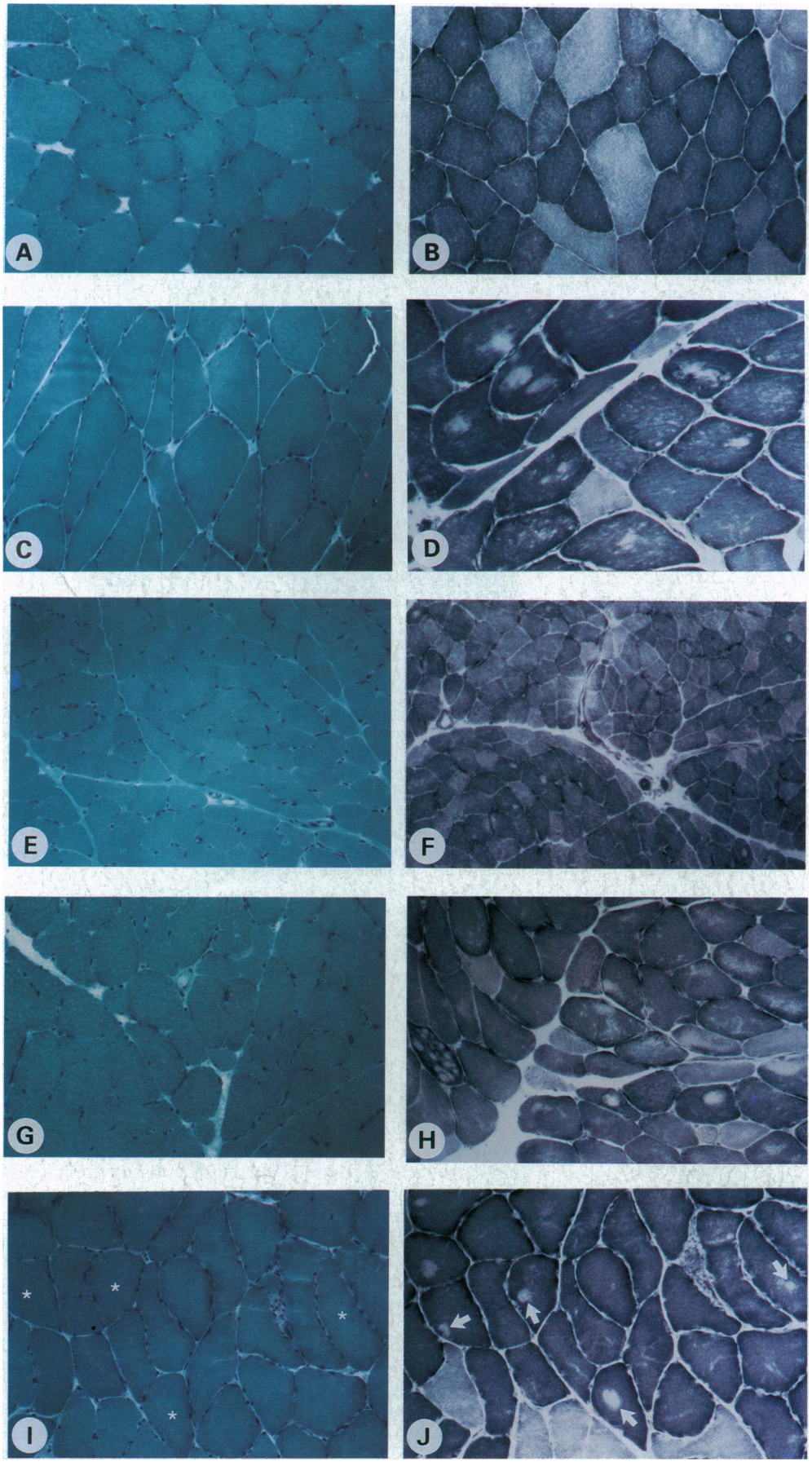

FIG. 2. Transverse sections of soleus muscle biopsies of the soleus muscles from a patient with HCM not linked to *MYH7* gene locus (*A* and *B*) and from patients with distinct *MYH7* missense mutations; an adult HCM patient (kindred 2755/I-10) with the L908V mutation (*C* and *D*); seemingly normal child (kindred 2755/IV-32) with the L908V mutation (*E* and *F*); an adult HCM patient (kindred 2002) with the R403Q mutation (*G* and *H*); and an adult HCM patient (kindred 2280/II-1) with the G256E mutation (*I* and *J*). *A*, *C*, *E*, *G*, and *I* were stained with trichrome; *B*, *D*, *F*, *H*, and *J* were stained with NADH. *I* and *J* are serial sections and demonstrate that cores are present in fibers (arrows) in the absence of degenerative features within the same fibers (asterisks). It is clear that the *MYH7* gene mutations are associated with hypertrophy of muscle fibers. In *E* the muscle fibers are small because of the patient's young age (5 years old). (×140.)

the Ca^{2+} channel of the sarcoplasmic reticulum, on chromosome 19q (23, 24). In swine, malignant hyperthermia (porcine stress syndrome) has been linked to a specific mutation (R615C) of the skeletal ryanodine receptor gene (27). CCD

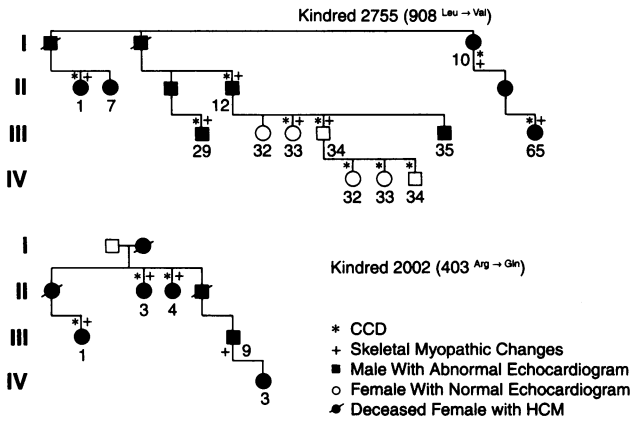

FIG. 3. Cosegregation of CCD and myopathy with HCM disease allele in subsets of kindreds 2755 and 2002. All patients with mutations from whom biopsies were obtained are identified by a number.

often causes hypotonia and weakness of proximal muscles in infancy, resulting in delayed motor milestones (20–24). There may be congenital hip dislocation, pes cavus, and kyphoscoliosis. None of the patients in our study had myoskeletal deformities and only two (kindred 2755, I-10; kindred 2002, III-9) had mild proximal muscle weakness. CCD has rarely been associated with dilated and hypertrophied heart (28, 29). Our study links CCD to mutations in the *MYH7* gene and demonstrates that *MYH7* gene-associated HCM is a disease involving striated (cardiac and skeletal) muscle. Thus, CCD results from mutations in two different genes that encode major proteins of the contractile apparatus: a calcium channel and a structural/enzymatic protein.

There is a growing literature of inherited mitochondrial myoencephalopathies that are sporadic or inherited in a maternal or a Mendelian pattern (30, 31). They result from point mutations or deletions in mitochondrial DNA or are due to mutations in nuclear-encoded proteins critical to mitochondrial stability and function. Primary mitochondrial diseases are only occasionally associated with cardiac abnormalities including cardiac hypertrophy (31–33). In our experience, β-myosin-associated HCM is not associated with encephalopathy and the mitochondrial abnormalities are by

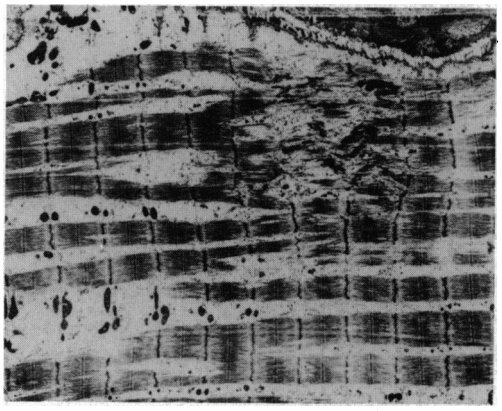

FIG. 4. EM examination of soleus muscle from a patient (kindred 2755/I-10) with the L908V mutation showing the typical findings of cores: relative or complete lack of mitochondria, changes in contractile apparatus consisting of smaller, less well-defined myofibrils, contracted sarcomeres and irregularity of Z disc (Z disc streaming), and loss of alignment of myofibrils within the core.

definition secondary. The mechanism(s) by which mutations in the *MYH7* gene lead to central cores and mitochondrial abnormalities is unknown. The contribution of the central cores and the associated mitochondrial abnormalities to the disease remain to be investigated.

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