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Localization of the gene encoding the α_2/δ-subunits of the L-type voltage-dependent calcium channel to chromosome 7q and analysis of the segregation of flanking markers in malignant hyperthermia susceptible families

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Malignant hyperthermia susceptibility (MHS) is an autosomal dominant disorder of skeletal muscle which manifests as a potentially fatal hypermetabolic crisis triggered by commonly used anaesthetic agents. The demonstration of genetic heterogeneity in MHS prompted the investigation of the roles played by calcium regulatory proteins other than the ryanodine receptor (RYR1), which is known to be linked to MHS in fewer than half of the European MHS families studied to date. Previously, we have excluded the genes encoding the skeletal muscle L-type voltage-dependent calcium channel α_1-, β_1- and γ-subunits as candidates for MHS. In this report, we describe the cloning and partial DNA sequence analysis of the gene encoding the α_2/δ-subunits, CACNL2A, and its localization on the proximal long arm of chromosome 7q. A new dinucleotide repeat marker close to CACNL2A was identified at the D7S849 locus and tested for linkage in six MHS families. D7S849 and flanking genetic markers were found to co-segregate with the MHS locus through 11 meioses in one, three-generation family. These results suggest that mutations in or near CACNL2A may be involved in some forms of this heterogeneous disorder.

INTRODUCTION

Malignant hyperthermia (MH) is a clinically heterogeneous (1), autosomal dominant, pharmacogenetic disorder of skeletal muscle (2). A fulminant MH crisis manifests as a hypermetabolic state characterized by some combination of hyperthermia, skeletal muscle rigidity, tachycardia or arrythmia, respiratory and metabolic acidosis, and muscle tissue breakdown as reflected by elevated serum creatine kinase activities and myoglobinuria (3). In susceptible individuals, an MH crisis can be triggered by volatile anaesthetics and depolarizing muscle relaxants (3), and unless treated promptly usually proves fatal (1). Presymptomatic assessment of MH risk is carried out using the standardized European *in vitro* contracture test (IVCT) protocol (4) which allows the following diagnoses: MH-susceptible (MHS), MH-normal (MHN) and MH-equivocal (MHE). Physiological and biochemical studies have indicated that MH is due to a breakdown in the mechanisms regulating sarcoplasmic calcium ion fluxes (5), and mutations in the calcium efflux channel of the skeletal muscle sarcoplasmic reticulum (RYR1) on chromosome 19q13.1 (6) have recently been associated with MHS and a related disorder, central core disease (7–10). However, several families have also been described where no linkage between markers for the RYR1 region and MHS exists, strongly suggesting genetic heterogeneity in MHS (11,12).

A close association is formed at the skeletal muscle triadic junctions between the ryanodine receptor and the L-type voltage-dependent calcium channel (13), also referred to as the dihydropyridine receptor (DHPR), and the two channel complexes are believed to function together in excitation–contraction coupling (14). The skeletal muscle DHPR calcium channel complex, which is located in the T-tubule membrane, is composed of five subunits: α_1, α_2, β_1, γ and δ (15,16), with the α_2- and δ-subunits being encoded by a single gene (17). The α_1-subunit forms the ion pore structure, binds calcium channel blockers, and functions both as a calcium channel and as a voltage sensor (18–20). The α_2/δ-, β_1- and γ-subunits do not themselves exhibit calcium channel activity but together may exert pronounced effects on current density, DHP pharmacology and voltage-dependence of channel activation and inactivation (21,22). Since the pathophysiology of an MH crisis involves a breakdown in the regulation of skeletal muscle calcium ion influx, we have

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+Z28599, Z28602, Z28605, Z28609, Z28613 and Z28696

investigated the potential roles played by the subunits of the DHPR complex in those forms of MHS not linked to RYR1.

The gene encoding the skeletal muscle isoform of the α_1-subunit has been localized to band q32.1 of human chromosome 1 (23,24), while the genes encoding the β_1- and γ-subunits are both located on the long arm of chromosome 17 (25,26). Genetic markers for CACNL1A3, CACNLB1 and CACNLG have recently been developed and tested for linkage in a number of European families whose MHS trait has been shown not to be linked to markers for chromosome 19 (25,27). Although the skeletal muscle DHPR β_1- and γ-subunit genes both map to the interval q11.2−q24 of chromosome 17, previously reported to carry the putative MHS2 locus (28), the results of these studies conclusively excluded all three subunits as candidate genes for MHS, and indeed, raised some questions as to the validity of the proposed MHS2 locus assignment on chromosome 17q11.2−q24 (28).

In this paper we report on the physical and genetic mapping of the gene encoding the DHPRα_2/δ-subunits, CACNL2A, to the proximal long arm of human chromosome 7. The gene and a neighbouring polymorphic dinucleotide repeat marker, D7S849, were also found to be linked to the gene encoding the hepatocyte growth factor (HGF; 29). Using a human chromosome 7-specific panel of human hamster somatic cell hybrids (30), we were able to place D7S849 within an interval spanning the boundary of bands q11.23 and q21.1.

In order to test the potential association of mutations in the CACNL2A gene with MHS, we tested D7S849 and adjacent markers for linkage in a group of six MHS families (11,12,25). Significantly, no recombinations were observed between MHS and D7S849 and two other markers through 11 meioses in one, well-characterized, three-generation pedigree. These results suggest that mutations in, or near the gene encoding the DHPRα_2/δ-subunits may in some cases be associated with MHS, and raise the possibility that as many as three different loci may be involved in the inheritance of this disorder.

RESULTS

Analysis of YAC clones containing the CACNL2A locus

A *Bam*HI fragment containing nt 1−2814 of the published rabbit skeletal muscle DHPRα_2-subunit cDNA sequence (31), which had previously allowed the assignment of CACNL2A to human chromosome 7 (data not shown), was used to screen a chromosome 7-specific YAC library (32). Two clones, designated HSCE1129 and HSCE520 (henceforth referred to as E1129 and E520), were identified by hybridization with the cDNA probe. The size of the inserts in each clone was determined by pulsed field agarose gel electrophoresis (PFGE; 33) and found to be approximately 660 kb for E1129 and 480 kb for E520 (Figure 1A). No evidence was found suggesting that the clones were chimeric. The identity of E1129 was confirmed by sequencing *Sau*3AI subclones which hybridized with the rabbit DHPRα_2 cDNA probe. Five different clones were isolated which contained putative exon sequences exhibiting 90−99% homology with nt 403−532, 1279−1368, 1369−1446, 1863−1939 and 2441−2506 of the published rabbit DHPRα_2-subunit cDNA sequence (31). In addition, a probe specific for the human HGF gene (29) also hybridized to E1129 and E520. The HGF gene was located on a 270 *Sal*I fragment present in both YACs, thus placing it within approximately 110−380 kb of CACNL2A (Figure 1B).

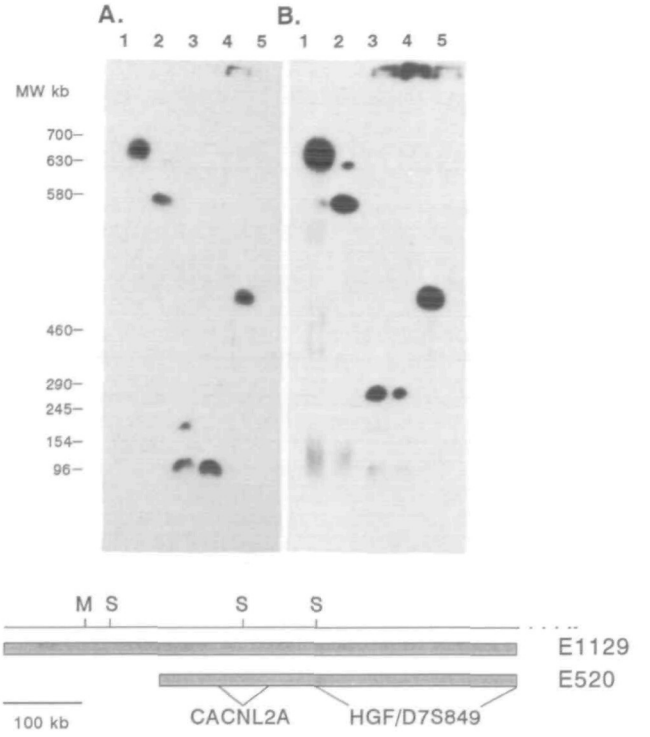

Figure 1. Southern analysis of YACs E1129 and E520. YAC DNAs were digested with *Mlu*I or *Sal*I and subjected to PFGE as described in materials and methods. Concatemeric λDNA and undigested DNAs from E1129 and E520 were used as markers. The order of samples in both panels is as follows: lane 1, intact E1129 DNA; lane 2, E1129/*Mlu*I; lane 3, E1129/*Sal*I; lane 4, E520/*Sal*I; lane 5, intact E520 DNA. Panel **A**, hybridization with a 2.8 kb *Bam*HI fragment from the rabbit skeletal muscle DHPRα_2-subunit cDNA. Panel **B**, hybridization with a 1.5 kb *Pst*I fragment from the human HGF gene. The approximate migration distances of molecular weight markers are depicted to the left of the figure. Two *Sal*I fragments from E520 hybridizing with the CACNL2A probe (panel A, lane 4) co-migrated under the conditions of PFGE, and so appear as a single heavy band. A restriction map showing the location of CACNL2A relative to HGF is depicted below the figure.

Table 1. Dinucleotide repeat polymorphism at the D7S849 locus

Allele	Size (bp)	Frequency
1	166	0.1
2	154	0.04
3	152	0.1
4	150	0.04
5	146	0.04
6	142	0.02
7	140	0.02
8	138	0.64

Allele frequencies were estimated from 50 unrelated individuals (Caucasians). Observed heterozygosity = 0.57.

Identification and physical mapping of a dinucleotide repeat marker at D7S849

The same subclones as used for the confirmation of the identity of E1129 were also screened for the presence of microsatellite repeat sequences potentially useful as genetic markers. One clone was found to contain an interrupted dinucleotide repeat and primers for its amplification were designed. A total of 50 individuals was screened for the presence and extent of

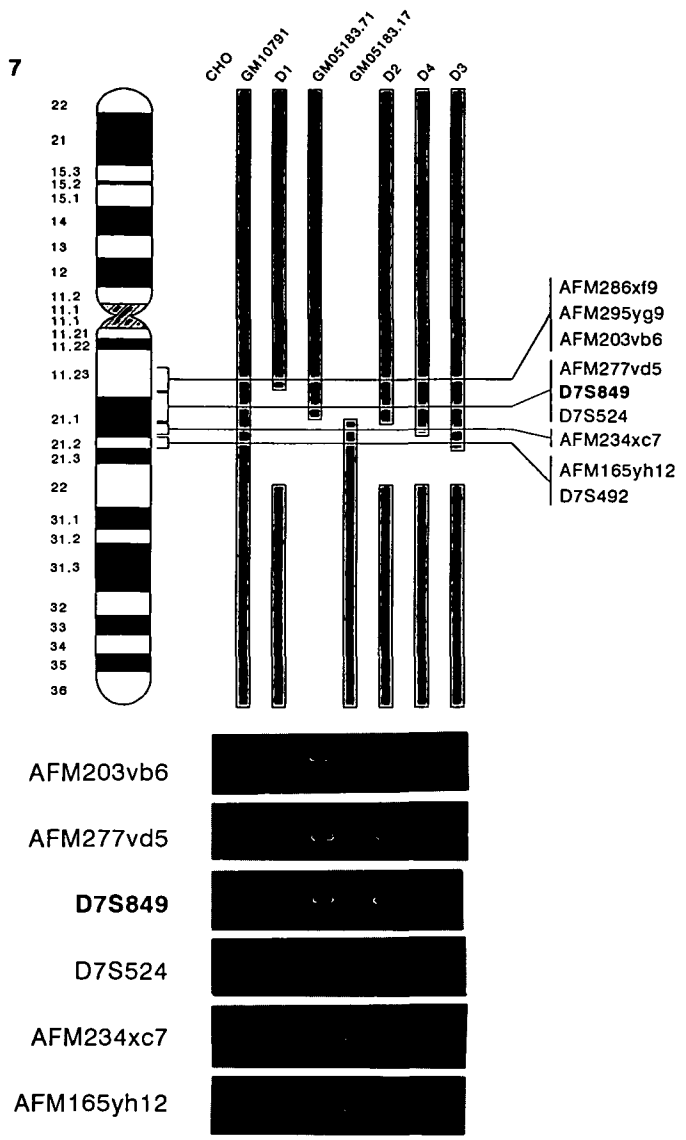

Figure 2. Physical mapping of D7S849 using human/hamster somatic cell hybrids. A schematic representation of the chromosome 7 content of the hybrid cell lines used in these studies is depicted in the upper panel and the identity of each cell line is indicated above the figure. The results of PCR analyses using markers from the 7q11.23−q22 region surrounding CACNL2A are shown in the lower panel. Amplification products were resolved on 2.5% agarose gels with the samples being loaded in the order indicated in the upper panel. The deduced order of markers in the four intervals across 7q11.23−q21 is depicted to the right of the figure. The position of AFM286xf9 relative to AFM295yg9 has not been determined in this study and is for illustrative purposes only.

polymorphism at the locus, designated D7S849, and as shown in Table 1, a total of eight alleles ranging in size from 138 to 166 nt were detected. The observed heterozygosity was 57%. Amplification of a panel of human/hamster somatic cell hybrids (30) containing defined fragments of human chromosome 7q (Figure 2) placed D7S849 at 7q11.23−q21.1 in the same interval as the markers D7S524 (34) and AFM277vd5, proximal to AFM234xc7 and distal to AFM203vb6. In addition, D7S849 was mapped to the same 270 kb *SalI* fragment as the HGF gene (data not shown). This result also places D7S849 within 110−380 kb of CACNL2A.

Physical mapping of CACNL2A by fluorescence *in situ* hybridization (FISH)

Total DNA from YAC E1129 was also used for FISH experiments to confirm the physical localization of the CACNL2A gene and D7S849. A total of 30 metaphase spreads were examined, and as shown in Figure 3, signals were unambiguously detected on the proximal long arm of chromosome 7, close to the boundaries of bands q11.23−q21.1.

Genetic mapping of D7S849 in MHS families

In order to make an approximate estimation of the genetic distance between D7S849 and other markers in the relevant region of chromosome 7q, we tested the new marker together with D7S524, D7S492 (34) and COL1A2 (35) in our group of six MHS families. All families together represented 63 meioses across three generations. Multipoint linkage analysis of the combined families generated a lod score of 7.34 at $\theta = 0.05$ between D7S849 and D7S524. The odds in favour of D7S849 being proximal to D7S524-D7S492-COL1A2 were 6.6×10^3 higher than a distal location. Two-point lod scores between D7S849 and MHS were negative in MH04, MH05, MH09, LMH03 and LMH08 (Table 2), indicating that CACNL2 is not a candidate gene for MHS in these families. In contrast, linkage between D7S849 and MHS could not be excluded in family MH06 and tight linkage was detected with the fully informative marker AFM203vb6 (Figure 4A). Haplotypes deduced from the genetic data obtained using the markers AFM295yg9, AFM203vb6, AFM277vd5, D7S849 and D7S524 (Figure 4B) in this family revealed recombinations between MHS and both AFM295yg9 and D7S524, thereby defining the 7q11.23−q21.1 region co-segregating with MHS. Multipoint linkage analysis of MHS against the marker set yielded a lod score of 2.91 at $\theta = 0$ for AFM203vb6, AFM277vd5 and D7S849 (Figure 5).

DISCUSSION

Since the demonstration of genetic heterogeneity in MHS (11,12), we have undertaken studies to elucidate the potential roles played in this disorder by subunits of the skeletal muscle DHPR calcium channel complex. We have previously isolated human genomic clones containing the skeletal muscle α_1-, β_1- and γ-subunit genes, and determined their chromosomal localizations (24−26). Genetic markers developed for each gene were subsequently used to exclude them as candidates for MHS in a number of European families (25,27). In the present study, we have determined the physical and genetic localization of the CACNL2A gene and investigated its association with those forms of MHS not linked to chromosome 19q13.1.

The α_2/δ-subunits are both derived from the proteolytic cleavage of a single precursor protein and are linked together by disulphide bonds (17). It has been shown that the two subunits together fulfil an essential functional role in DHPR calcium channel activity, by perhaps being involved in the insertion of the α_1-subunit into the membrane (36), the regulation of current density, and together with the β_1-subunit, in the pronounced increase in the voltage-sensitivity of channel activation and inactivation (21,22). In contrast to the complex tissue-specific expression patterns of the α_1- and β-subunits (15,16), only a single isoform of the α_2-subunit protein has been detected in skeletal muscle (15,16), with several alternatively spliced isoforms of the protein found in brain being derived from the same gene (36,37).

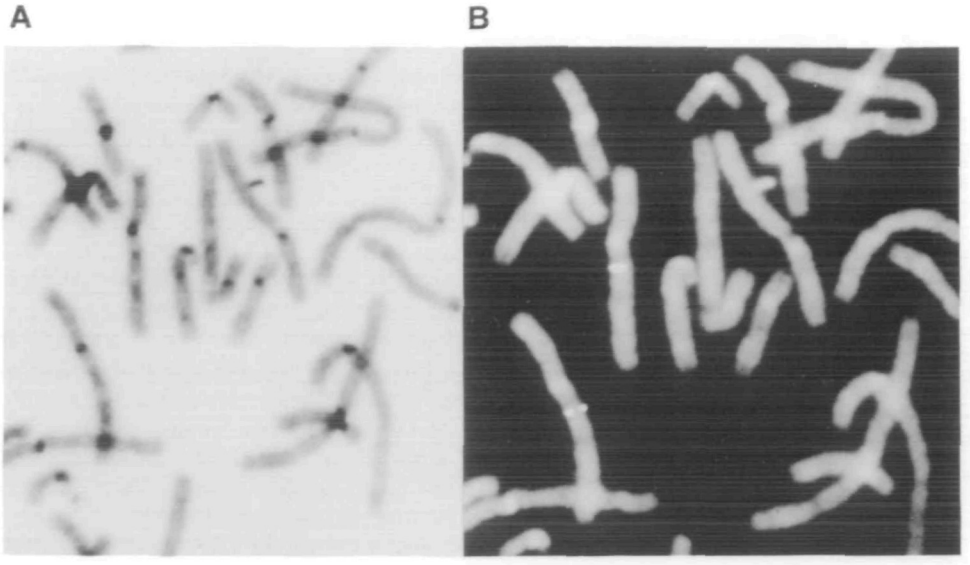

Figure 3. Localization of CACNL2A by FISH. **(A)** Positively imaged metaphase chromosomes counterstained with DAPI for chromosome identification and **(B)** chromosomes stained with propidium iodide showing hybridization of YAC E1129 DNA to the proximal long arm of chromosome 7 in the region q11.23−q21.1.

Table 2. Two point lod scores MHS vs. D7S849

	Recombination fraction					
	0.0	0.05	0.1	0.2	0.3	0.4
MH04	**−1.69**	−1.10	−0.74	−0.34	−0.13	−0.03
MH05	**−0.97**	−0.51	−0.32	−0.13	−0.05	−0.01
MH09	**−1.62**	−0.94	−0.61	−0.26	−0.09	−0.02
LMH03	**−1.48**	−0.95	−0.71	−0.42	−0.23	−0.1
LMH08	**−1.71**	−1.08	−0.70	−0.31	−0.12	−0.02

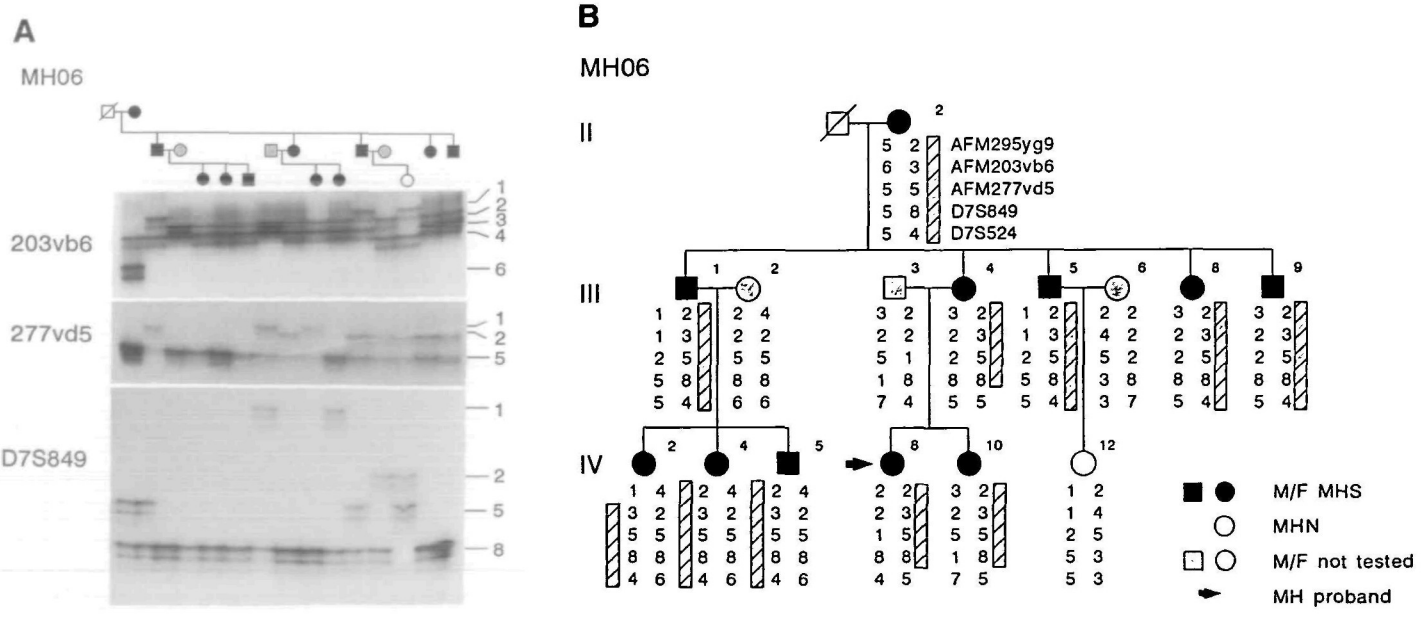

Figure 4. Co-segregation of markers for 7q11.23−q21.1 with MHS in family MH06. **(A)** Autoradiographs of alleles detected for AFM203vb6, 277vd5 and D7S849. The structure and MHS status of the family is illustrated at the top of the figure, with the order of lanes corresponding to the family structure. Allele numbers are indicated to the right of each panel. **(B)** Haplotypes deduced for MH06. Shaded bars indicate the grandmaternal haplotype co-segregating with MHS in this family. Recombinations between MHS and AFM295yg9, and between MHS and D7S524 appear to have occurred in individuals IV02 and III04 respectively.

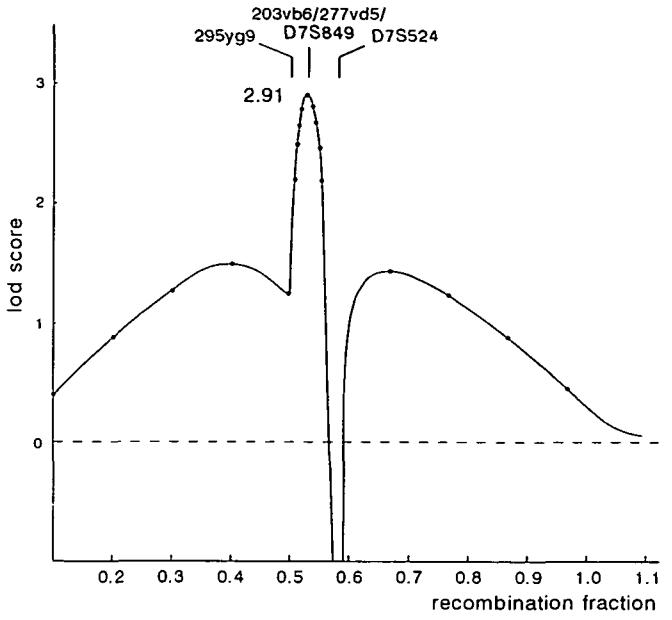

Figure 5. Results of multipoint linkage analysis for MHS on chromosome 7q11.23−q21.1 in MH06. Lod scores were calculated using the LINKMAP package of LINKAGE 5.2 assuming an autosomal dominant disorder, with untested individuals being assigned an unknown disease status. The frequency of MHS was assumed to be 0.0001, penetrance was assumed to be 0.98 in MHS individuals and 1.0 for the proband.

Preliminary results (not shown) indicating that the human DHPRα_2/δ gene was located on chromosome 7 led us to screen a human chromosome 7-specific YAC library for clones containing the CACNL2A locus. The identity of clones hybridizing with the rabbit DHPRα_2-subunit cDNA probe was confirmed by DNA sequencing, and a search for genetic markers within the YACs resulted in the identification of the microsatellite marker D7S849. Using a high resolution somatic cell hybrid mapping panel for chromosome 7 (30), we were able to place the new marker in a narrow interval spanning 7q11.23−q21.1 (Figure 2). This assignment was confirmed by our FISH experiments in which specific signals were consistently detected on the proximal long arm of chromosome 7 (Figure 3). In another recent study, CACNL2A was localized to the 7q21−q22 region by somatic cell hybrid analysis (38). The HGF locus has previously been assigned to band q21.1 of chromosome 7 (39), and since we have demonstrated the physical proximity of CACNL2A and HGF, we conclude that the most likely location of CACNL2A is in the proximal region of band 7q21.1.

Genetic mapping of D7S849 in our families generated a maximum multipoint lod score of 7.34 at a recombination fraction of 0.05 relative to D7S524, with odds strongly in favour of a proximal location for our marker relative to the linkage group D7S524-D7S492-COL1A2. Interestingly, AFM277vd5, D7S849 and D7S524 were all placed within the same interval of the mapping panel (Figure 2), but AFM277vd5 has also been shown to lie on the same YAC contig (40) as the thrombospondin gene (CD36) previously mapped to 7q11.2 (41). By combining genetic mapping data, the YAC contig data and our physical mapping data, the order of markers across the region 7q11.23−q21.3 would therefore appear to be 7cen-(AFM286xf9-AFM295yg9)-AFM203vb6-(CD36-AFM277vd5)-(CACNL2A-D7S849-HGF)-D7S524-AFM234xc7-AFM165yh12-D7S492-7qter.

The markers D7S849, D7S524, D7S492 and COL1A2 were tested for linkage with MHS and multiple recombinations between the marker set and MHS were observed in five families, therefore excluding CACNL2A as a candidate gene for MHS in these cases. However, only a single recombination between MHS and D7S524, D7S492 and COL1A2 was observed in individual III04 of family MH06 (Figure 4B), while no recombinations appeared to have occurred between MHS and D7S849. More detailed linkage analyses in MH06 also revealed linkage with the markers AFM203vb6 and AFM277vd5 (Figure 4A), but an apparent recombination between AFM295yg9/AFM286xf9 and MHS in individual IV2 (Figure 4B) limits the region co-segregating with the trait to the 9 cM interval between D7S524 and AFM295yg9 (J.Weissenbach, personal communication).

We have previously speculated that the high incidence of MHS in MH06 may be due to the presence of a second MHS allele in this family (11). However, our data clearly show that the same 9 cM region of 7q11.23−q21.1 has been passed on by the MHS grandmother to all 11 MHS individuals, and that the unusual MHS segregation pattern in this family can be explained by the inheritance of a single grandmaternal haplotype. The lod score of 2.91 at a recombination fraction of 0.0 in favour of linkage with MHS is probably lower than the theoretical maximum for this family of 3.3 because not all meioses were fully informative and not all clinical and genetic data were available. Although evidence in favour of linkage is not in itself conclusive of a causal relationship between mutations in CACNL2A and MHS, the presence in the immediate vicinity of the linked region of a gene so important in excitation−contraction coupling and the regulation of sarcoplasmic calcium fluxes is interesting. We are therefore seeking to identify mutations in the CACNL2A gene that may be associated with MHS in this family.

Whereas porcine MH appears to be associated with a single Arg615Cys substitution in the ryanodine receptor gene (42), the situation in the human disorder is clearly more complex. To date at least six different point mutations in RYR1 have been associated with MHS and the allelic disorder CCD (7−10), but of these, three have been detected only in single families, and two of the three, a Gly248Arg (7) and an Ile403Met (9) substitution, seem to be restricted to small, two-generation families. Nevertheless, several different mutations in the same gene do appear to underly both MHS and CCD. Since MHS does not seem to map to either chromosomes 19q or 7q in five families analysed here, our data suggesting linkage between markers for 7q11.23−q21.1 and MHS in MH06 imply that mutations in three or more genes may underlie clinically similar disorders which manifest as an adverse reaction to commonly used anaesthetic agents. This degree of genetic heterogeneity in MHS also implies that diagnosis of MHS by means of genetic testing rather than by the *in vitro* contracture test (4) will be difficult to validate. Further studies on additional families are being carried out to assess the extent of involvement of a chromosome 7q locus in MHS and to define the proportion of MHS families for which it can account, and also to determine the extent of genetic heterogeneity in MHS by scanning for other chromosomal regions linked to MHS throughout the genome and identifying candidate genes within those regions.

MATERIALS AND METHODS

Materials

The plasmid vector pGEM4 was obtained from Promega and propagated in Escherichia coli DH5α. Yeast cells were cultured in AHC medium (containing per litre 8 g yeast nitrogen base [Difco 091-15], 20 g glucose [Merk, 8337] 55

mg adenine hemisulphate [Sigma, A 9126], 55 mg l-tyrosine [Sigma, T 3754] and 1.4 g casamino acids [Difco, 0288], pH 5.8), and bacterial cells were cultured in Luria-Bertani medium containing ampicillin at a concentration of 50 μg/ml. T7 and SP6 sequencing primers were obtained from Promega. T7 DNA polymerase and custom synthesized oligonucleotides were purchased from Pharmacia Benelux (Roosendaal, NL). Restriction enzymes, T4 polynucleotide kinase (PNK) and large fragment (Klenow) DNA polymerase were provided by BRL-Life Technologies. GELaseTM was obtained from Epicentre Technologies. All procedures using the enzymes were carried out according to the manufacturer's instructions. Zymolase was obtained from Kyrin Breweries, Japan, and AmpliTaqTM DNA polymerase was obtained from Perkin Elmer Cetus (Hoffmann-La Roche). Low melting temperature (LM) agarose was purchased from SeaPlaque (50101), and α[^{32}P]dCTP and γ[^{32}P]ATP were supplied by Amersham International. Small DNA fragments (0.2−5.0 kb) were isolated from agarose gels using a GeneClean kit (Bio 101). Dulbecco's minimal essential medium and fetal calf serum were purchased from GibcoBRL-Life Technologies.

Restriction analysis of YAC clones containing the CACNL2A locus

A *Bam*HI fragment from the rabbit DHPRα_2-subunit cDNA containing 2.8 kb of coding sequence was used to screen a human chromosome 7-specific YAC library (31) using standard procedures (43). Two clones, HSCE1129 and HSCE520, hybridized with the probe and were taken for further analysis. Single yeast colonies were inoculated into 50 ml AHC medium, cultured for 60 h at 30°C, and harvested by centrifugation (500 *g*, 10 min). Procedures for the preparation and lysis of yeast spheroplasts in 0.5% LM agarose plugs were carried out essentially as described (33). For restriction enzyme analysis, the LM agarose plugs containing yeast DNA were first equilibrated in several changes of a restriction enzyme buffer for 1 h at 0°C, incubated for 2 h on ice in 1 volume of buffer containing 20 units enzyme (*Sal*I or *Mlu*I) per μg DNA, and then overnight at 37°C. The buffer and enzyme were then refreshed, and digestion was allowed to continue for a further 8 h. Plugs were equilibrated in several volumes of 0.5×TBE prior to resolution of DNA fragments by PFGE (33) for 24 h in 1% agarose gels, using a field strength of 200 V and a switch time of 40 s. Undigested yeast DNA and λDNA concatemers were used as markers. Visualization of DNA fragments, Southern blotting and hybridization were carried out as described (39).

Subcloning and partial sequence analysis of CACNL2A

Restriction analysis of clone E1129 showed that a 580 kb *Mlu*I fragment, which apparently overlapped clone E520, contained the CACNL2A locus. This fragment was isolated by preparative PFGE. Approximately 50 μg E1129 DNA in LM agarose plugs was digested to completion with *Mlu*I and applied to a preparative 1% LM agarose gel in 0.5×TBE. PFGE was carried out as described above using undigested DNA and *Mlu*I-digested DNA as markers. The marker lanes were removed and stained with ethidium bromide to localize the 580 kb *Mlu*I band in the preparative portion of the gel, which was not exposed to UV light. The band was removed and the DNA was recovered intact by GELase^{TM} digestion followed by concentration and dialysis against 10 mM Tris-HCl pH 7.6, 1 mM EDTA in an Amicon 10 concentrator. Random *Sau*3AI fragments from the isolated DNA were subcloned into pGEM4, and subclones hybridizing with the rabbit DHPRα_2-subunit cDNA probe were sequenced using double-stranded plasmid DNA as template and T7 DNA polymerase as described (25). All DNA sequences were analysed using Intelligenetics Suite 5.4 software and the University of Nijmegen CAMMSA package. The sequences of five subclones containing putative exons of CACNL2A have been deposited in the EMBL database (accession nos. Z28599, Z28602, Z28605, Z28609 and Z28613).

Identification and characterization of the D7S849 locus

Screening the 580 kb *Mlu*I fragment subclones for the presence of potentially polymorphic microsatellite repeats as described previously (25) yielded the structure $(A)_5(AC)_{13}AA(AC)_6AA(AC)_{11}AA(AC)_5AA(AC)_6GA$ within subclone p1129Re (EMBL accession number Z28696), and primers (AC strand 5′-AAGGCCTGT-TAAAAATCACC, TG strand 5′-GACCCTGGGCAAGTCATTA) were synthesized for its amplification. The expected size of the amplification product was 152 bp (GDB accession no. G00-251-863). A 125 bp *Bam*HI/*Alu*I fragment from p1129Re was used as a probe on the Southern blot of the YAC clones to position D7S849 relative to CACNL2A.

Fluorescence *in situ* hybridization

Total YAC E1129 DNA (1 μg) was labelled with biotin-14-dATP (Sigma) using a bio-nick labeling system (Life Technologies), separated from unincorporated label by Sephadex G-50 column chromatography and ethanol precipitated in the presence of a 200-fold excess of sonicated human genomic DNA. Probe diluted to a final concentration of 5 ng/μl was denatured, preannealed and hybridized to high resolution chromosomal preparations, with immunocytochemical detection and visualization of hybridized probe DNA were carried out as described (44,45). Digital images were recorded using a Photometrics high performance CH 250/a cooled CCD camera controlled by TCL-image image analysis and processing software (TNO, Delft, the Netherlands; modified by Biological Detection Systems, Pittsburgh, USA) running on a Macintosh computer.

Somatic cell hybrids and polymerase chain reaction (PCR) analysis

The generation and propagation of human/hamster somatic hybrid cell lines containing fragments of human chromosome 7 have been reported in detail elsewhere (30). In the present study, the following cell lines were used: GM10791, entire human chromosome 7; D1, 7pter−q11.2::q22.1−qter; GM05183.71, 7pter−q21.1; GM05183.17, 7q21.1−qter; D2, 7pter−q21.1::q22.1−qter; D4, 7pter−q21.12::q22.1−qter; D3, 7pter−q21.2::q22.1−qter. Chinese hamster ovary cell line DNA was used as control. PCR analyses of somatic hybrid cell lines were carried out in 50 μl volumes containing 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 50 pmol each primer, 100 ng DNA template and 1 U *Taq* DNA polymerase using a 'hot start' procedure as described (34). The reactions were processed through 35 cycles of denaturing at 94°C for 40 s, annealing at 55°C for 30 s and extension at 72°C for 1 min. The reaction products were analysed by electrophoresis on 2.5% agarose gels.

Analysis of genomic DNA

For the analysis of microsatellite repeats on human genomic DNA, reactions were carried out in 25 μl volumes using the same buffer as described above, but with only 50 ng DNA template, 0.5 U *Taq* DNA polymerase and 10 pmol each primer, with one primer from each pair being labelled with ^{32}P (46). The conditions for amplification were as described above except that the extension time at 72°C was reduced to 15 s and the number of cycles was reduced to 30. Alleles were separated on 6% denaturing polyacrylamide sequencing gels and visualized by autoradiography.

MHS families and *in vitro* contracture test

Clinical details of the families MH04, MH05, MH06, MH09, LMH03 and LMH08 have been published previously (11,12,24,47). The MHS status of all persons investigated was determined using the standardized European *in vitro* contracture (IVCT) test protocol (4), which classifies MH risk as either MH-susceptible (MHS), MH-normal (MHN) or MH-equivocal (MHE).

Linkage analyses

Pairwise and multipoint lod scores were calculated using the MLINK and LINKMAP programs of the LINKAGE 5.2 software package run on a Sun Sparc10 under SunOS 4.1.3 at the University of Nijmegen CAOS/CAMMSA centre. For multipoint analyses, alleles were reduced where necessary, but correct allele frequencies were retained to avoid possible distortion of lod scores (48). The sex-average genetic distance between markers was based on published (35) and unpublished data (J.W.) and on the results of this study. The sex-average distances (in cM) between markers were as follows: AFM295yg9-3-AFM203vb6-0.1-AFM277vd5-1-D7S849-5-D7S524. MHS penetrance was assumed to be 1 for probands, 0.98 for those individuals diagnosed as MHS and 0.02 for all those diagnosed as MHN in the IVCT. The frequency of the MHS allele was assumed to be 0.0001 without mutation and an unknown disease status was assigned to all MHE and untested indviduals.

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