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Myotonia levior is a chloride channel disorder

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The group of dominant non-dystrophic myotonias, comprising disorders characterized by clinically similar forms of myogenic muscle stiffness, is genetically inhomogeneous. Dominant myotonia congenita (Thomsen's disease) is linked to *CLCN1*, the gene encoding the major muscle chloride channel, localized on chromosome 7q35. In contrast, dominant myotonias sensitive to potassium are caused by point mutations in *SCN4A* on chromosome 17q, the gene for the α subunit of the adult skeletal muscle sodium channel. No linkage or molecular genetic data are as yet available on 'myotonia levior' characterized by milder symptoms and later onset of myotonia than in Thomsen's disease, and absence of muscle hypertrophy. We report a *CLCN1* Gln-552-Arg substitution for a family with dominant inheritance previously diagnosed to have myotonia levior. Thus, this disorder appears as a variant of Thomsen's disease due to mutations leading to low clinical expressivity. In addition, we report a novel Ile-290-Met *CLCN1* mutation for a typical Thomsen pedigree. In another family previously diagnosed as having Thomsen's disease, we unexpectedly found a *CLCN1* 14 bp deletion known to cause recessive myotonia, and a rare Trp-118-Gly polymorphism.

INTRODUCTION

When the molecular genetic basis for the group of non-dystrophic myotonias was revealed, a number of long-standing questions about these diseases were answered. For instance, it was shown in 1992, that both the dominant Thomsen's disease (1) and the recessive Becker-type myotonia (2) are linked to the same gene, *CLCN1*, located on chromosome 7q35, encoding the major muscle chloride channel (3,4). This linkage of myotonic disorders to the chloride channel gene, however painstaking and elegant its demonstration, did not come as a great surprise to many experts, as electrophysiological and pharmacological experiments had demonstrated for animal models, the myotonic goat (5) and the ADR mouse (6) and consequently also for several human patients (7,8), that in these diseases the chloride conductance of the skeletal muscle fibre membrane is reduced. To date, *CLCN1* mutations have been discovered in six Thomsen families including Dr Thomsen's own family (9,10,11) and several other *CLCN1* mutations that cause the recessive Becker-type (for review see 12).

Another discovery about the dominant form of myotonia, made also around 1992, came less expected. Prior to this, Becker himself, trying to classify all the many myotonic kinships available to him into having either the dominant or the recessive form, had discovered that many families for which the disease clearly followed the dominant mode of inheritance, exhibited a clinical picture which did not quite fit with the classical form of Thomsen's disease (2). This long-standing puzzle was clarified by molecular biology when it was shown that several mutations in the gene encoding the α subunit of the muscle sodium channel are responsible for the atypical conditions. It turns out that these atypical Thomsen cases, now classified as potassium-aggravated myotonias, are more common than Thomsen's disease. To date, sodium channel mutations have been reported for 14 dominant myotonia families with myotonia fluctuans, myotonia permanens, acetazolamide-responsive myotonia, or similar syndromes (Ser-804-Phe: 13,14; Gly-1306-Ala/Val/Glu: 14–17; Val-1589-Met: 18; Ile-1160-Val: 19).

In this now clarified map of the dominant myotonias there is still one 'blank spot', i.e. myotonia levior, first described by DeJong (20). Chloride conductance measurements have been performed in two related patients of a myotonia levior family, and since myotonia is very mild in this form, led to ambiguous results (patients MyC5A,5B in 21). We therefore decided to systematically search for a *CLCN1* mutation in this family. In addition, we screened a newly detected Thomsen kinship and one of Becker's pedigrees with Thomsen's disease.

RESULTS

Family DM-5 (myotonia levior)

This kinship was previously diagnosed as having myotonia levior (for details see Patients section). An abnormality in the electrophoresis mobility of single-stranded DNA fragments of the *CLCN1* transcript was found in exon 15 (Fig. 1A) but in none of the other 22 exons. This abnormality was present neither in the unaffected family members nor in other families with dominant or recessive myotonia congenita nor in 100 healthy controls.

Direct sequencing exhibited an A-to-G transition at position 1655 of the major skeletal muscle chloride channel cDNA, leading to a Gln-552-Arg substitution (Fig. 1B). This glutamine is preserved in all known members of the ClC family (Fig. 2 top).

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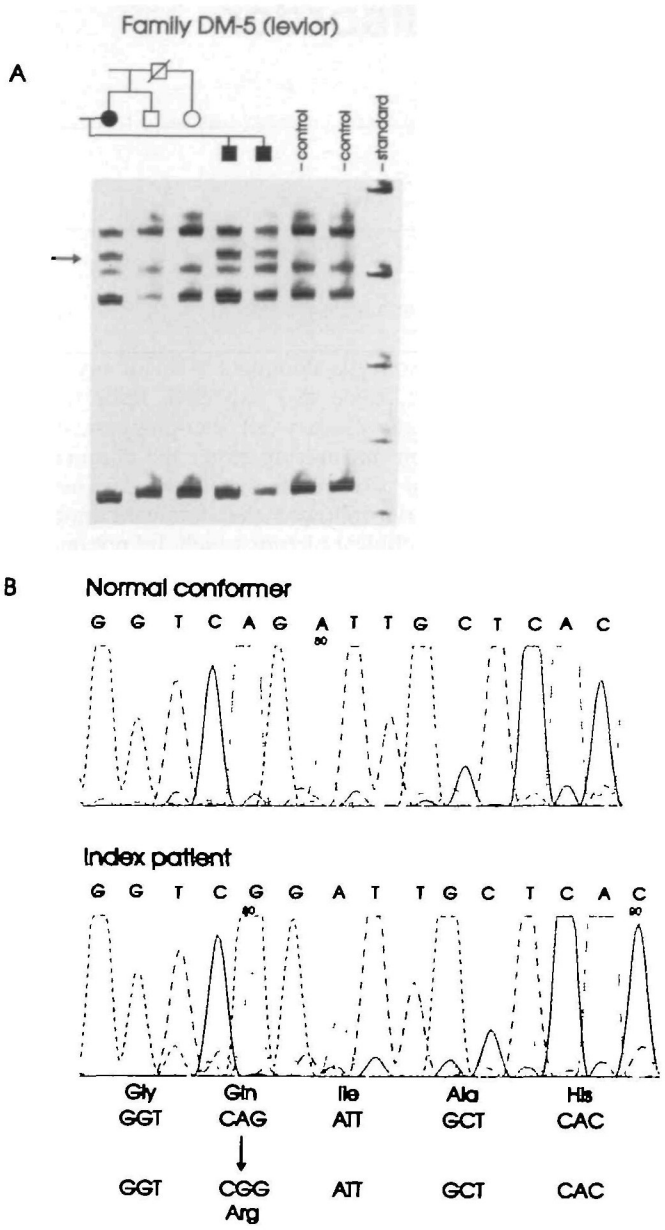

Figure 1. (A) Pedigree of family DM-5 (myotonia levior). The ethidium bromide-stained polyacrylamide gel below shows single-strand conformational polymorphisms of exon 15 of *CLCN1* encoding the major skeletal muscle chloride channel. The affected family members (filled symbols) revealed aberrant bands (arrows). (B) A comparison of the *CLCN1* sequences of a part of exon 15 from a control and an affected family member. Note the A-to-G exchange predicting the substitution of Arg for Gln. Far right: molecular weight standard (100 bp ladder, Pharmacia).

Family DMC-6 (Thomsen)

Aberrant single-stranded DNA fragments of the *CLCN1* transcript were discovered only in exon 8 for several members of the typical Thomsen myotonia family (Fig. 3A). These fragments were present in the affected family members and absent in all other myotonia patients as well as in 56 healthy controls screened by use of SSCP analysis.

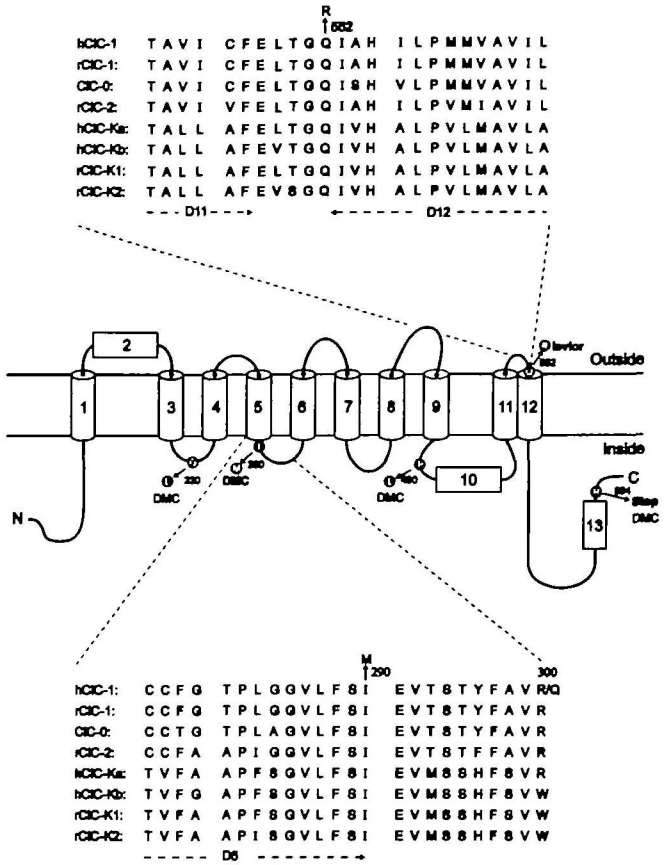

Figure 2. Schematic drawing of the major skeletal muscle chloride channel modified after Middleton and co-workers (22). The novel Gln-to-Arg mutation leading to myotonia levior is situated at the extracellular end of domain 12; the novel Ile-to-Met mutation causing Thomsen's disease (DMC = dominant myotonia congenita) is located at the intracellular end of the fourth transmembrane segment (domain 5). The upper inset shows an alignment of the predicted chloride channel sequence from various species over parts of the extracellular loop between domains 11 and 12 and the adjacent domain 12; the lower inset the corresponding information on parts of domain 5 and the intracellular loop between domains 5 and 6; hClC-1 (or *CLCN1*) and rClC-1, major human and rat skeletal muscle chloride channel; ClC-0, *Torpedo marmorata* electroplax chloride channel, rClC-2, ubiquitously expressed chloride channel of rat; hCl-Ka and hClC-Kb, human kidney chloride channels; rClC-K1 and rClC-K2, rat kidney chloride channels (23). Note the high conservation of glutamine-552 and isoleucine-290 among all species.

By sequencing the DNA eluted from the aberrant band found for family DMC-6, a C-to-G base exchange at *CLCN1* cDNA position 870 was discovered; it predicts a substitution of methionine for isoleucine-290 (Fig. 3B). This isoleucine is situated in the intracellular loop between domains 5 and 6, and is highly conserved among all known members of the ClC family (Fig. 2 bottom).

Loss of a *Taq*I restriction site by the base exchange made it possible to easily screen genomic DNA from an additional 44 healthy controls. *Taq*I (palindromic recognition sequence: TCGA) yielded three fragments for PCR products from control DNA (47 bp, 79 bp, and 133 bp) and an additional band of 126 bp due to loss of a restriction site for the DMC-6 patients (Fig. 4). Since the patients carry a normal and an affected

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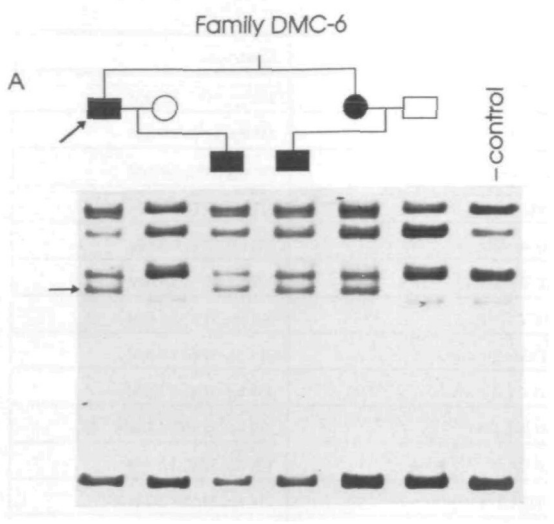

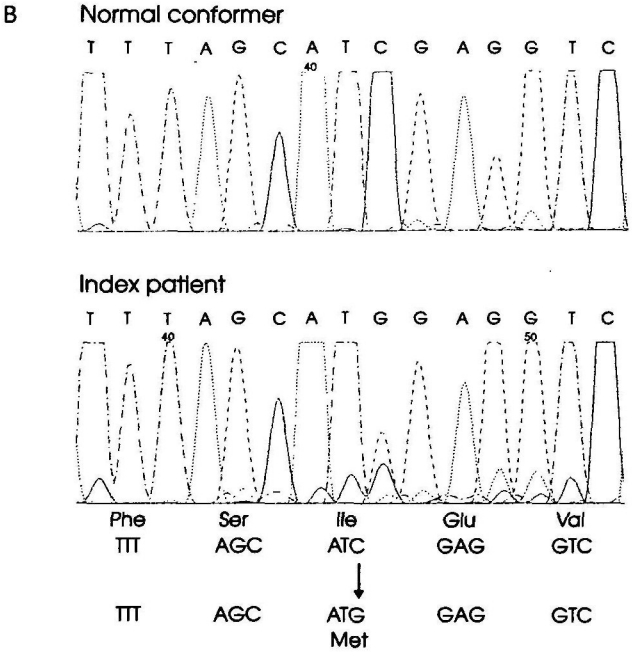

Figure 3. (**A**) Pedigree of family DMC-6. The index patient is indicated by an arrow. The affected family members (filled symbols) revealed aberrant bands (arrows) suggesting a mutation in *CLCN1* exon 8. For technical details see Figure 1 and Methods section. (**B**) A comparison of the *CLCN1* sequence of a fragment of exon 8 from a control and the index patient of family DMC-6. Note the presence of both the normal base, C, and the C-to-G base exchange predicting the substitution of Met for Ile.

CLCN1 gene, the DNA fragments of the normal gene were visible with lower density than those obtained from controls.

Family MC-3 ('dominant' myotonia congenita)

Dominant pattern of inheritance in this family was demonstrated by P.E.Becker (2) who had examined the index patient and his mother. Thus, we were surprised to find the index patient homozygous for a deletion in exon 13 of *CLCN1* (Fig. 5). Direct sequencing of the PCR product revealed a 14 bp

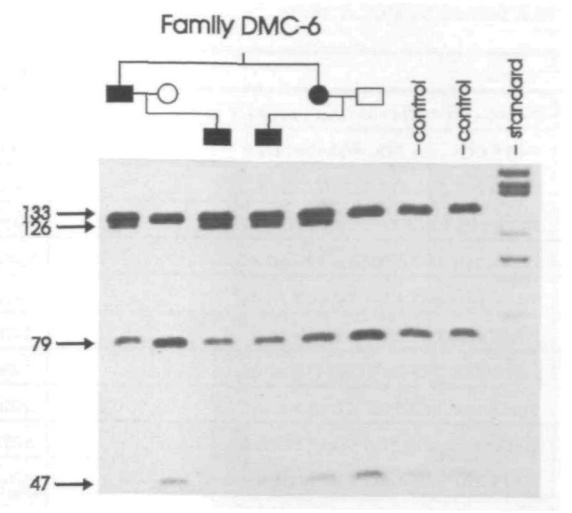

Figure 4. Pedigree of family DMC-6 with corresponding polyacrylamide gel displaying PCR-amplified genomic DNA fragments following digestion with *Taq*I and staining with ethidium bromide. PCR products of normal individuals show complete digestion yielding three fragments (47 bp, 79 bp, and 133 bp) whereas patients' DNA shows an additional band of 126 bp due to loss of a *Taq*I restriction site between the 47 and 79 bp fragments. Far right: molecular weight standard (pBR322/*Msp*I digest, New England Biolabs).

deletion (nc 1437–1450; not shown) leading to premature stop codons identical to the deletion reported by Meyer-Kleine *et al.* (24). In a heterologous pattern, this deletion was observed in both non-myotonic sons of the index patient (Fig. 5) and in two index patients with the clinical diagnosis of recessive myotonia congenita (not shown).

In addition, the MC-3 index patient was found to be homozygous for a T-to-G transversion at nucleotide 352 located in exon 3 predicting a glycine substitution for tryptophan-118. His sons, who had neither clinical nor EMG myotonia, as well as four other unrelated myotonia index patients of different ethnic origin and seven out of 205 healthy controls were heterozygous for this base exchange.

DISCUSSION

Both the A-to-G transition in myotonia levior family DM-5 predicting a Gln-552-Arg substitution and the C-to-G transversion in Thomsen family DMC-6 predicting an Ile-290-Met substitution, fulfil the criteria of a causal mutation. This being: (i) absence of the substitution in a great number of controls (100 unrelated individuals with no known neuromuscular disease); (ii) segregation with the clinical status of the family members; (iii) presence in a gene primarily or exclusively expressed in the affected tissue (skeletal muscle); and (iv) location in a highly conserved gene region (in all members of the ClC family including that of the electric organ of *Torpedo marmorata*).

According to the channel model of Middleton and co-workers (22), the two previously published Thomsen mutations (Gly-230-Glu and Pro-480-Leu) (9,10) as well as the novel Ile-290-Met substitution would be situated at the intracellular side of the membrane (Fig. 2). In contrast, Gln-552-Arg would be located at the extracellular side close to the membrane and

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Table 1. PCR primers for *CLCN1* exons

Exon	Upstream	Downstream	Conditions
1	5'-GGG GCT CGG GGG GAG GGA AT-3'	5'-CCT CAT TTT CAC CAG TCT CT-3'	272 bp, 56°C, 1.5 mM
2	5'-CTT CCA CAA GGC AGA CAC TG-3'	5'-ATG CCC AAG TTA TTC TCC TA-3'	234 bp, 51°C, 2.0 mM
3	5'-TTT TCC CTC ATC TCT TCC TA-3'	5'-CCA TAA CAC ACC CTG CTT AC-3'	256 bp, 53°C, 3.0 mM
4	5'-CGG TGG ACA CGG CTG CTC AG-3'	5'-GCC GAG TCT GGT GGC AAG TT-3'	211 bp, 59°C, 1.5 mM
5	5'-TAA TCT TTC AAC GCT TTT AG-3'	5'-ATC CAC TTC CAC TCC CAG AG-3	248 bp, 53°C, 2.0 mM
6	5'-CCT CTG TGT AAC TCC CGT AT-3'	5'-ATT TCA CTG TCT CAA CCT TA-3'	227 bp, 53°C, 1.5 mM
7	5'-ACC CAC CCT GTT TCT CTG TC-3'	5'-GCT ATT CTC GTA AGT AAC CT-3'	227 bp, 51°C, 2.0 mM
8	5'-TGC CCC CAA CCA CAC TTC TG-3'	5'-GCC CAT TAT TCC TTT TCT GA-3'	259 bp, 55°C, 1.5 mM
9	5'-ACT GGC CTT TCC ATC CTA CA-3'	5'-GCC TCA CTC CAC ACC CTG CT-3'	157 bp, 54°C, 1.5 mM
10	5'-TCC AAG AGA TGA GGA TTT CA-3'	5'-GAC AAA AAG GGA GGA ACT CT-3'	229 bp, 52.5°C, 1.5 mM
11	5'-ATT TAC TGT GAG TTG GCT GA-3'	5-'GTG CTG TTT CCT CTT TAC CA-3'	178 bp, 52°C, 1.5 mM
12	5'-GAC CAC CTT CTG CTT CTT CC-3'	5'-TGG AGG TTT AGG GTG TTG CT-3'	214 bp, 54.5°C, 2.0 mM
13	5'-CAG AGT TGA AAG GGT ATT CC-3'	5'-CCT TAT GTT TCC TGT ATC CT-3'	176 bp, 51°C, 1.5 mM
14	5'-ATC TCG TAA CAC CTT CCT TC-3'	5'-ATG GGA GAG TTT AAG TGT GG-3'	221 bp, 51°C, 1.5 mM
15	5'-CAG GCG TAT TCC TGT GTC AT-3'	5'-ATT CCG TCT AGT GCC CCT GA-3'	260 bp, 56°C, 1.5 mM
16	5'-GGC TAA CCC ACC ATG CTT CT-3'	5'-ATT CAG GTC ACT AGC TCT GG-3'	209 bp, 55°C, 1.0 mM
17	5'-GCC TCT CCT GTT CCT TCT CA-3'	5'-AGA CGA CGC TTT CCC TTG CT-3'	389 bp, 59°C, 1.5 mM
18	5'-CCA GGC TGA GAC TTC TTA CT-3'	5'-AGG GGT GAG TTG GGG TGC AT-3'	179 bp, 55°C, 2.0 mM
19	5'-CAT CCA CTC ACC TGT CCT CT-3'	5'-GGG TCT CTG CCT TCC TGA TT-3'	197 bp, 54.5°C, 1.0 mM
20	5'-GGA AGA AAA GGG AAA GAA CT-3'	5'-GAC AAC ACA ATA AAG AAG GT-3'	171 bp, 47°C, 1.5 mM
21	5'-TGA TTT TCG TGA CTT TCC TC-3'	5'-CCC CAC AGC CCT GAG CAG TC-3'	172 bp, 54°C, 2.0 mM
22	5'-ACC TGT GCT CTT CAT CCT CA-3'	5'-CCC TCC TGC TGC TCA AAT GG-3'	144 bp, 55°C, 2.0 mM
23A	5'-TTT CCA ACT TTT TAC CCT CT-3'	5'-TGG GGA GGC AGC AAT CAC AT-3'	229 bp, 55°C, 1.5 mM
23B	5'-ACT GGA ACA GGG GAT GTG AT-3'	5'-TTT ATG AGG AGG TCG TGG GA-3'	244 bp, 58°C, 2.0 mM

could alter the voltage dependence of channel gating by the addition of an extra positive residue. Thus, this dominant mutation could effect gating of the homo-oligomeric channel complex in a way suggested by Steinmeyer and co-workers (10).

Gln-552-Arg was found to be present in a kinship earlier diagnosed as having myotonia levior (21). The family fulfils all diagnostic criteria of the disease by showing later age of onset than in Thomsen's disease and by exhibiting mild to moderate myotonia of bulbar muscles and minimal myotonia of arm and leg muscles, in the absence of muscle hypo- and hypertrophy (20,25). Until now, it was unclear if such patients have a sodium or a chloride channel myotonia. Our finding of a chloride channel mutation is in agreement with the observation that the patients' muscles are insensitive to low temperature and high potassium similar to Thomsen and Becker patients (26). In contrast, myotonias caused by sodium channel mutations are sensitive to potassium and cold environment (13–19).

The debate about the inheritance is between whether it is a dominant trait with low clinical expressivity (20) or a recessive disease which causes mild symptoms in heterozygous carriers of the mutation (2). Since the disease-causing mutation is a missense mutation and since male and female heterozygous carriers exhibit the same clinical expressivity we consider it as having dominant effects. In contrast, heterozygous carriers of the recessive Becker-type myotonia show male predominance of latent myotonia (27). In conclusion, myotonia levior seems to be a mild form of Thomsen's disease. The fibre type

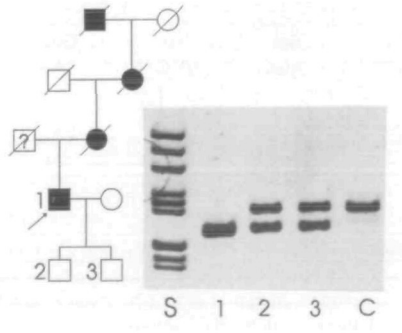

Figure 5. Pedigree of family MC-3, modified after Becker (1977), shows compatibility with dominant mode of inheritance. However, the ethidium bromide-stained non-denaturating polyacrylamide gel of the *CLCN1* exon 13 PCR product suggests a homozygous deletion in the index patient (1; arrow) and a heterozygous deletion in the available family members (2, 3). Left: (S) molecular weight standard (pBR322/*Msp*I digest, New England Biolabs); right: normal control.

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IIa deficiency observed in the here reported family stands in contrast to the well-known type IIb deficiency in other myotonia patients but may not be a specific finding.

The genotype of the seven dominant myotonia families reported by Iaizzo and co-workers (21) is now identified. Four of them carry a *CLCN1* mutation: the here reported three families and Dr Thomsen's own family (10; MyC-7 in 21); and in the remaining three kinships, point mutations in the *SCN4A* gene encoding the α subunit of the adult muscle sodium channel have been discovered: for MyC-1 a Gly-1306-Ala substitution (14); for MyC-2 a Val-1589-Met (18); and for MyC-4 a Val-1293-Ile (unpublished results).

The recessive *CLCN1* 14 bp deletion reported here for a family diagnosed to have 'dominant' Thomsen myotonia (MC-3) was completely unexpected. It is now obvious that the index patient is homozygous for a recessive mutation. Clinical findings and *in vitro* results are in agreement with the 'new' diagnosis of recessive myotonia congenita (Becker-type): (i) the myotonia of the index patient was much more severe than that of his heterozygous mother and that of most patients with Thomsen's disease; (ii) his stature exhibited the typical disproportion characterized by poorly developed neck, shoulder, and arm muscles and hypertrophied leg muscles (2); (iii) he displayed transient weakness, a symptom also characteristic for the recessive type (28), and (iv) his muscle fibres revealed a significantly reduced chloride conductance of 40 μS/cm^2 as compared to values of normal controls (175 μS/cm^2) and the other index patients with dominant forms of myotonia (77 to 214 μS/cm^2) (21).

The question arises why his mother also exhibited myotonia. Although latent myotonia, i.e. myotonic signs only present in the EMG, has been recorded from some parents of recessive myotonia congenita patients, clinical signs of myotonia have not yet been reported (29,30). A dominant mutation in the maternal ancestors in addition to the 14 bp deletion, the former not inherited by the MC-3 index patient or not detected by SSCP analysis, could explain the dominant inheritance. Unfortunately, since the genotypes of the deceased family members are unknown we cannot verify this hypothesis. Alternatively, the T-to-G transversion reported here resulting in a Trp-118-Gly substitution inherited by the index patient from both parents could have an aggravating effect on chloride channel dysfunction. However, both sons were heterozygous on the same chromosome for both the deletion and the substitution and exhibited no myotonia. Since the Trp-118-Gly substitution did not segregate with the disease in other myotonia families in whom it was not combined with the 14 bp deletion, we consider it as a polymorphism.

MATERIALS AND METHODS

Patients

The patients have given informed consent for a thorough clinical and genetic investigation. Index patients agreed to a vastus medialis muscle biopsy. The study was approved of by the Ethics Committee of the University of Ulm. The presence of prolonged CTG repeats within the myotonic dystrophy gene was excluded. Abnormal single-strand conformational polymorphisms were not found in the 24 exons of the muscle sodium channel gene.

Family DM-5 (levior): The two brothers (pedigree see Fig. 1), now 27 and 25 years of age, were labeled as patients MyC-5A and B in an earlier study (21). Since the age of approximately 5 years, they complained of impeded muscle relaxation which was pronounced when exercise was initiated, which was similar to the degree of their mother's myotonia. Clinical examination of the two brothers showed normotrophic skeletal muscles, lid lag, percussion myotonia, mild myotonia (pronounced in the forearm muscles) with warm-up phenomenon but no transient or permanent weakness. The EMG revealed myotonic runs. Neither cooling of the forearm nor oral potassium load (80 mmol) affected myotonia or force. Serum CK levels were normal. Biopsies of the vastus medialis muscle showed almost normal morphology with fibre type IIa deficiency. CT scans of thigh and leg muscles were normal.

Family DMC-6: The pedigree is shown in Figure 3A. In an earlier study, the index patient was labeled as dominant myotonia congenita patient MyC-6 (21). This male patient is a 54 year old metal worker with hypertrophic muscles. Clinical features were lid lag phenomenon, percussion and slight generalized myotonia. Grip myotonia was present after rest and diminished with repetitive movements (warm-up phenomenon). Muscle stiffness did not worsen after oral ingestion of 80 mmol potassium. Muscle weakness (transient or permanent) and cataract were absent. His son, sister and nephew also had slight myotonia. Myotonic runs were recorded in all investigated muscles of the index patient. The serum CK level was normal. A biopsy of the biceps brachii showed slight hypertrophy and fibre type IIb deficiency. The family was included in the analysis showing genetic linkage to the *CLCN1* locus (3).

Family MC-3 (pedigree shown Fig. 5): In studies also performed at a time when *CLCN1* had not yet been located, this family was diagnosed as having autosomal dominant myotonia congenita Thomsen (MyC-3 in 21). In 1964, the index patient, born in 1927, and his mother were examined by P.E.Becker (kindred Fa. in 3) and classified to have moderate myotonia (index, onset of myotonia at age of 3 years) and very mild myotonia (mother). His maternal grandmother and great-grandfather were also reported to suffer from myotonia. In several recent clinical examinations, the index patient showed percussion myotonia, moderate to severe myotonia of arm and leg muscles with warm-up phenomen and transient muscle weakness. No muscle atrophy was noted, but the musculature of the arms, especially distally, was found to be poorly developed in contrast to the hypertrophic leg muscles. CK was normal and no cataract was found. Myotonic symptoms were successfully treated with mexiletine (200 mg 3 times a day). Myotonic runs were recorded in all muscles. A biopsy from the vastus medialis displayed slight hypertrophy and a fibre type IIb deficiency. Clinical examinations of his sons were normal as well as the EMG performed in one of them.

Molecular genetics

Genomic DNA was extracted and screened for mutations from anticoagulated blood obtained from members of the three families following their informed consent. In addition, DNA from unrelated 100 (205) controls with no neuromuscular disease were screened.

Polymerase chain reaction (PCR): Samples of genomic DNA were amplified by PCR with primers specific for the 23 exons encoding ClC-1, the major muscle chloride channel protein. The primers used are listed in Table 1. The experimental conditions were optimized for each primer. The reaction mixture with a final volume of 100 μl contained: 100 ng DNA, 50 pmol of each PCR primer, 50 μM of each deoxynucleotide triphosphate, 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 1.5 units of Ampli*Taq* polymerase. Amplification conditions were as follows: denaturation of probes at 96°C for 10 min; 35 cycles at 94°C for 30 s, 55°C for 45 s, and 72°C for 1 min followed by a terminal elongation at 72°C for 5 min.

Single-strand conformational polymorphism (SSCP) analysis: PCR products were precipitated with ethanol and resuspended in 30 μl of distilled water. Six microlitres of the amplified samples were diluted with 1 μl of gel-loading dye (40% sucrose, 0.05% bromophenol blue, 0.05% xylene cyanol), denaturated at 96°C and loaded onto 5% polyacrylamide gels. Gels were run at 300 V for 4 to 5 h in a buffer containing 90 mM Tris borate (pH 8.3) and 2 mM EDTA. After electrophoresis, the gels were stained with 0.5 μg/ml ethidium bromide.

Direct PCR sequencing: Single bands were cut directly from gels under UV light and eluted for 15 min in 100 μl of distilled water at 80°C. A 10 μl aliquot was used for asymmetric PCR re-amplification with the same PCR primers and conditions except for the primer ratio (5–50 pmol) and the number of amplification cycles (n = 50). PCR products were purified with centricon-100 dialysis concentrators (Amicon) and 30–50% of the retentate sequenced with the dideoxy termination method using *Taq* polymerase, upstream or downstream primers, and fluorescently tagged dideoxynucleoside triphosphates on a 373 DNA sequencer (Applied Biosystems, Foster City, CA).

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Digestion with TaqI: A 10 µl aliquot of the amplified sample was digested with 5 U *TaqI* for 16 h at 65°C under mineral oil in a solution of 10 mM Tris acetate (pH 7.5), 10 mM magnesium acetate, and 50 mM potassium acetate (total volume 20 µl). Reaction was stopped by adding 5 µl of blue sucrose solution (30% sucrose, 50 mM EDTA, 0.25% bromophenol blue, 0.1% SDS). Five microlitres of the digested sample were directly loaded onto 5% polyacrylamide gels. The gels were run at 300 V for 2 h in a buffer containing 90 mM Tris–borate (pH 8.3) and 2 mM EDTA. After electrophoresis, the gels were stained with 0.5 µg/ml ethidium bromide. Digestion was done on 44 control samples.

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