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Novel Muscle Chloride Channel Mutations and Their Effects on Heterozygous Carriers

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Summary

Mutations within *CLCN1*, the gene encoding the major skeletal muscle chloride channel, cause either dominant Thomsen disease or recessive Becker-type myotonia, which are sometimes difficult to discriminate, because of reduced penetrance or lower clinical expressivity in females. We screened DNA of six unrelated Becker patients and found four novel *CLCN1* mutations (Gln-74-Stop, Tyr-150-Cys, Tyr-261-Cys, and Ala-415-Val) and a previously reported 14-bp deletion. Five patients were homozygous for the changes (Gln-74-Stop, Ala-415-Val, and 14-bp deletion), four of them due to parental consanguinity. The sixth patient revealed compound heterozygosity for Tyr-150-Cys and Tyr-261-Cys. Heterozygous carriers of the Becker mutations did not display any clinical symptoms of myotonia. However, all heterozygous males, but none of the heterozygous females, exhibited myotonic discharges in the electromyogram suggesting (i) a gene dosage effect of the mutations on the chloride conductance and (ii) male predominance of subclinical myotonia. Furthermore, we report a novel Gly-200-Arg mutation resulting in a dominant phenotype in a male and a partially dominant phenotype in his mother. We discuss potential causes of the gender preference and the molecular mechanisms that may determine the mode of inheritance.

Introduction

When A. J. Thomsen (1876) first described myotonia congenita, he stated the dominant mode of inheritance as one of the characteristic features of the disease. In the late 1950s, Becker (1957) discovered that in many of his families the mode of inheritance was recessive. In general, Becker-type myotonia is more severe than Thomsen disease and shows 3:1 male predominance in propositi (Becker 1977), suggesting reduced penetrance or milder clinical expressivity in females. Characteristic repetitive discharges in the electromyogram (EMG) commonly used to diagnose myotonic disorders have been detected not only in Becker-type patients but also in some of their clinically unaffected parents. This subclinical phenomenon was termed *latent myotonia* (Becker 1977; Zellweger et al. 1980; Streib and Sun 1982). To date, no information on the genotype-phenotype relationship of latent myotonia and its gender distribution is available.

According to the low chloride conductance theory of myotonia first developed for an animal model, the dominant myotonic goat (Bryant 1969), a reduction of the membrane chloride conductance was found in excised muscle fibers from patients with dominant and recessive myotonia (Lipicky et al. 1971; Rüdel et al. 1988) as well as for the recessive myotonic ADR mouse (Mehrke et al. 1988). However, the conductance measurements did not yield a clear-cut difference between dominant and recessive phenotypes. Later, when Thomsen disease and Becker-type myotonia were found to be allelic disorders, it became obvious that the different inheritance modes must result from different effects of the mutations on the muscle chloride channel function (Koch et al. 1992).

To date, 10 missense mutations, 2 nonsense mutations, and 2 deletions in various exons of *CLCN1* have been described. Four point mutations cause Thomsen disease (for review, see Hoffman et al. 1995), and an additional mutation was found in a family with myotonia levior, a mild form of Thomsen disease (Lehmann-Horn et al. 1995). The other point mutations (Koch et al. 1992; George et al. 1994; Heine et al. 1994; Lorenz et al. 1994) and the deletions (Heine et al. 1994; Meyer-Kleine et al. 1994) were detected in Becker-type patients. Most of these (~60) patients were heterozygous for one mutation and probably carry a second heterozygous mutation that in combination (a condition termed compound heterozygosity) causes the recessive disease. Of a total of seven Becker patients, some of whom were offspring of consanguineous parents, the mutation in five was homozygous (Koch et al. 1992; Heine et al. 1994; Meyer-Kleine et al. 1994; Lehmann-Horn et al. 1995) and in two was compound heterozygous (George et al. 1994; Lorenz et al. 1994).

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Insight into the effects of so far four *CLCN1* mutations was gained by the study of channel mutants expressed in heterologous cells (Lorenz et al. 1994; Steinmeyer et al. 1994; Fahlke et al. 1995). However, functional expression is so time-consuming that it cannot compete with the rapidly increasing number of *CLCN1* mutations. For the determination of the functional consequences of five novel mutations on muscle fiber chloride conductance, we studied clinically unaffected heterozygous carriers.

Patients and Methods

The patients and their relatives have given informed consent for a thorough clinical, electromyographic, and genetic investigation. Six unrelated propositi, all male, were diagnosed as having recessive Becker-type myotonia (RMC), since (i) onset of the disease was between 4 and 9 years of age, (ii) myotonic stiffness began in the leg muscles and then ascended to arm and bulbar muscles within several years, (iii) leg muscle hypertrophy and transient muscle weakness developed, and, most important, (iv) none of the parents exhibited clinical signs of myotonia. However, all fathers as far as available for clinical and EMG examination (i.e., fathers of patients RMC-32/37/48) and the brother of RMC-62 revealed latent myotonia; that means myotonic discharges in the EMG in at least one of three muscles examined. In contrast, neither the sister of RMC-62 nor any of the four mothers available for EMG displayed latent myotonia. Consanguinity was present in four of the six families.

Another male propositus (MC-34) was diagnosed as having dominant Thomsen disease because of (i) mild myotonia with age at onset at <2 years and (ii) absence of both muscle hypertrophy and transient weakness, and (iii) his mother reported myotonic stiffness of leg muscles when pregnant and on several occasions (e.g., when tired or hungry) showed equivocal myotonic symptoms and displayed myotonic discharges in the EMG, and (iv) his father had neither clinical nor latent myotonia.

Molecular Genetics

Genomic DNA was extracted and screened for mutations from anticoagulated blood obtained from 36 index patients with Thomsen or Becker-type myotonia, with their informed consent. In addition, DNA from ≥ 100 (≤ 205) German and 50 Turkish controls with no neuromuscular disease were screened.

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 primer sequences used were given in the study by Lehmann-Horn et al. (1995). 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, 25 μM of each deoxynucleotide triphosphate, 10 mM tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 1.5 U of AmpliTaq polymerase. Amplification conditions were denaturation of probes at 96°C for 10 min and 30 cycles at 94°C for 30 s, at annealing temperature for 45 s, and 72°C for 1 min, followed by a terminal elongation at 72°C for 5 min.

SSCP analysis.—PCR products were precipitated with ethanol and resuspended in 30 μl of distilled water. Six microliters 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 4–5 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 (Yap and McGee 1992).

Direct PCR sequencing.—Direct PCR sequencing was performed on both double- and single-stranded PCR products of DNA of all family members indicated in the pedigrees. 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 reamplification 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).

Results

SSCP screening was performed on all 23 *CLCN1* exons from genomic DNA. Data obtained from direct sequencing of single-stranded or double-stranded PCR products of patients' DNA was compared to those from ≥ 100 control subjects of comparable ethnic origin. Control DNA showed no abnormalities, if not indicated otherwise.

Gln-74-Stop

Abnormal electrophoresis mobility of single-stranded DNA was found in exon 2 for the German family RMC-62 (fig. 1*A*). DNA of the patient, his unaffected daughter, and siblings revealed an aberrant band. In contrast to them, the patient's DNA displayed loss of a normal conformer, suggesting homozygosity.

By sequencing the DNA eluted from the aberrant band in patient RMC-62, a C-to-T transition at position 220 of the *ClCN1* cDNA sequence was shown to cause a

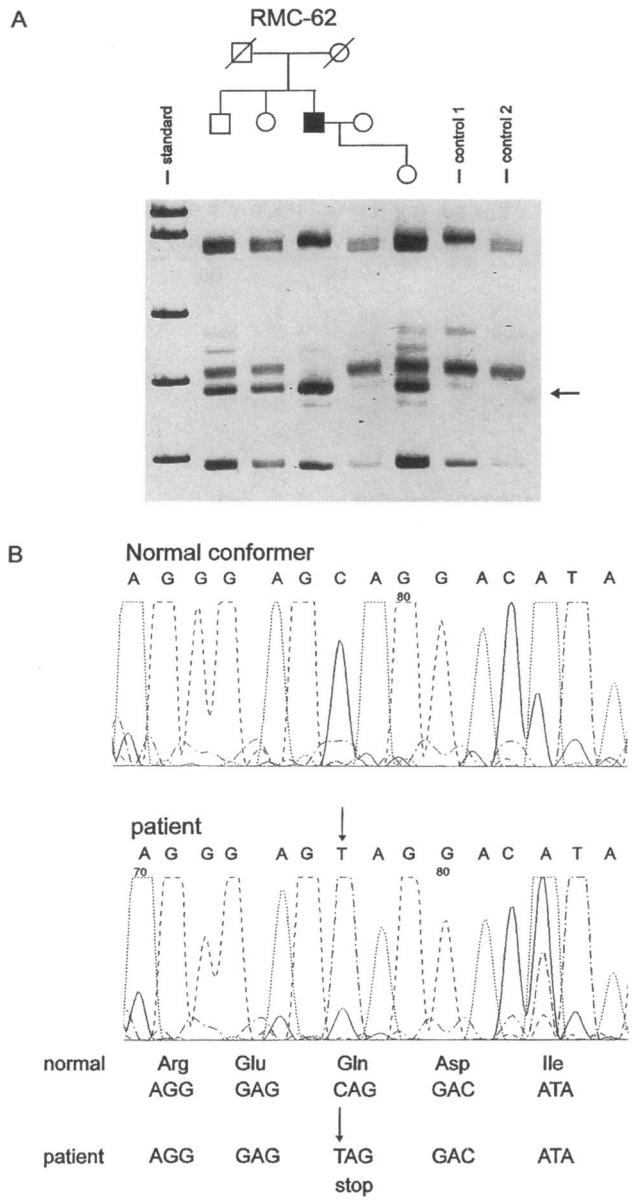

Figure 1 *A,* Pedigree of family RMC-62. The ethidium bromide–stained polyacrylamide gel below shows single-stranded conformers of exon 2 of *CLCN1* encoding the major skeletal muscle chloride channel. The DNA of the patient (filled symbol) revealed an additional band (arrow) and loss of others as compared to healthy controls. Another abnormal conformer due to a polymorphism was found for spouse, daughter, and siblings of the patient (for details, see text). *Far left,* DNA size standard illustrated by pBR/*Msp*I digest. *Far right,* Control DNA revealing the polymorphism. *Bottom,* Double-stranded PCR product. *B,* Comparison of the *CLCN1* sequences of a part of exon 2 from a control and the patient. Note that sequencing the double-stranded PCR product of the patient's DNA yielded high fluorescence for base T, confirming homozygosity.

termination codon replacing Gln-74 (fig. 1*B*) located in the N-terminus of the chloride channel (fig. 2). By sequencing the double-stranded PCR product, the patient was confirmed to be homozygous for this nonsense mutation, while his daughter and his siblings, a male with latent myotonia and a female without latent myotonia (table 1), were heterozygous carriers. A silent mutation causing an abnormal SSCP conformer was found in the same exon for spouse, daughter, and siblings of the patient, as well as for numerous normal controls, due to a polymorphism at cDNA position 261 (threonine-87).

Tyr-150-Cys and Tyr-261-Cys

Abnormal single-stranded DNA conformers were found for the Turkish patient RMC-48 and his father in exon 4 as well for the patient and his mother and brother in exon 7 (for pedigree, see fig. 3*A;* SSCP not shown). A-to-G base exchanges were detected at cDNA positions 449 and 782 in the DNA eluted from the aberrant bands, predicting substitutions of cysteine for tyrosine-150 (fig. 3*B*) and tyrosine-261 (not shown). Direct sequencing of the double-stranded PCR products for exons 4 and 7 demonstrated that the patient inherited both mutations. Similar to previously reported mutations (Asp-136-Gly, Phe-167-Leu, Val-327-Ile, Arg-338-Gln, Phe-413-Cys, and Gln-552-Arg), tyrosine-150 and tyrosine-261 are highly conserved among ClC-1 of various species but poorly conserved among the newly detected members of the ClC family such as ClC-Ka/2/3/4, which exert different functions (fig. 2, top, left).

Introduction of a *Bss*HII restriction site by the A-449-G base exchange made it possible to screen genomic DNA from 54 Turkish controls easily. *Bss*HII (recognition sequence: G.CGCGC) did not digest the 211-bp PCR product from control DNA but yielded two additional fragments of 138 bp and 73 bp because of introduction of a restriction site on the mutated allele for the patient and his father (fig. 3*A*). The patient's father had latent myotonia, whereas his mother, who was heterozygous for the A-782-G base exchange, did not display myotonic discharges in the EMG (table 1).

Ala-415-Val

Aberrant single-stranded DNA conformers were discovered in exon 11 for three patients (RMC-32/36/38), corresponding to a C-to-T base exchange at position 1244, which results in a substitution of valine for alanine-415 (fig. 4). The SSCP pattern suggested homozygosity in agreement with the consanguinity of the parents of each patient. This suggestion was confirmed by sequencing of the double-stranded PCR product. Of the consanguineous parents of patient RMC-32, the father presented with latent myotonia, whereas the mother revealed normal EMG. Alanine-415 is conserved among most members of the ClC channel gene family (fig. 2, top, right).

14-bp Deletion

The Turkish patient, son of second-grade consanguineous parents (grandparents were cousins; pedigree not

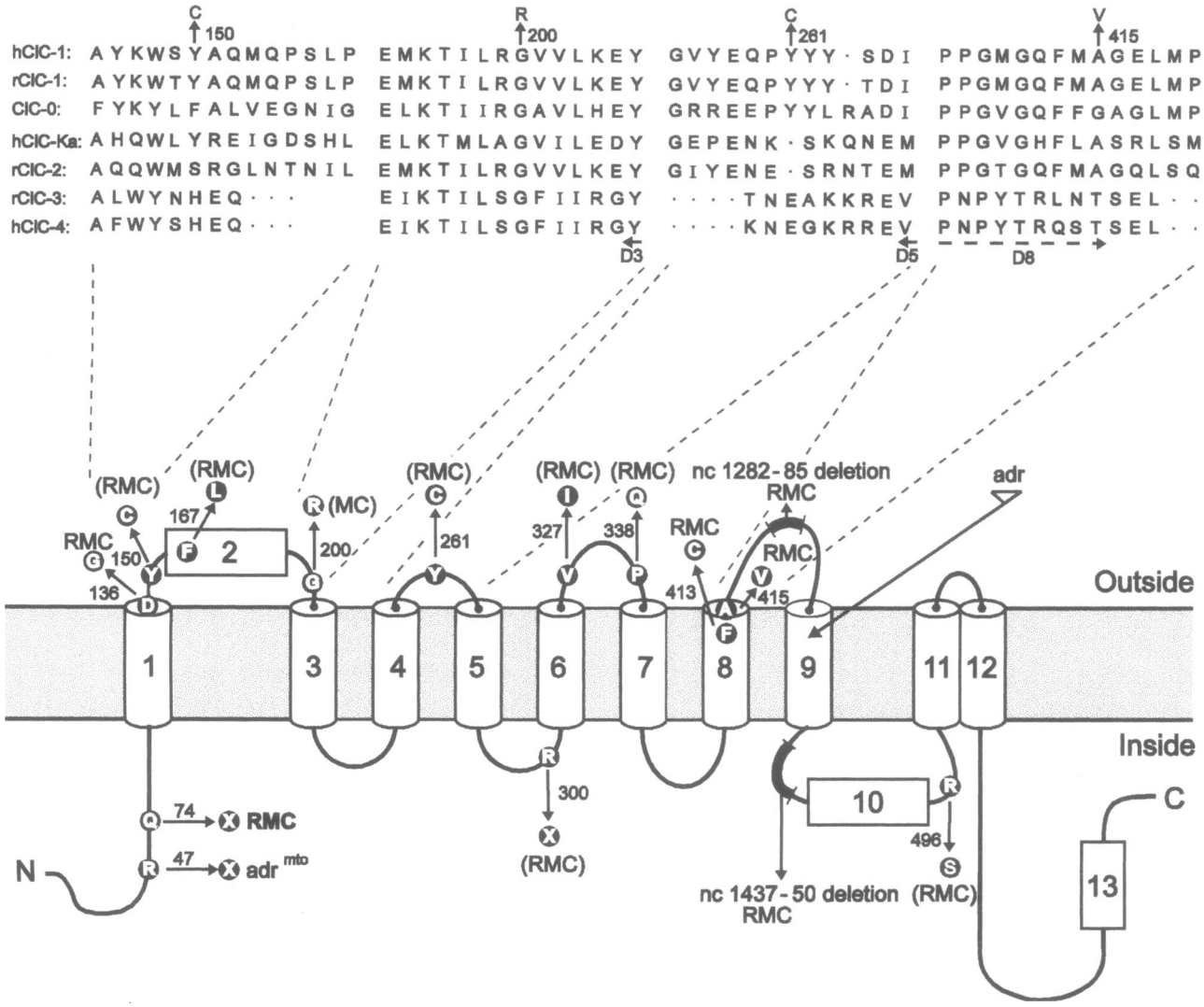

Figure 2 Schematic drawing of the major skeletal muscle chloride channel modified after the scheme of Middleton et al. (1994). The novel mutations, as well as the previously reported recessive myotonia congenita mutations and deletions, are included. RMC = recessive myotonia congenita in homozygous carriers; (RMC) = recessive myotonia congenita in patients with (definite or assumed) compound heterozygosity; MC = myotonia congenita with dominant inheritance as discussed in the text; adr = insertion of an ETn family transposon in the ADR mouse (Steinmeyer et al. 1991). The insets show alignments of the predicted chloride channel sequences from various species over regions in which the novel missense mutations are situated: hClC-1 (or *CLCN1;* GenBank accession number Z25884) and rClC-1 (accession number X77197) = major human and rat skeletal muscle chloride channel, respectively; ClC-0 = *Torpedo marmorata* electroplax chloride channel (Thiemann et al. 1992); hClC-Ka = human kidney chloride channel (Kieferle et al. 1994); rClC-2 = ubiquitous rat chloride channel activated by cell swelling (Thiemann et al. 1992); rClC-3 = rat chloride channel mainly expressed in cerebrum and cerebellum (Kawasaki et al. 1994); hClC-4 (or *CLCN4*) = human chloride channel also expressed in skeletal muscle (Van Slegtenhorst et al. 1994).

shown), was found to be a homozygous carrier of the previously published 14-bp deletion in exon 13 (Meyer-Kleine et al. 1994). In addition, the patient is a heterozygous carrier of a T-to-G transversion at nucleotide 352 located in exon 3, predicting a glycine substitution for tryptophan-118. This base exchange has been recently reported as a rare polymorphism (Lehmann-Horn et al. 1995). It must have occurred recently in family RMC-37, since it was present in the patient's father but not in the consanguineous mother. As in the other recessive families, of the heterogenous parents only the father showed latent myotonia.

Gly-200-Arg

SSCP analysis of exon 5 revealed an abnormal pattern for both the Turkish patient (MC-34) and his minimally affected mother. The detected G-to-A base exchange at cDNA position 598 predicts a substitution of arginine for glycine-200 (fig. 3*C, D*), which is highly conserved among

Table 1

***CLCN1* Mutations and Their Effects**

Family Code	Base Exchange	Amino Acid Substitution	Clinical Phenotype	Patient	Myotonia in Heterozygous Males	Myotonia in Heterozygous Females
RMC-62	C-220-T	Gln-74-Stop	Recessive	Homozygous	Latent	Absent
RMC-48	A-499-G	Tyr-150-Cys	Recessive	Compound	Latent	?
	A-782-G	Tyr-261-Cys		Heterozygous	?	Absent
RMC-32-36-38	C-1244-T	Ala-415-Val	Recessive	Homozygous	Latent	Absent
RMC-37	1437-50	Deletion	Recessive	Homozygous	Latent	Absent
MC-34	G-598-A	Gly-200-Arg	Dominant	Heterozygous	?	Borderline
RMC-43	1282-85	Deletion	Recessive	Homozygous	Latent	Absent
RMC-07	A-407-G	Asp-136-Gly	Recessive	Homozygous	?	Absent

NOTE.—Data are taken from this report (upper part) and study by Heine et al. (1994) (lower part).

all known members of the ClC gene family (fig. 2, top, middle). Both patient and mother were heterozygous for this missense mutation as suggested by sequencing of the double-stranded PCR product. In contrast to the recessive families, the patient's father revealed no latent myotonia, and, as stated in the Patients and Methods section, the patient's mother exhibited not only latent myotonia but also minimal clinical myotonia and a positive history. Therefore, we consider Gly-200-Arg as a mutation having dominant effects with low clinical expressivity in females, similarly to other *CLCN1* mutations.

Discussion

Genotype-phenotype correlations in six typical Becker families led to the unexpected finding that all clinically unaffected heterozygous males, but no females, revealed latent myotonia, although some of them had the same mutation. The cause of this apparent sex predominance of subclinical myotonic signs is unclear. Sex hormones or the product of another muscle chloride channel gene, *CLCN4* (Kawasaki et al. 1994, 1995), encoded on the X-chromosome, could contribute to this preference. Although probably not of importance in normal muscle fibers (i.e., in the presence of abundant ClC-1 channels), chloride channels encoded by *CLCN4* expressed on both X-chromosomes could have a compensatory effect when the number of functional ClC-1 channels is reduced. A copy of *CLCN4* on the Y-chromosome has been excluded (Van Slegtenhorst et al. 1994), and its location on distal Xp may allow it to escape X-chromosomal inactivation (Disteche 1995).

The effects of the four novel Becker mutations on the membrane chloride conductance seem to be similar (i.e., they led to latent myotonia in heterozygous males), although three of them are missense mutations and one (Gln-74-Stop) is a nonsense mutation. Gln-74-Stop destroys the coding potential of the gene for all membrane-spanning domains and is therefore the human equivalent of the recessive adr^{mto} mouse mutation (Gronemeier et al. 1994; see fig. 2). If one assumes a tetrameric structure of the functional chloride channel complexes (Steinmeyer et al. 1994), the *CLCN1* chloride channels of the individuals heterozygous for Gln-74-Stop must be exclusively composed of nonmutated channel proteins that are encoded by the second (normal) allele. Assuming unchanged expression rate of the normal gene, apparently half the number of channel complexes is sufficient to generate a normal clinical phenotype. This is in agreement with the effects of chloride channel blockers, which induce myotonic stiffness only when they reduce the membrane chloride conductance to $\leq 30\%$ of its normal value (Palade and Barchi 1977). According to the EMG recordings that revealed latent myotonia in the relatives heterozygous for the Gln-74-Stop mutation, a chloride conductance reduced to 50% of the normal amount by blocking agents is able to reduce the stability of the resting membrane potential (Kwiecinski et al. 1988).

Since latent myotonia was also detected in male individuals heterozygous for Becker missense mutations (see table 1), the resulting reduction of chloride conductance may be similar to that in Gln-74-Stop. A reduction of 50% has been shown for the Arg-496-Ser mutation when coexpressed with normal ClC-1 proteins in an 1:1 ratio (Lorenz et al. 1994). Missense mutations, which allow the mutant protein to interact with normal ClC-1, may destroy the function of the complex. If three monomers of the tetrameric complex, proposed by Steinmeyer et al. (1994), need to be mutants for a loss of function, a heterozygous mutation leaves 68% ([1 + 4 + 6]/16), of the complexes functional and exerts recessive effects. A reduction of the chloride conductance to this value might be yielded by some Becker missense mutations and also result in slight hyperexcitability of the muscle fiber membrane, meaning latent myotonia.

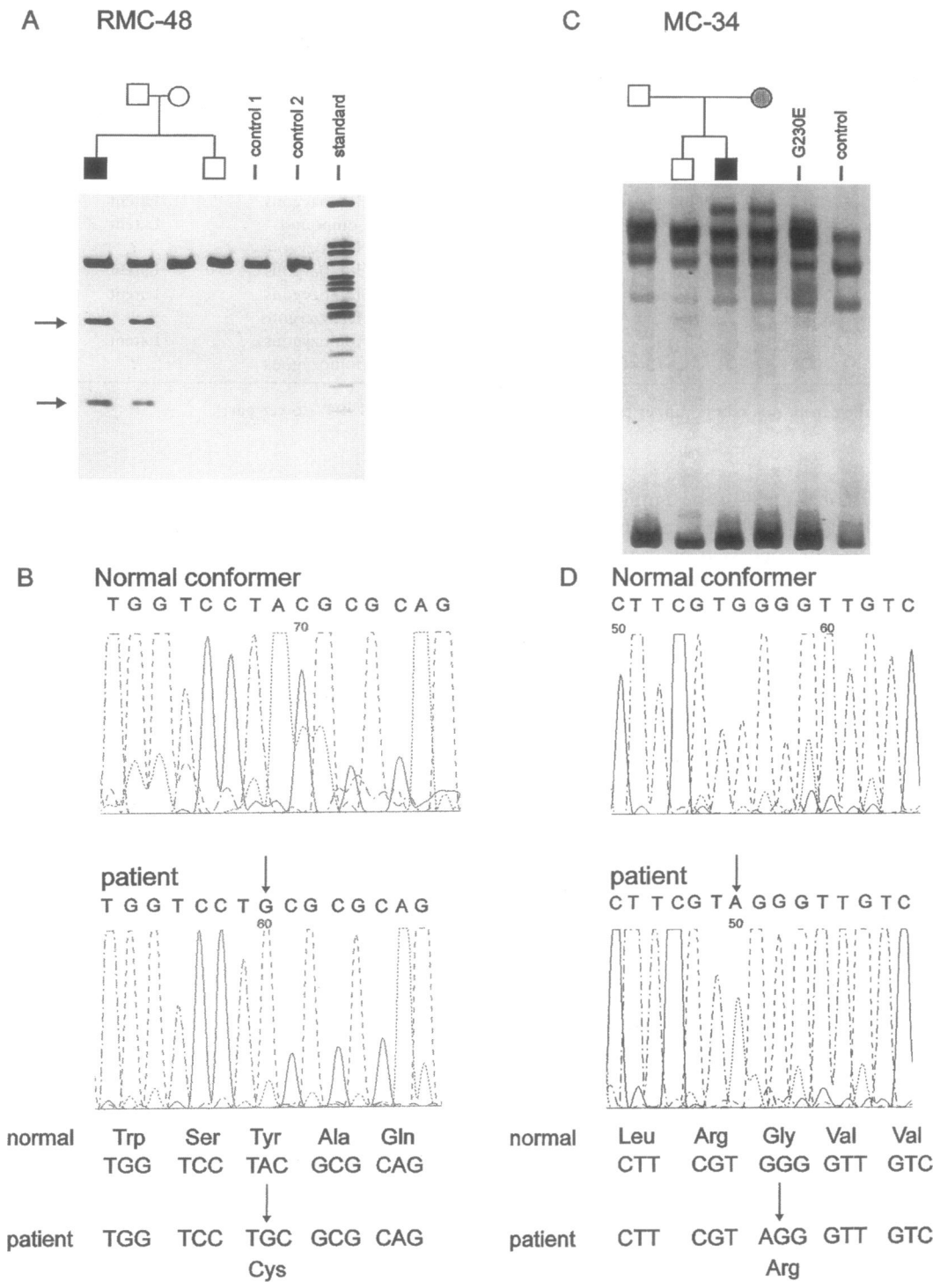

Figure 3 *A*, Pedigree of family RMC-48 with corresponding polyacrylamide gel displaying PCR-amplified genomic DNA fragments following digestion with *Bss*HII and staining with ethidium bromide. The *Bss*HII restriction site was introduced by the base exchange shown in *B*. The patient (filled symbol) and his unaffected father revealed additional fragments (arrows). *Far right*, DNA size standard illustrated by pBR/MspI digest. *B*, Comparison of the *CLCN1* sequence of a part of exon 4 from a control and the patient. Note the A-to-G base exchange in the abnormal single-stranded conformer predicting a substitution of cysteine for tyrosine-150. *C*, Pedigree of the dominant myotonia family MC-34. The index patient and his minimally affected mother revealed an aberrant band (*top*), suggesting a mutation in *CLCN1* exon 5. The pattern was slightly different from that resulting from the previously published G689A base exchange (George et al. 1993) in the same exon (second lane from right). For technical details, see fig. 1 and Patients and Methods. *D*, Comparison of the *CLCN1* sequence of a part of exon 5 from a control and the patient. Note the G-to-A base exchange predicting a substitution of arginine for glycine-200.

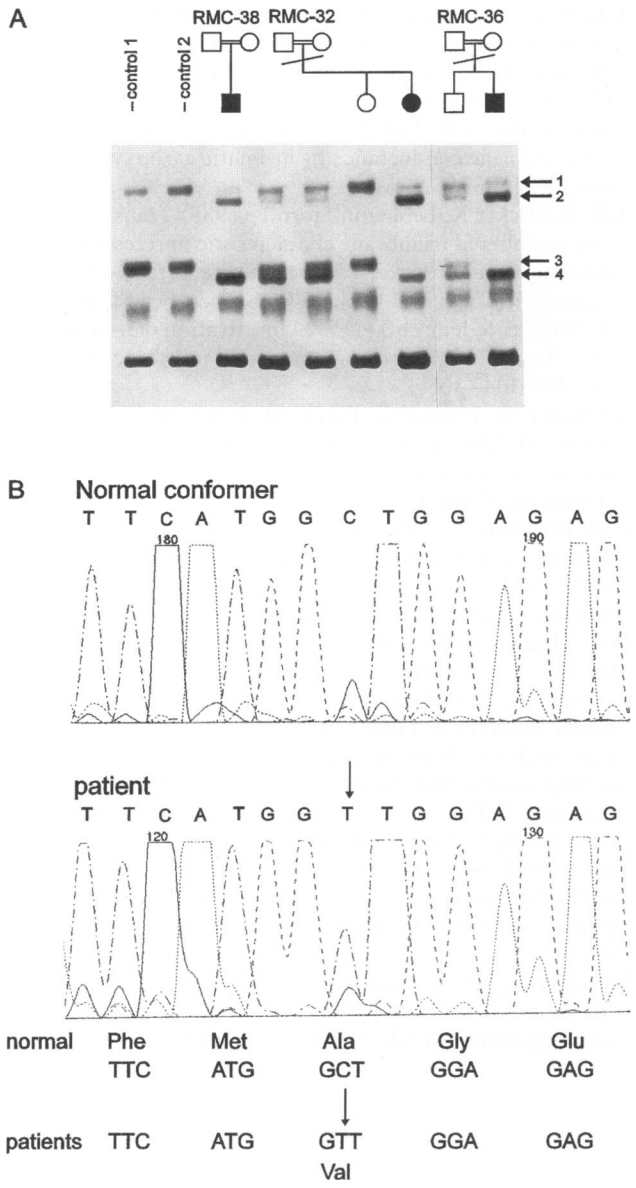

Figure 4 *A*, Pedigrees of families RMC-32/36/38. Individuals shown above oblique slashes were not tested. Aberrant single-stranded DNA conformers (bands 2 and 4) were discovered in exon 11 for patients and some family members. Conformer 3, which was regularly present in normal controls and unaffected family members, was absent in the three patients with consanguineous parents, suggesting homozygosity. All four conformers appeared in the consanguineous parents of patient RMC-32 and in the brother of patient RMC-36, suggesting heterozygosity. For details, see fig. 1 legend, Patients and Methods, and Results. *B*, Comparison of the sequence of a PCR product of exon 11 from a control and the patients.

In contrast, if one or two mutant monomers are sufficient to cause loss of function, only 1/16 or 5/16 of complexes will be functional. Such mutations exert either strong (chloride conductance reduced to 6%) or mild dominant effects (reduction to 30%; Steinmeyer et al. 1994). Because of the low clinical expressivity in the patient's mother, the reported novel Gly-200-Arg mutation seems to have mild dominant effects. In another family with a mild dominant mutation (myotonia levior; Lehmann-Horn et al. 1995), reexamination revealed a similar partially dominant effect in female carriers of the Glu-552-Arg mutation. We hypothesize that the same sex pattern may be present in the families carrying the "Canadian" mutations (Gly-230-Glu; George et al. 1993).

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