

## A New Variant of Charcot-Marie-Tooth Disease Type 2 Is Probably the Result of a Mutation in the Neurofilament-Light Gene

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Charcot-Marie-Tooth (CMT) disease is the most common inherited motor and sensory neuropathy. The axonal form of the disease is designated as “CMT type 2” (CMT2). Although four loci known to be implicated in autosomal dominant CMT2 have been mapped thus far (on 1p35-p36, 3q13.1, 3q13-q22, and 7p14), no one causative gene is yet known. A large Russian family with CMT2 was found in the Mordovian Republic (Russia). Affected members had the typical CMT2 phenotype. Additionally, several patients suffered from hyperkeratosis, although the association, if any, between the two disorders is not clear. Linkage with the CMT loci already known (CMT1A, CMT1B, CMT2A, CMT2B, CMT2D, and a number of other CMT-related loci) was excluded. Genomewide screening pinpointed the disease locus in this family to chromosome 8p21, within a 16-cM interval between markers D8S136 and D8S1769. A maximum two-point LOD score of 5.93 was yielded by a microsatellite from the 5' region of the neurofilament-light gene (*NF-L*). Neurofilament proteins play an important role in axonal structure and are implicated in several neuronal disorders. Screening of affected family members for mutations in the *NF-L* gene and in the tightly linked neurofilament-medium gene (*NF-M*) revealed the only DNA alteration linked with the disease: a A998C transversion in the first exon of *NF-L*, which converts a conserved Gln333 amino acid to proline. This alteration was not found in 180 normal chromosomes. Twenty unrelated CMT2 patients, as well as 26 others with an undetermined form of CMT, also were screened for mutations in *NF-L*, but no additional mutations were found. It is suggested that Gln333Pro represents a rare disease-causing mutation, which results in the CMT2 phenotype.

### Introduction

Charcot-Marie-Tooth neuropathy (CMT) is one of the most common hereditary disorders, affecting 10–40/100,000 individuals, although the prevalence varies in different populations (Skre 1974; Emery 1991). CMT involves the peripheral nerves: it gives rise to symmetrical, progressive weakness and atrophy of the distal muscles, with diminished or absent tendon reflexes, and frequently results in deformity of the feet. The disorder can be divided into two large groups, CMT1 and CMT2, on the basis of nerve conduction velocity (NCV) criteria. CMT1 is characterized by a considerable decrease in motor and sensory NCVs, with an upper limit of 38 m/s for the motor NCV of the median nerve (Harding and Thomas 1980). CMT1 usually results from myelin alteration, and histological examination often reveals extensive segmental demyelination and remyelination (Dyck et al. 1993). CMT2 is characterized by an NCV

that is only slightly decreased (>38 m/s) or even normal (De Jonghe et al. 1998). It is thought that the primary lesion in CMT2 is localized within the axon, and this form of the disorder is often termed an “axonopathy.” In both forms of CMT, the sensory nerves are usually affected also, resulting in some sensory impairment.

Genetically, CMT is a very heterogeneous group of diseases. Seventeen loci are already known to be implicated in different forms of CMT and related neuropathies (De Jonghe et al. 1997b), and further genetic heterogeneity seems very likely. However, thus far, only three of the genes involved have been identified, those for peripheral myelin protein 22 (*PMP22*), on chromosome 17p11.2 (Lupski et al. 1991; Patel et al. 1992; Timmerman et al. 1992; Roa et al. 1993), for myelin protein zero (*MPZ/P0*), on 1q22-23 (Hayasaka et al. 1993), and for connexin 32 (*Cx32/GJB1*), on Xq13.1 (Bergoffen 1993). These three genes correspond to three loci that have been designated “CMT1A” (MIM 118220), CMT1B (MIM 118200) and “CMT1X” (MIM 302800). Recently, mutations in the early growth response 2 (*EGR2*) transcription-factor gene have also been identified in some patients with CMT1 (Warner et al. 1998). All these proteins are closely related to myelin, and mutations in the genes that encode them result

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mainly in CMT1. In rare cases, patients with an NCV that is only slightly decreased also have mutations in either *Cx32* (Timmerman et al. 1996; Birouk et al. 1998) or *MPZ* (Marrosu et al. 1998; De Jonghe et al. 1999). However, such patients show signs of a myelin disorder and could be considered to have a milder form of CMT1.

Four loci implicated in autosomal dominant CMT2 have also been mapped. CMT2A (MIM 118210) maps to chromosome 1p35-p36 (Ben Othmane et al. 1993; Timmerman et al. 1996), CMT2B (MIM 600882) to 3q13-q22 (Kwon et al. 1995; De Jonghe et al. 1997a), and CMT2D (MIM 601472) to 7p14 (Ionasescu et al. 1996). The fourth locus, which is implicated in the proximal dominant form of axonal hereditary motor and sensory neuropathy, has been assigned to the 3q13.1 region (Takashima et al. 1997, 1999). In addition to the typical features of CMT2, CMT2B is further characterized by severe sensory abnormalities and ulceration. In families with CMT2D families, the disorder commences in the upper limbs and predominantly affects them, whereas patients with proximal axonal neuropathy manifest an elevated creatine kinase level, hyperlipidemia, and diabetes mellitus. However, no causative gene has yet been identified for any of these disorders. In this report, we describe a large family, from the Mordovian Republic (Russia), that has autosomal dominant CMT2. We assign the gene responsible for the disorder in this family to chromosome 8p21, and we have termed this locus “CMT2E.” Two neurofilament genes, those for neurofilament light (*NF-L* [GenBank accession number X05608]) and neurofilament medium (*NF-M* [GenBank accession number Y00067]), are also known to be localized to the same region of chromosome 8p, and a missense mutation in *NF-L* was found to segregate with the disorder in this family.

## Subjects and Methods

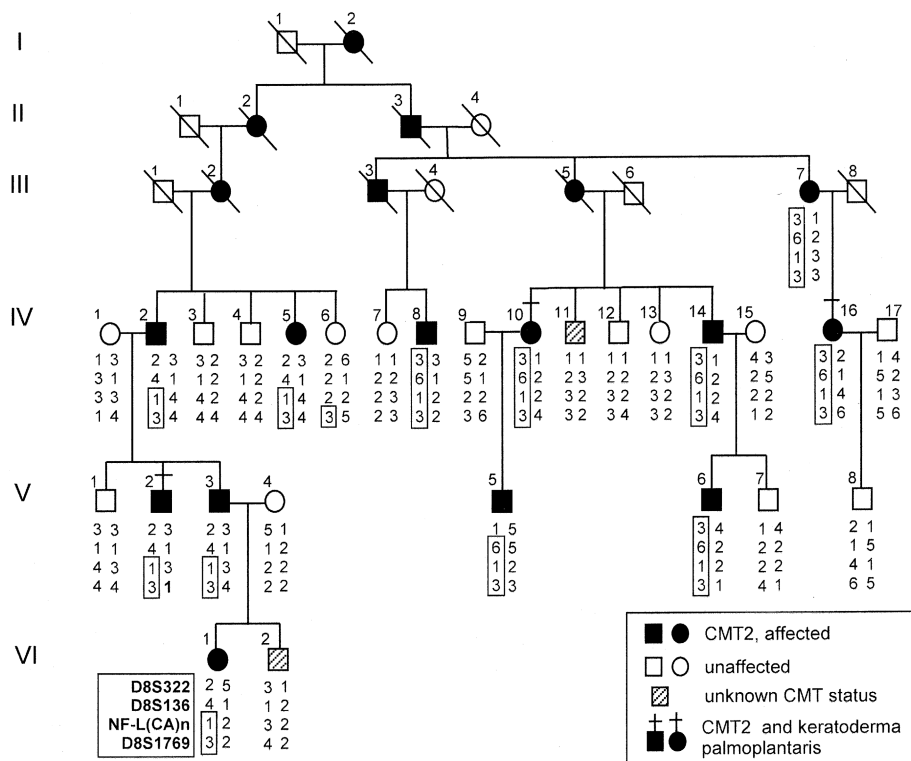
### *Diagnostic Criteria and Pedigree Description*

A large, six-generation family with autosomal dominant CMT2 was encountered in the Mordovian Republic (Russia). All members of this family considered themselves to be Russians. There was a clear history of male-to-male transmission, confirming the autosomal dominant nature of the disorder. Twenty-eight members of the family, extending over four generations, were investigated clinically: the diagnosis of CMT2 was confirmed in 12 individuals and was clearly rejected in 14 others (fig. 1). All these persons were included in the genetic analysis. The status of one adult patient (individual IV-11 in fig. 1) was considered to be unknown, since the diagnosis of CMT was complicated by alco-

holic neuropathy. An 8-year-old boy (individual VI-2 in fig. 1) showed no signs of CMT, either clinically or after electromyography (EMG) examination, but, since he had not reached the average age at onset, he too was considered to be of “unknown” status. However, his 10-year-old sister (individual VI-1 in fig. 1) is considered as being affected, despite her age, because she showed initial signs of the disorder, and the diagnosis was clearly confirmed on EMG investigation. Blood samples for molecular testing were collected after informed consent was obtained, and the study was approved by the Scientific Council of the Research Centre for Medical Genetics (Moscow).

The diagnosis of CMT2 was based on the diagnostic criteria agreed by the European CMT Consortium (De Jonghe et al. 1998). Clinically, the most important of these criteria are (1) a slowly progressive, symmetrical wasting and weakness of the muscles, predominantly of the distal part of the lower limbs; (2) decreased or absent tendon reflexes; and (3) sensory disturbance with different degrees of sensory loss, including lack of sensitivity to pain. A slightly reduced motor median NCV (38–52 m/s) also was typical.

In this family, CMT2 usually manifests between the 2d and 3d decades of life, with difficulty in walking and weakness of the leg muscles, followed by wasting of the lower limbs and a variable degree of deformity of the feet (all patients >20 years of age showed pes cavus, to a greater or lesser extent). Ten family members, including seven affected individuals, were subjected to EMG investigation: the muscle action-potential amplitude was reduced, and there was EMG evidence of denervation of the distal limb muscles. Six of 12 affected individuals showed spontaneous fasciculation in limb-girdle muscles. One affected individual, age 68 years, showed significantly impaired balance, together with ptosis. Affected individuals did not exhibit palpably enlarged nerves, and there were no instances of ulcerated feet, hearing impairment, or paralysis of the vocal cords and/or diaphragm. We had previously described the association of CMT and hyperkeratosis in this family (Evgrafov et al. 1997). However, further dermatologic investigation of the affected members of the family confirmed the diagnosis of hyperkeratosis (palmoplantar keratoderma) in only two individuals (individuals IV-10 and V-2 in fig. 1). In other individuals, the dermatologic changes were mild or absent and did not constitute clinically obvious hyperkeratosis. One individual with apparently severe involvement of the skin (individual IV-16 in fig. 1) was not examined dermatologically, and a second, said by the family to have been similarly affected, had died recently. Thus, there is no strict proof of an association between CMT and the specific dermatologic changes in this family, although its existence could be suspected.



**Figure 1** Segregation analysis of markers from the *NF-L* - *NF-M* region on 8p21 in the CMT2E family. Only family members implicated in the linkage studies are shown. The haplotypes segregating with the disease are boxed. The markers are presented in order, from telomere (top) to centromere (bottom). The recombination event between D8S136 and the disease that occurred in the family identifies D8S136 as a telomeric flanking marker, and recombination between *NF-L(CA)<sub>n</sub>* (microsatellite from the 5' region of *NF-L*) and D8S1769 in unaffected individual IV-6 places D8S1769 centromeric to the CMT2E locus.

*Genotyping*

DNA was extracted from peripheral white blood cells by a standard technique and was amplified via PCR. The following microsatellite markers were used to test linkage with known CMT loci: D1S2667 (AFMa224wg9), for CMT2A; D3S1278 (AFM164xc5), D3S1292 (AFM199xd6), D3S3606 (AFMb020zb9), D3S3694 (AFMc028yb9), and D3S1279 (AFM164yg9), for CMT2B; D7S435 (Mfd20), for CMT2D; D17S122 (RM11GT), D17S921 (AFM191xh12), and D17S261 (Mfd41), for CMT1A; and ApoA2 and D1S431 (AFM212xf6), for CMT1B. The reaction was performed in a 25- $\mu$ l reaction mix containing 0.1–0.5  $\mu$ g of genomic DNA, 0.25  $\mu$ M of each primer, 200  $\mu$ M of each dNTP, 1  $\times$  buffer consisting of 67 mM Tris-HCl (pH 8.8), 16.6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01% Tween-20, and 1 U of *Taq* DNA polymerase (BioMaster). The concentration of MgCl<sub>2</sub> was selected individually for every primer pair. Reaction products were separated on nondenaturing 8% PAGE and were stained with ethidium bromide prior to visualization under UV light.

*Linkage Analysis*

Two-point linkage analysis was performed by the programs MLINK and ILINK (LINKAGE package, version 5.1) (Lathrop et al. 1984). The disorder was coded as fully penetrant and dominant. Disease-gene frequency was defined as .0001. Equal rates of male and female recombination were assumed. Marker-allele frequencies were assumed to be distributed in accordance with the Genome Database frequency data, in Europeans, for the marker under investigation. When this information was not available, a uniform distribution was assumed. The FASTLINK package (version 4.1P) was used for multi-point analysis of data.

*Mutation Analysis*

Mutation screening of *NF-L* and *NF-M* was performed via SSCP analysis. Since this approach is most efficient when short fragments of DNA are being analyzed, long coding stretches of the first exon of *NF-L* were amplified in six overlapping DNA fragments, the third exon in two overlapping DNA fragments. In the

case of *NF-M*, exon 1 was amplified in three overlapping fragments, exon 3 in five overlapping fragments. Additionally, long PCR products were fragmented by restriction endonucleases before SSCP analysis (*HaeIII* for *NF-M* exon 1 part 1, *PvuII* for *NF-M* exon 1 part 2, *RsaI* for *NF-M* exon 1 part 3, *Sau3AI* or *TaqI* for exon 3 part 1, *Sau3AI* for *NF-M* exon 3 part 2, and *PstI* for *NF-M* exon 3 part 4). Oligonucleotide primer sequences and PCR conditions used for the analysis of *NF-L* and *NF-M* are shown in table 1.

For SSCP analysis, PCR products were subjected to alkaline denaturation: an aliquot of 15  $\mu$ l of each amplified PCR product (or PCR product after restriction-endonuclease digestion) was mixed with 5  $\mu$ l of 0.5 M NaOH and 0.5  $\mu$ l of 0.5 M EDTA (pH 8.0) and was heated at 42°C for 10 min. Then 4  $\mu$ l of loading buffer (95% formamide, 0.5% bromophenol blue, and 0.5% xylolcyanol) was added to each probe, and the samples were loaded on nondenaturing 8% PAGE (acrylamide:methylene-bisacrylamide ratio 29:1) either with or without 5% glycerol and were electrophoresed in 0.5 x Tris-borate EDTA at 110 V at room temperature for 18–24 h. Finally, the PAGE was stained with either ethidium bromide or silver nitrate, by means of the Silver sequence kit (Promega), according to standard protocol.

A band that showed altered mobility was cut off the PAGE stained by ethidium bromide, and the corresponding DNA fragment was eluted and amplified through 15 cycles. This PCR product and a normal sample were sequenced by the dideoxy chain-termination method, with both forward and reverse PCR primers (NEFL1F6 and NEFL1R6; see table 1). DNA sequencing was performed by means of the ABI Prism dRhodamine Dye Terminator Cycle Sequencing Ready Reaction Kit and an ABI 310 DNA sequencer (PE Biosystems), according to the standard protocols.

Since the identified mutation results in destruction of the *AluI* site, the corresponding endonuclease was used to test for the mutation in all family members. In addition, 180 chromosomes from 90 unrelated, unaffected individuals of Russian ethnicity were screened for mutations in the same gene fragment, by SSCP and by RFLP analysis using *AluI*.

## Results

### Linkage Study

As a preliminary analysis, family members were screened for linkage with known CMT2 and other

**Table 1**

**Oligonucleotide Primers and PCR Conditions Used for Analysis of *NF-L* and *NF-M***

GENE	PRIMER SEQUENCE (5'→3')		SIZE (bp)	PCR ANNEALING TEMPERATURE (°C)		[MgCl <sub>2</sub> ] (mM)
	Forward	Reverse				
<i>NF-L:</i>						
Exon 1:						
Part 1:	NEFL1F1: GCACACAGCCATCCATCCTCCC	NEFL1R1: GATCCAGAGCTGGAGGAGTAGC	276	68	1.5	
Part 2:	NEFL1F2: CTTCTCGCTGTCCGTGCGCC	NEFL1R2: GCTCGTACAGCGCCCGGAAGC	279	65	1.5	
Part 3:	NEFL1F3: CAGAAGCACTCCGAGCCATCC	NEFL1R3: CCTCGGCGTCTCGCGGCTC	196	63	1.5	
Part 4:	NEFL1F4: GGAGGAGACCCTGCGCAACC	NEFL1R4: CATCTCCACGGAGATCTGCGC	241	60	1.5	
Part 5:	NEFL1F5: CCGAAGTGCAGGCGCAGATCC	NEFL1R5: CTCCGACACCTCGTCTTGGC	226	63	1.5	
Part 6:	NEFL1F6: GAGAGCGCCGCAAGAACACC	NEFL1R6: CCCTGTGTTTCTGGCCGTGCC	205	63	1.5	
Exon 2:	NEFL2F: TAATAGAAGGGATTATGCTCGG	NEFL2R: TCCTAAGGTTAATGGCTGCTG	225	60	2.0	
Exon 3:						
Part 1:	NEFL3F1: CCTCACCTTGACCTGCATCC	NEFL3R1: TTCGGTCTGCTCCTCTTGGAC	215	65	1.5	
Part 2:	NEFL3F2: CAGCTCTATCTGATGTCACC	NEFL3R2: CACCCAGTTTACTTGAAGTTGC	248	62	2.0	
Exon 4:	NEFL4F: ACTGGACTTACCCTGGATTTCG	NEFL4R: CCTGATTTCGGGAGAATTATTCC	241	57	1.5	
<i>NF-M:</i>						
Exon 1:						
Part 1:	NEFM1F1: ACGCTGTGACAGCCACACGCC	NEFM1R1: TCTGCTGCTCCAGGTAGTGCAC	411	65	1.5	
Part 2:	NEFM1F2: GGGCTGAACGACCGCTTTGCC	NEFM1R2: TGCGATGCCTGGATCTGGGCC	440	65	1.5	
Part 3:	NEFM1F3: CGAGGAGGAGGTGGCCGACC	NEFM1R3: CTGGCCGAGGCCGCGTTCC	390	66	1.5	
Exon 2:	NEFM2F: CTGTTTGCAAGGATGAGTCTGG	NEFM2R: CCACGCACGTAGTAAGCATCG	195	62	1.5	
Exon 3:						
Part 1:	NEFM3F1: ATGTAATGAAGCTCAGAAGGCC	NEFM3R1: GCCTTCTTCTCCTCTTTTCC	459	60	1.5	
Part 2:	NEFM3F2: CTCCAGTGAAAGCAACTGCACC	NEFM3R2: GATTTTGGCACAGGAGACTTGGC	328	65	1.5	
Part 3:	NEFM3F3: AGGAGCTGGTGGCAGATGCC	NEFM3R3: TCCTCTTCTGTTCACCTTTCC	316	68	6.0	
Part 4:	NEFM3F4: CACCAGTGAAGAGGCAAAGTC	NEFM3R4: CTCAAGTCTAGGCCATTGGTGAC	457	58	1.5	
Part 5:	NEFM3F5: GGAGGGAAGAGGAGAAAGGC	NEFM3R5: GCTTAACCTTTTGCAATGGACTC	315	60	2.5	

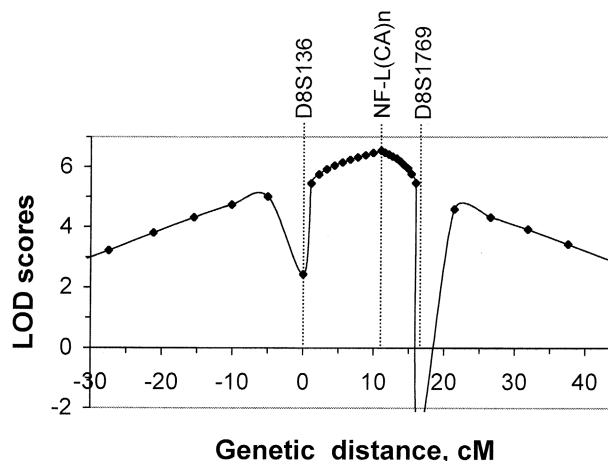
<sup>a</sup> MgCl<sub>2</sub> concentration in 1 x PCR reaction buffer.

CMT loci. After exclusion of linkage with CMT1A (17p11.2-p12), CMT1B (1q21-q23), CMT2A (1p35-p36), CMT2B (3q13-q22), and CMT2D (7p14), a genome-wide search was started. Possible candidate loci were investigated first. After 25% of the genome had been excluded in this way, significant linkage to markers on chromosome 8p21 was detected (table 2). Recombination and multipoint analyses indicated that the most probable location of the CMT2E gene was within a 16-cM interval flanked by markers D8S136 and D8S1769 (see figs. 1 and 2). This interval contains both *NF-L* and *NF-M*. No recombination was detected between the disease locus and a polymorphic microsatellite repeat from the 5' region of *NF-L*, with a two-point LOD score of 5.93. *NF-L* and, to a lesser extent, *NF-M* were considered as candidate genes and were screened for mutations.

### Mutation Analysis

The entire coding regions of *NF-L* and *NF-M*, including splice sites and the promoter region, were screened for mutations, by SSCP analysis. One mobility shift, which cosegregated with the disorder, was found in the first exon of *NF-L*. Sequencing revealed an A→C substitution in CAG codon 333, which results in a missense mutation, Gln333Pro. This alteration destroys the *AluI* restriction site, and this enzyme was used for confirmation of mutation and for the screening procedure (fig. 3). An A998→C nucleotide substitution was found in all affected members of the family but in none of their unaffected relatives, nor was it found in 180 chromosomes from 90 healthy unrelated individuals. No other DNA alterations in either *NF-L* or *NF-M* were found that segregated with the disorder.

After this procedure, the entire coding region of *NF-L* was screened for possible mutations in 46 unrelated individuals with CMT who did not have either the CMT1A duplication or mutations in *MPZ* or *Cx32*. Twenty of these individuals had been diagnosed as having CMT2, and the diagnosis had been confirmed by EMG. In the other 26 individuals, EMG data were not available, and these individuals were considered to represent an unspecified type of CMT. None of these individuals was found to have a mutation in *NF-L*.



**Figure 2** Multipoint localization of *CMT2E*. The peak multipoint LOD score of 6.54 was yielded by a microsatellite marker from the 5' region of *NF-L(NF-L[CA]<sub>n</sub>)*. The marker loci D8S136 and D8S1769 were fixed at a distance of 16.38 cM, and a microsatellite marker from the 5' region of *NF-L* was localized between markers D8S136 and D8S1769, at the approximate distance of 11 cM from D8S136. The genetic distances are inferred from the Marshfield Chromosome 8 Sex-Averaged Linkage Map (D8S136 and D8S1769) and the NIH/CEPH Chromosome 8 Linkage Map (1992) (*NF-L[CA]* repeat and D8S136), both of which are available from the Genome Database. Kosambi's formula was applied to convert recombination fractions into genetic distances.

### Discussion

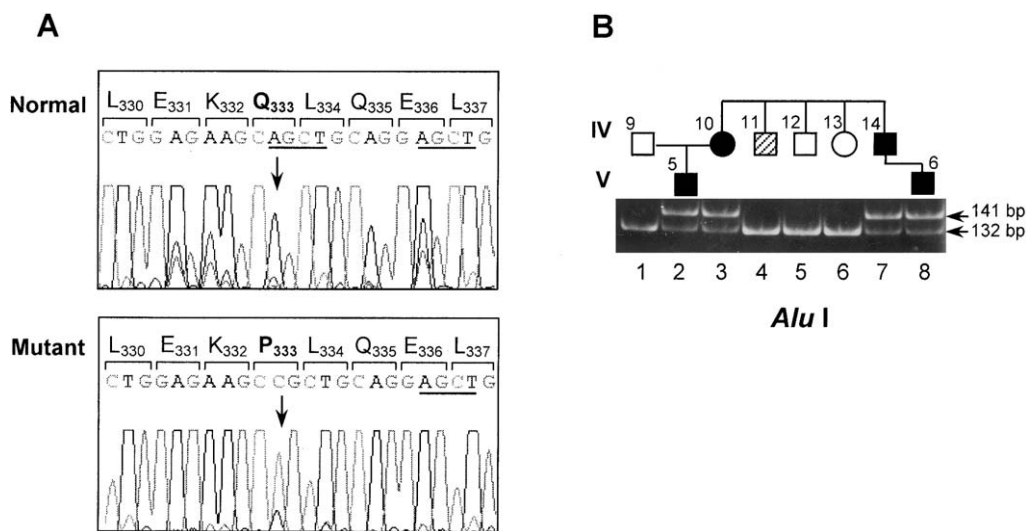
The clinical resemblance between different types of CMT suggests some degree of similarity in the pathological process. The most common type of inherited peripheral neuropathy, CMT1A, is caused either by a duplication of *PMP22* (Lupski et al. 1991; Nelis et al. 1999) or, in some instances, by point mutations in this gene (Valentijn et al. 1992; Roa et al. 1993; Nelis et al. 1999); the mouse *Trembler* and *Trembler-J* mutations are animal models of such point mutation (Suter et al. 1992a, 1992b). The disorder results in demyelination of the peripheral nerves, accompanied by a reduction in NCV (Adlkofer et al. 1995; Magyar et al. 1996; Thomas et al. 1997). However, demyelination is not the direct cause

**Table 2**

**Two-Point LOD Scores between CMT2E Locus and Microsatellite Markers on 8p21**

MARKER	LOD SCORE AT RECOMBINATION FRACTION OF								MAXIMUM LOD SCORE	MAXIMUM RECOMBINATION FRACTION
	.00	.01	.05	.10	.20	.30	.40	.50		
D8S322	-∞	.51	1.61	1.83	1.60	1.08	.47	.00	1.830	.108
D8S136	1.55	3.93	4.20	3.94	3.05	1.91	.71	.00	4.208	.040
<i>NF-L(CA)<sub>n</sub></i> <sup>a</sup>	5.93	5.83	5.40	4.84	3.67	2.40	1.05	.00	5.932	.000
D8S1769	-∞	2.34	2.75	2.67	2.16	1.48	.72	.00	2.754	.058

<sup>a</sup> Microsatellite from 5' region of *NF-L*.



**Figure 3** Detection of A998→C nucleotide substitution in codon 333 of *NF-L*. *A*, Automated fluorescent sequence analysis of normal (*top*) and mutant (*bottom*) alleles. The mutant allele was sequenced as “hemizygote” by means of separation, by SSCP method, of single-strand DNA with altered mobility followed by its elution and PCR amplification. Sequencing was performed with templates of this PCR product of the mutant allele, as well as the PCR product of normal homozygous DNA. The nucleotide substitution is denoted by the downward-pointing arrow (↓). Amino acids with codon numbers are indicated above the sequence. Recognition sites for the restriction endonuclease *AluI* are underlined. *B*, Detection of A→C transversion in codon 333, by restriction-enzyme analysis in a part of the family with CMT2E. *AluI* digestion of the wild-type *NF-L*-sequence yields restriction fragments of 132, 9, and 64 bp (designations 1, 4, 5, and 6; 9-bp and 64-bp fragments are not shown). The A998C substitution destroyed one of *AluI* sites, and, as result, the mutated allele is digested, with *AluI*, into 141 and 64 bp. Both fragments—a normal one of 132 bp and an uncut one of 141 bp—are presented in the heterozygous affected members of the family with CMT2E (designations 2, 3, 7, and 8). On the pedigree fragment, presented above the electrophoregram, the numbers of the individuals correspond to those in the complete pedigree (see fig. 1).

of the muscle atrophy and weakness found in CMT; it appears to act by initiating a chain of events resulting in axonal loss and muscle denervation. It has been shown that local axonal demyelination in *Trembler* mice results in decreased neurofilament phosphorylation, slow axonal transport, and reduced axonal diameter, whereas myelinated regions of the same axon have normal parameters (de Waegh et al. 1992). Investigation of the *PMP22* duplication gives similar results (Sahenk et al. 1999). Another example of the relationship between myelin components and axonal function is demonstrated by a mouse with a null mutation in the myelin-associated glycoprotein gene (*MAG*). This mouse develops chronic atrophy of myelinated peripheral-nervous-system axons, which results in axon degeneration (Yin et al. 1998). It is important to note that the absence of *MAG* correlates with both a decrease in neurofilament phosphorylation and reduced axonal caliber. Thus, mutations in *PMP22* and *MAG*, the genes for myelin proteins, can both act as signals that initiate a similar pathological process in the axons.

Thus, neurofilament abnormality appears to be the primary cause underlying defective axonal transport, decreased axonal diameter, and further pathological processes. In higher eukaryotes, there are three neuro-

filament proteins—the light, medium, and heavy neurofilaments (NF-L, NF-M, and NF-H, respectively). All three have a similar, central coiled domain, which is responsible for the assembly of 10-nm filaments. Of the three neurofilament proteins, NF-L seems to play the most important role. In the first place, it is the only neurofilament protein capable of organizing filaments on its own (Geisler and Weber 1981; Carpenter and Ip 1996). Targeted disruption of *NF-L* in mice has shown that the NF-M and NF-H subunits cannot form 10-nm neurofilament in the absence of NF-L (Zhu et al. 1997). Second, NF-L plays a part in regulating the expression of the other neurofilament proteins. “Shaky” quail (*Coturnix coturnix*), which have defects in *NF-L*, also have a severely reduced level of *NF-M* and *NF-H* expression (Ohara et al. 1993). Axons in these quail vary more in diameter, presumably affecting neuronal transmission. Similarly, *NF-L* knockout mice attain only 5% of the normal level of *NF-M* and *NF-H* expression and, like the quail, exhibit reduced axonal radial growth. Nerve regeneration is also delayed (Zhu et al. 1997).

It is clear that neurofilament-protein expression must be balanced. Overexpression of either *NF-M* or *NF-H* results in both accumulation of neurofilament proteins in cell bodies and a decrease in axon caliber. However,

a combined increase of *NF-L* with either *NF-M* or *NF-H* promotes radial axonal growth. (Xu et al. 1996).

It is worth noting that *NF-L* null mutations are not lethal in mice and do not result in a CMT-like phenotype. However, it is also significant that, at age 2 mo, these mice exhibit a 15%–20% reduction in axon number. The absence of clinical CMT features may be explained either by the difference in longevity or by absolute differences in axonal length. The nature of different mutations also may be important in determining the resulting phenotype. In this regard, it is worth mentioning that the Leu394Pro mutation in mice has consequences that are far more severe than those resulting from a null mutation (Lee et al. 1994), despite the fact that both amino acids belong to the same group of neutral amino acids with nonpolar side chains. As early as 18 d after birth, transgenic mice with this mutation display abnormal gait, with reduced activity and weakness of both upper and lower limbs. There is a permanent loss of mostly large, neurofilament-rich motor axons (Lee et al. 1994; Cleveland et al. 1996), such that this mutation could be considered an instance of gain of function. Another interesting model is a transgenic mouse with the human *NF-H* gene, which also can be considered as a mutated version of the mouse *NF-H* gene (Collard et al. 1995). Despite a low level of expression, human protein gives rise to neurological defects, muscle atrophy, and neuronal neurofilament accumulation, usually explained as the effect of the human protein on the cell.

All these data indicate that neurofilaments are important for the structure and function of axons—particularly the larger axons—and that they may be responsible for effective transport, axon regeneration, and axonal longevity. Neurofilament proteins are evidently key targets in axonal pathology, and the corresponding genes could be considered candidate genes for CMT.

Neurofilament proteins are involved in the pathogenesis of several other neurological disorders—for example, giant axonal neuropathy (MIM 256850 [Flanigan et al. 1998]), amyotrophic lateral sclerosis (ALS [MIM 105400] [Hirano 1991; Julien 1995]), Parkinson disease (MIM 168600 and MIM 168601 [Goldman et al. 1983; Schmidt et al. 1991b]), and Alzheimer disease (MIM 104300 [Lee et al. 1988; Schmidt et al. 1991a; Pollanen et al. 1994]). The common feature in all these disorders is the accumulation of neurofilament aggregates during the disease process. A rare form of axonal hereditary motor and sensory neuropathy with neurofilament accumulation also has been reported (Vogel et al. 1985). A recombinant mouse strain, which overexpresses *NF-H*, shows signs of ALS-like plaques in the brain tissue (Cote et al. 1993). Overexpression of *NF-L* above a certain, specific level also results in a similar

ALS-like phenotype (Xu et al. 1993). Neurofilament accumulation may trigger the pathological process, although there is no direct evidence of this. Figlewicz et al. (1994) reported an association between specific alleles of *NF-H* with a deletion in the KSP-motif region and ALS, but this has not been confirmed by other investigators (Rooke et al. 1996; Vechio et al. 1996). Recently, five additional mutations (four novel codon deletions and one insertion of 84 bp) have been found in the COOH domain of *NF-H* in patients with ALS (Tomkins et al. 1998; Al-Chalabi et al. 1999). This suggests that alterations in *NF-H* could be the primary cause of ALS, but only in a small percentage (~1%) of cases (Julien 1999). Furthermore, it recently has been shown that, at least in cases of dystonia musculorum and SOD1-linked familial ALS, neurofilament proteins do not play a prominent role in pathogenesis (Eyer et al. 1998). A similar situation can be found in the field of investigation of polyglutamine disorders, where the role of polyglutamine aggregation is not yet known (Zoghbi and Orr 1999). Despite the clearly different cellular mechanism in the polyglutamine disorders, the investigative pathway in both fields is surprisingly similar, and the general structure of the still unknown gain-of-function mechanism in both cases could be similar.

The missense mutation, Gln333Pro, is located in an *NF-L* region that is conserved in mice, rats, pigs, and cows, and this region shows high similarity in the quail and *Xenopus*. Glutamine is found in the same position in all available sequences of either *NF-L* or corresponding proteins, even in such remote species as *X. laevis*. This degree of conservation suggests the importance of this particular amino acid for the structure or function of the protein. Glutamine is a neutral amino acid with a polar side chain, whereas proline has a nonpolar side chain. The two also differ quite significantly in size. The site of the mutation is surrounded by the protein-rod domain, which is responsible for neurofilament assembly (Carpenter and Ip 1996). More precisely, Gln333 is located in the coil 2B domain, the last and largest of four coil domains that form the rod region. The severe Leu394Pro *NF-L* mutation in the mouse also is located in the same coil domain. In addition, *NF-L* is directly or indirectly responsible for the expression of other neurofilament proteins, and this mutation also could affect the proper regulation of such proteins. It therefore seems probable that this mutation is the true cause of the disorder in this family. The fact that we failed to find any *NF-L* mutation in other patients indicates that mutation in this gene may be only a rare cause of hereditary motor and sensory neuropathy of the axonal variety. However, this is not unexpected. To date, four loci for CMT2 have been assigned, but none has been shown to be a major genetic locus for the disorder (De Jonghe et al. 1998).

It is still possible that Gln333Pro is merely a rare genetic variant, which does not cause the disorder. At least one more sequence variant—Asp469Asn—is known, in the tail of the protein (subdomain B), but this does not seem to confer a pathological phenotype (Vechio et al. 1996). However, mutations in structural genes can have different phenotypes, and we believe that the arguments in favor of the pathological phenotype of Gln333Pro are very convincing. The high LOD score strongly confirms the localization of the pathological gene as being within the vicinity of *NF-L* and *NF-M*. Of all the genes and expressed sequence tags from this region, the neurofilament genes are the only plausible candidate genes. The only known DNA alteration in these genes is inherited with the disorder in this family, and this alteration is a missense mutation in the conserved region of *NF-L*. A mutation in the same domain in mice results in a pathological phenotype similar to CMT. All these arguments strongly support the conclusion that this mutation in *NF-L* is the true cause of the CMT2 phenotype in the investigated family.

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## Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, <http://www.ncbi.nlm.nih.gov> (for *NF-L* [accession number X05608] and *NF-M* [accession number Y00067])  
 Genome Database (GDB), <http://www.gdb.org> (for marker frequencies)  
 Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim> (for Alzheimer disease [MIM 104300], ALS [MIM 105400], CMT1B [MIM118200], CMT2A [MIM 118210], CMT1A [MIM 118220], Parkinson disease [MIM 168600 and MIM 168601], giant axonal neuropathy [MIM 256850], CMT1X [MIM 302800], CMT2B [MIM 600882], and CMT2D [MIM 601472])

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