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Genetics

Myelin protein zero gene mutated in Charcot–Marie–Tooth type 1B patients

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ABSTRACT **Autosomal dominant of Charcot–Marie–Tooth disease (CMT), whose gene is type 1B (CMT1B), has slow nerve conduction with demyelinated Schwann cells. In this study the abundant peripheral myelin protein zero (MPZ) gene, *MPZ*, was mapped 130 kb centromeric to the Fc receptor immunoglobulin gene cluster in band 1q22, and a major *MPZ* point mutation was found to cosegregate with *CMT1B* in one large CMT1B family. The *MPZ* point mutation in 18 of 18 related CMT1B pedigree 1 patients converts a positively charged lysine in codon 96 to a negatively charged glutamate. The same *MPZ* locus cosegregates with the *CMT1B* disease gene in a second CMT1B family [total multipoint logarithm of odds (lod) = 11.4 at $\theta = 0.00$] with a splice junction mutation. Both mutations occur in MPZ protein regions otherwise conserved identically in human, rat, and cow since these species diverged 100 million years ago. MPZ protein, expressed exclusively in myelinated peripheral nerve Schwann cells, constitutes >50% of myelin protein. These mutations are anticipated to disrupt homophilic MPZ binding and result in CMT1B peripheral nerve demyelination.**

Charcot–Marie–Tooth disease [CMT; hereditary motor and sensory neuropathy (HMSN)] is the most common genetic neuropathy with an incidence of 1 in 2600 (1). Genetically heterogeneous CMT subtypes are clinically similar with pes cavus, distal muscle weakness and atrophy, absent or diminished deep tendon reflexes, and mild sensory loss. CMT type I (CMT1, HMSNI) is a demyelinating peripheral neuropathy with slower nerve conduction velocities, while type II (CMT2, HMSNII) is a nondemyelinating neuronal disorder with nearly normal nerve conduction velocities (2). The more severe CMT1 tends to be manifest in late childhood or adolescence and progresses slowly but inexorably (3). Loci for CMT1 have been mapped to chromosome 1 (4, 5), chromosome 17 (6, 7), chromosome X (8, 9), and another autosomal locus (11). The locus for CMT1A on chromosome 17 is usually associated with duplication or mutation of peripheral myelin protein PMP-22 (12–14). This study found that the *CMT1B* locus on chromosome 1 (5, 17) cosegregates with mutations of the *MPZ* gene (18, 19),¶ which encodes the most common peripheral myelin transmembrane protein.

Myelin loss results in both peripheral and central neuropathies such as Guillain–Barre syndrome and multiple sclerosis, respectively (18, 20). Abnormal myelin protein like PMP-22 results in demyelinating neuropathy. Mapping the gene for mouse peripheral myelin protein (designated *Mpp; MPZ* used for simplicity here) to chromosome 1 in a region syntenic to human chromosome 1q (21) made *MPZ* a candidate gene for CMT1B. The MPZ protein functions as a double adhesion molecule with extracellular and cytoplasmic interactions that hold together the myelin sheath (22). Since MPZ is expressed exclusively in the peripheral nervous system in myelinating Schwann cells and composes >50% of the myelin protein, abnormal MPZ would likely cause the peripheral demyelination seen in CMT1B. This study found abnormal *MPZ* genes in two CMT1B families.

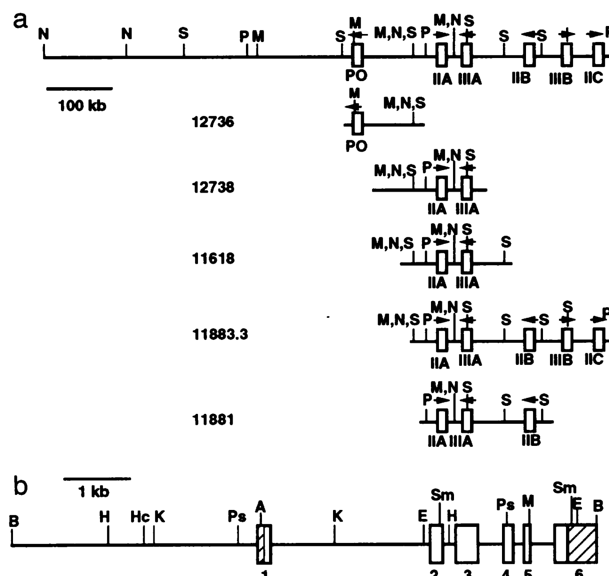

FIG. 1. (*a*) Physical map of human chromosome 1q22 *MPZ* gene region. Restriction sites common to human genomic DNA (top line) and YACs (bottom) are indicated. PO, *MPZ*; IIA, FcγRIIA (*FCGR2A*); IIIB, FCγRIIIB (*FCGR3B*); M, *Mlu* I; P, *Pme* I; N, *Nru* I; S, *Sfi* I. Boxes indicate gene positions including the five human *FCGR* genes with their class II or III designations. Arrowheads denote transcriptional orientations of *FCGR* (27) and *MPZ* genes. Gene locations are consistent with a homologous mouse linkage map (21). (*b*) Exon–intron and restriction map of human *MPZ*. Boxes indicate exons, and cross-hatching denotes untranslated regions. A, *Apa* I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; K, *Kpn* I; M, *Mlu* I; Ps, *Pst* I; Sm, *Sma* I.

MATERIALS AND METHODS

Population and MPZ Analysis. Peripheral blood samples were obtained with informed consent from 88 normal unrelated individuals and 37 CMT pedigrees selected to exclude all but 7 CMT1A duplication pedigrees (5, 17, 23). Lympho-

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Abbreviations: MPZ, myelin protein zero; CMT, Charcot–Marie–Tooth disease; YAC, yeast artificial chromosome; lod, logarithm of odds.

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¶The recommended standard *MPZ* designation (19) replaces Po, BR, PO, humspm, MRP, myp0, and myelin Po-protein and avoids confusion with another Po gene.

Table 1. Linkage analysis: two-point linkage analysis for A → G point mutation

	Locus/$\log_{10}$ likelihood for the recombination values vs. *CMT1B*									
θ	0.00	0.01	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40
MPZ	6.92	6.81	6.37	5.78	5.17	4.53	3.85	3.12	2.36	1.55

cyte DNAs were extracted directly from blood or from Epstein–Barr virus-transformed lymphocyte cultures (5). Originally 12 primer sites were chosen from within the six mouse *MPZ* gene exons (GenBank accession numbers M62857-60 and M62427) (24) by the computer program PRIMER VERSION 0.5 (Whitehead Institute for Biomedical Research, Cambridge, MA, 1991). Exon 3 primers MMPZ3F: TTC CAC TAT GCC AAG GGA CAA C and MMPZ3R: CTG GTG GGT TTT TGA CAT CAC AT or exon 6 primers MMPZ3′5F: GCG GCA GGT TAG TAG GGC TT and MMPZ3′6R: TAT CCT TGC GAG ACT CCC CC amplified human DNA. To 50 pmol of each primer pair were added 100 ng of DNA in 16.6 mM $(NH_4)_2SO_4$/67 mM Tris·HCl, pH 8.8/6.7 mM $MgCl_2$/10 mM 2-mercaptoethanol/170 μg of bovine serum albumin per ml/6.8 μM EDTA buffer/1.67 mM of each dNTP/2% dimethyl sulfoxide. The DNA was amplified by denaturing for 40 sec at 94°C, annealing for 1 min at 60°C, and elongating for 1 min at 72°C for each of 35 cycles. Amplified product was sequenced by the method of Sanger or digested according to the manufacturer's recommended conditions with 40 units of *Bst*BI at 65°C for 4 hr (New England Biolabs), resolved by 9% acrylamide gel electrophoresis, stained with ethidium bromide, and photographed. Subsequently, primers for human *MPZ* exons 1 and 2 (GenBank accession nos. D10537 and D90501; ref. 25)—HMPZ1F: CTC AAC CCC ACA GAT GCT C, HMPZ1R: CCA AAG AAG AGA AGA GCA GC, HMPZ2F: AGT GCT GTC CCC GGC CC, and HMPZ2R: CCG AAA TGG CAT CTC TGC C—were synthesized and amplified by using similar PCR conditions, but [^{32}P]dCTP was included in the reaction mixture to label the PCR product. The product was denatured and tested for single-stranded conformation polymorphism on 0.5× MDE (AT Biochem, Malvern, PA) gel and 0.5× MDE plus 10% (vol/vol) glycerol at 4°C and 22°C according to the AT Biochem protocol. Linkage data were handled by the data management program LIPIN (Tables 1 and 2) (26).

Yeast Artificial Chromosome (YAC) Analysis. YACs containing genes for IgG Fc receptors II and III (*FCGR2* and *FCGR3*) were isolated from the St. Louis YAC library as indicated (27). Additional YACs were derived from a Centre d'Etude du Polymorphisme Humain (CEPH) library by walking with a probe from the centromeric end of YAC 11618. The YACs included in this report are identified as follows: from St. Louis, 11618 = A104C1, 11881 = A298B5, and 11883.3 = A298C11; from CEPH, 12736 = 194H7 and 12738 = 248H4. Restriction analyses of human and yeast DNAs were as described (27).

***MPZ* Cloning.** YAC 12736 was isolated in low-melting agarose and digested with *Bam*HI. A library of these fragments was made in pBluescript and screened with a cloned human *MPZ* exon 6 probe made by PCR of total human DNA using mouse primers. A 10.2-kb cloned segment 13075 was characterized that contained the full MPZ coding region. Restriction enzyme analysis with oligonucleotide probes based on the human MPZ cDNA sequence (25) generated a restriction map (Fig. 1*a*) and an exon–intron map (Fig. 1*b*) during studies to completely sequence the human genomic *MPZ* gene (Y.S., unpublished data). Restriction enzyme analysis of YAC 12736 carrying human *MPZ* determined the transcriptional orientation of the *MPZ* exons relative to the reported *FCGR* orientation (27). *In situ* hybridization and pulsed-field gel electrophoresis were as described (17).

RESULTS

Previously we reported that the gene encoding autosomal dominant CMT1B disease in a large pedigree (pedigree 1; Fig. 2) cosegregated with the IgG Fc receptor locus *FCGR* [multipoint logarithm of odds (lod) = 5.63; θ = 0.00] (5, 17). Both Na^+/K^+-ATPase genes near the chromosome 1 centromere were excluded as candidate genes by recombination with *CMT1B* (5). Three candidate Fcγ receptor II genes (*FCGR2*) were found to be essentially normal in CMT1B patients by sequencing all 24 PCR-amplified exons from genomic DNA with primers in adjacent introns (not shown).

MPZ, which produces >50% of the myelin protein (22), was mapped with cloned mouse *MPZ* gene within 500 kb of the mouse (*Fcgr*) and human (*FCGR*) Fcγ receptor loci on chromosome 1 (21). Thus, we constructed a fine map of the candidate human *MPZ* gene. YACs constituting a 900-kb contig (group of clones with contiguous nucleotide sequences) encoding five human *FCGR* genes were screened with the amplified mouse *MPZ* exon 6 probe. One identified YAC, 12736, hybridized to probes for all six amplified mouse *MPZ* exons, so it contains the complete human *MPZ* gene. A physical map of the YAC contig and normal human lymphoblast DNA localized human *MPZ* 130 kb centromeric to *FCGR2A* (Fig. 1*a*). The entire coding region for human *MPZ* was subcloned from this YAC (Fig. 1*b*), and the resulting plasmid localized uniquely to human chromosome 1q22 by fluorescence *in situ* hybridization (not shown). The human *MPZ* exon–intron organization and orientation were determined by Southern and DNA sequence analyses (Fig. 1*b*) with primers based on the human cDNA sequence.

Candidate *MPZ* genes were sequenced in both reported CMT1B pedigrees (5) to test whether the *MPZ* gene mutation cosegregates with *CMT1B*. While single-stranded conformation polymorphism analysis of exons 1 and 2 revealed only normal sequences in CMT1B patients, an A → G transition mutation in *MPZ* exon 3 in pedigree 1 (Fig. 3 *Top*) was found to generate a *Bst*BI restriction enzyme site (Fig. 3 *Middle*). *Bst*BI PCR analysis found this mutation in 18 of 18 CMT1B patients in pedigree 1 (Figs. 2 and 3 *Middle*). All other 282 unrelated chromosomes tested did not have this mutation in

Table 2. Linkage analysis: multipoint linkage analysis for A → G point mutation

Loci condition: most → least likely	Recombination fractions between adjacent loci θ = 0.5					lod score	Likelihood
1. *FY-ATP1A2-CMT1B-MPZ-FCGR2-D1S42*	0.05	0.04	0.00	0.00	0.15	9.26	1
2. *CMT1B-FY-ATP1A2-MPZ-FCGR2-D1S42*	0.05	0.05	0.04	0.00	0.15	6.26	1,000
3. *FY-ATP1A2-MPZ-FCGR2-D1S42-CMT1B*	0.05	0.04	0.00	0.15	0.04	5.42	6,918
4. *FY-CMT1B-ATP1A2-MPZ-FCGR2-D1S42*	0.01	0.04	0.04	0.00	0.15	4.66	39,810

The lod score is the maximum multipoint lod within that interval. The multipoint lod score is defined as the $\log_{10}$ difference between the disease being unlinked or within the multipoint map. Since *CMT1B* is perfectly linked to both *FCGR2* and *MPZ*, there is only one interval from *ATP1A2* to *D1S42*, and it includes all three of the aforementioned loci.

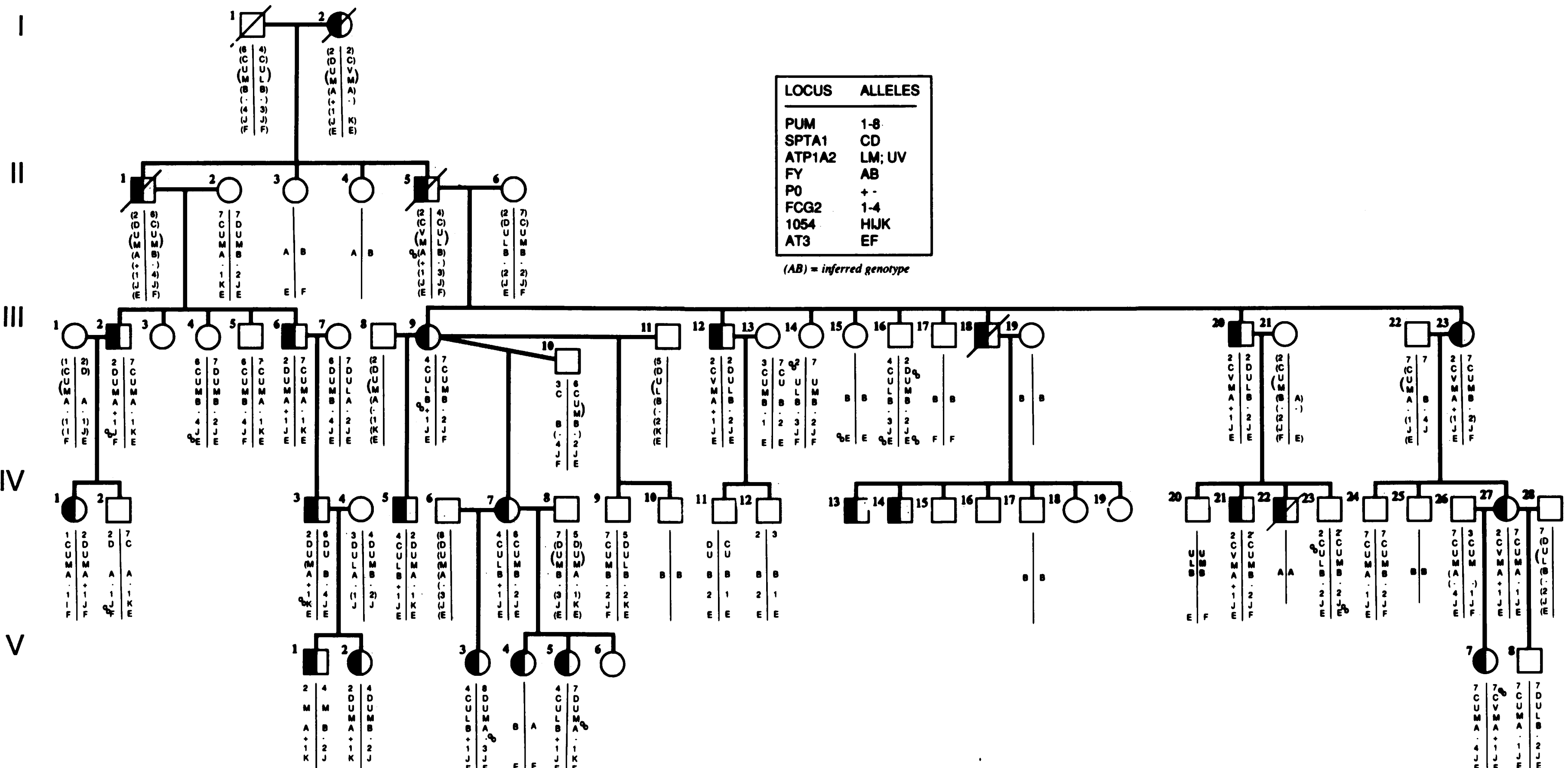

FIG. 2. CMT1B pedigree 1. Segregation of the most informative polymorphic loci tested in the *CMT1B* region is illustrated (see *Inset*). Half-filled symbols indicate autosomal dominant *CMT1B*. Inferred genotypes are in parentheses. The *MPZ* locus cut by *Bst*BI (+) cosegregates with *CMT1B*. Allelic polymorphisms of mucin (*PUM*; symbol now withdrawn and replaced by *MUC1*), α-spectrin (*SPTA1*), Na^+/K^+-ATPase (*ATP1A2*), Duffy erythrocyte antigen (*FY*), Fcγ receptor II (*FCGR2*), *D1S42* (probe CRI-L1054), and antithrombin III (*AT3*) have been defined (5). The *MPZ* locus is either cut (+) or not cut (−) by *Bst*BI with the (+) allele segregating with *CMT1B*. Putative recombinations are indicated.

18 normal pedigree 1 members, 7 unrelated CMT1A patients with a chromosome 17 duplication (23), 28 unrelated CMT patients without a *CMT1A* duplication, including CMT1B pedigree 2 (23), and 88 unrelated normal individuals.

Computer linkage analysis of the *Bst*BI *MPZ* mutation in pedigree 1 reflected concordant segregation between *MPZ* and *CMT1B* (lod = 6.92 at $\theta = 0.00$). Linkage analysis of this mutation along with the four other most informative CMT1B markers (17) confirmed the suggested *CMT1B* gene location in the 3-Mb chromosome region between the flanking *ATP1A2* and *D1S42* loci (multipoint lod = 9.26; $\theta = 0.00$, Table 1) (5, 17). The 95% confidence interval based on the -1 lod rule extends 3 centimorgans (cM) proximal and 8 cM distal to the *FCGR2-MPZ* combined locus. This *MPZ* locus adjacent to the FCGR2 locus also cosegregated with *CMT1B* in pedigree 2 because both flanking loci and *FCGR2* cosegregated with *CMT1B* (multipoint lod = 2.1) (5). Thus, the *MPG* locus cosegregates with *CMT1B* in each informative meiosis (total multipoint lod = 9.3 + 2.1 = 11.4; $\theta = 0.00$).

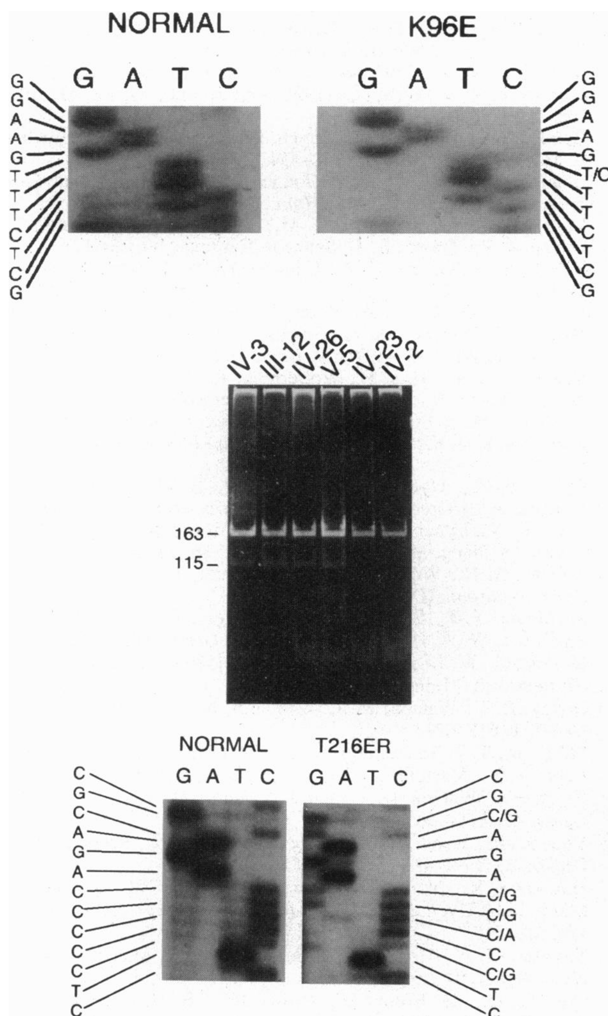

FIG. 3. (*Top*) Normal and mutant *MPZ* sequences. An A → G point mutation at nucleotide 115 in *MPZ* exon 3 is carried by the abnormal *CMT1B* chromosome in pedigree 1 (Fig. 2). (*Middle*) *Bst*BI PCR restriction enzyme analysis of pedigree 1 CMT1B patients. The *MPZ* A → G transition at base pair 115 of exon 3 converts a TTCAAA sequence to the palindromic *Bst*BI restriction site TTCGAA. Thus, the normal amplified 163-bp-amplified sequence does not cut (−), but the mutated sequence is digested to 115- and 48-bp fragments (+) in CMT1B pedigree 1 patients. The light 48-bp fragment is not visible. (*Bottom*) A 9-bp sequence with five nucleotide substitutions results in the *CMT1B* mutation in pedigree 2.

Genomic PCR sequencing found that three of three tested CMT1B patients in pedigree 2 (III-1; IV-2; affected son of III-6 in ref. 17) had five *MPZ* nucleotide substitutions (Fig. 3*C*). These mutations disrupted the splice site between intron 5 and exon 6 (Fig. 4, codon 216) and may have created a new putative splice site three bases earlier. The original and new splice sites both satisfy the general criteria of 3′ vertebrate splice sites: $\overset{96}{N}$-$\overset{97}{Y}$-$\overset{98}{N}$-$\overset{99}{N}$-(A $\overset{100}{\text{or}}$ G)-$\overset{\downarrow 55}{G}$, where Y is any pyrimidine nucleoside and the arrow indicates the splice site, with pyrimidine-rich 5′ sequences (28). This new putative splice site would replace a neutral threonine with a positively charged arginine and a negatively charged glutamic acid in a 20-amino acid MPZ region otherwise conserved perfectly in human, rat, and cow (Fig. 4). This major charge change is very likely to interfere with serine phosphorylation (22) six amino acids upstream. The original exon 6 splice junction is certainly abolished. If the putative new splice site is not functional, more severe protein structure disruption would result in extensive MPZ protein modification. This mutant 9-bp sequence is identical to a mouse intron 1 sequence. Thus, a recombination between the homologous human *MPZ* intron 1 and the *MPZ* intron 5 splice acceptor site could have generated this *CMT1B* allele.

These major MPZ mutations in pedigrees 1 and 2 occur in MPZ regions with identical amino acid sequences in three mammalian orders: human (primate), rat (rodent), and cow (artiodactyl) that diverged about 100 million years ago (Fig. 4) (15). That these mutations occurred in protein regions conserved identically in three unrelated mammals emphasizes the importance of maintaining these protein structures. Missense mutation Lys-96 → glutamic acid in CMT1B pedigree 1 substitutes a negatively charged glutamic acid for a positively charged lysine in this conserved extracellular protein region required for adhesive binding to another MPZ protein (Fig. 4). This probably disrupts homophilic extracellular attachment. Likewise, substitution of two charged amino acids in the MPZ intracellular domain in CMT1B pedigree 2 very likely disrupts intracellular homophilic attachment as well as serine phosphorylation and perhaps myelin regulation.

DISCUSSION

MPZ, encoding a major structural peripheral myelin protein, is an excellent candidate gene for demyelinating CMT1B neuropathy. Peripheral myelin MPZ protein expressed exclusively in the peripheral nervous system in myelinating Schwann cells accounts for >50% of the myelin protein (16).

```
          50        60 61            75 76            90 91 K96E     105
HUMAN..CSFWSSEWVSD DISFTWRYQPEGGRD AISIFHYAKGQPYID EVGTFKERIQWVGDP
COW   ..CSFWSSEWVSD DLSFTWRYQPEGGRD AISIFHYAKGQPYID EVGTFKERIQWVGDP
RAT   ..CSFWSSEWVSD DISFTWRYQPEGGRD AISIFHYAKGQPYID EVGTFKERIQWVGDP
CMT1B1 (Ped.1)                                   ...EVGTFEERIQWVGDP

          196          210 211 T216ER    225 226           240 241  248
HUMAN. .AMEKGKLHKPGKDAS KRGRQ TPVLYAMLDH SRSTKAVSEKKAKGL GESRKDKK
COW  . .AMEKGKLHKTAKDAS KRGRQ TPVLYAMLDH SRSTKAASEKKTKGL GESRKDKK
RAT  . .AMEKGKFHKSSKDSS KRGRQ TPVLYAMLDH SRSTKAASEKKSKGL GESRKDKK
CMT1B2  (Ped.2) ...KDAS KRGRQERPVLYAMLDH SRSTKA...

      RAT:  total of 94% identity to human in 248 aa overlap
      COW:  total of 93% identity to human in 219 aa overlap

      AA# 1-153    Extracellular domain in myelin intraperiod line
      AA# 154-179  Transmembrane Domain
      AA# 180-248  Cytoplasmic domain in major dense line

      UNDERLINED sequences are identical to normal human sequence
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FIG. 4. Amino acid comparison of *MPZ* genes. Identity of codon sequences 50–105 spanning the pedigree 1 *CMT1B* mutation (upper set of four sequences) and 207–231 spanning the pedigree 2 *CMT1B* mutation (lower set of four sequences) in normal human, rat, and cow emphasizes the otherwise complete sequence identity maintained for 100 million years.

The Schwann cell lays down insulating cytoplasmic membrane sheets, with each sheet including an intracellular major dense line between adjacent single membranes and an extracellular intraperiod line between pairs of cytoplasmic membranes. The mature MPZ protein has a cytoplasmic domain in the major dense line (15), a transmembrane domain, and an extracellular domain in the intraperiod line (15) that hold together the myelin membranes. Cultured HeLa cells transfected with the *MPZ* gene express MPZ on the cytoplasmic membrane that migrates to the point of membrane contact between touching cultured cells to act as an adhesive to maintain cell contact (22). Cultured Schwann cells infected with retrovirus carrying antisense MPZ cDNA ensheathe axons but are unable to myelinate (29). Together these data strongly suggest that MPZ mutation would result in abnormal myelinated Schwann cells in CMT1B patients.

Positional cloning projects generally proceed from linkage analysis and physical mapping to isolating and identifying candidate disease genes in the chromosome region. Occasionally the process is expedited by discovering a previously reported candidate gene sequence that maps directly to the linked chromosome region like the mutant human keratin 5 gene that results in epidermolysis bullosa (10). In this instance of autosomal dominant CMT1B, comparison of available mammalian MPZ amino acid sequences emphasizes the importance of major MPZ amino acid changes in both CMT1B pedigrees that are otherwise identical in human, rat, and cow. Structural proteins like MPZ that bind to each other are predicted to result in autosomal dominant genetic disease by generating multimeric abnormal structural proteins.

That MPZ protein is a double adhesion molecule maintaining myelin membrane is widely accepted. Furthermore, the extracellular MPZ domain shares homology with the adhesion molecules including myelin-associated glycoprotein (MAG) and neural cell adhesion molecule L1 that makes MPZ a member of the nervous system immunoglobulin superfamily (15). Thus, glycosylated peripheral nerve cell surface molecules exposed by myelin membrane dissociation might elicit an autoimmune response that results in peripheral neuropathy like that reported for abnormal myelin-associated glycoprotein. Similarly MPZ might act as an ion channel because the transmembrane region of the human Na^+ channel β-1 subunit shares 25% identity and 45% homology to 44 MPZ amino acid sequences. Other potential functions for MPZ as a signaling molecule or receptor for neurotropic viruses like polio await experimental verification.

In conclusion, several forms of evidence support *MPZ* as a candidate gene for CMT1B: (*i*) *MPZ* is located in chromosome band 1q22 in the *CMT1B* gene region, (*ii*) *MPZ* point mutations found in two CMT1B families cosegregate with *CMT1B*, (*iii*) the *MPZ* point mutations in both pedigrees occur in adhesive MPZ regions otherwise perfectly conserved in three mammalian orders, (*iv*) MPZ is expressed only in peripheral nerve Schwann cells where all peripheral myelin is synthesized and contributes >50% of the myelin protein, (*v*) MPZ functions as an adhesion molecule to maintain myelin structure and integrity, (*vi*) CMT1B is a peripheral neuropathy that results from demyelinated Schwann cells, and (*vii*) MPZ is a structural protein that binds to itself, which explains the autosomal dominant transmission of CMT1B.

Note. Hayasaka *et al.* (30) have also reported the *MPZ* gene to be mutated in the families reported here (here designated pedigree 1 and pedigree 2, designated K1568 and K1521, respectively, by Hayasaka *et al.*) and Kulkens *et al.* (31) have reported a similar finding in another family (which they designated NL-47). Both this paper and Hayasaka *et al.* report the same mutation in pedigree 1 (K1568) but this paper reports a splicing mutation in pedigree 2 (K1521), whereas Hayasaka *et al.* report a conservative point mutation.

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