A leucine-to-proline mutation in the putative first transmembrane domain of the 22-kDa peripheral myelin protein in the trembler-J mouse

(myelin/Schwann cell/growth regulation/peripheral neuropathy)

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ABSTRACT Peripheral myelin protein PMP-22 is a potential growth-regulating myelin protein that is expressed by Schwann cells and predominantly localized in compact peripheral myelin. A point mutation in the *Pmp-22* gene of inbred trembler (*Tr*) mice was identified and proposed to be responsible for the *Tr* phenotype, which is characterized by paralysis of the limbs as well as tremors and transient seizures. In support of this hypothesis, we now report the fine mapping of the *Pmp-22* gene to the immediate vicinity of the *Tr* locus on mouse chromosome 11. Furthermore, we have found a second point mutation in the *Pmp-22* gene of trembler-J (*Tr^J*) mice, which results in the substitution of a leucine residue by a proline residue in the putative first transmembrane region of the PMP-22 polypeptide. *Tr* and *Tr^J* were previously mapped genetically as possible allelic mutations giving rise to similar, but not identical, phenotypes. This finding is consistent with the discovery of two different mutations in physicochemically similar domains of the PMP-22 protein. Our results strengthen the hypothesis that mutations in the *Pmp-22* gene can lead to heterogeneous forms of peripheral neuropathies and offer clues toward possible explanations for the dominant inheritance of these disorders.

Myelin is a unique structure that consists of specialized plasma membranes of either Schwann cells (in the peripheral nervous system; PNS) or oligodendrocytes (in the central nervous system; CNS) wrapped around axons (reviewed in ref. 1). The resulting multilaminated structure facilitates electrical conduction of myelinated nerve fibers (2). The major protein components of the compact myelin sheath include myelin basic protein (MBP) and myelin-associated glycoprotein (MAG), which are both present in the CNS as well as the PNS. Proteolipid protein (PLP) is only found in CNS myelin, whereas protein zero (P₀) is only found in the PNS (reviewed in ref. 3).

We have recently described the cloning and characterization of an additional myelin protein, 22-kDa peripheral myelin protein, PMP-22 (SR13/PasII/gas3/CD25; refs. 4 and 5). A rat cDNA (SR13) encoding this polypeptide was isolated by a differential hybridization approach using cDNA libraries constructed from injured versus contralateral, noninjured sciatic nerves (6). PMP-22 mRNA and protein levels are dramatically reduced in the distal nerve stump after sciatic nerve crush but recover during axonal regeneration (5, 7). PMP-22 protein expression is predominantly confined to the compact myelin sheath of PNS fibers, whereas greatly re-

duced levels of PMP-22 mRNA can also be detected in the CNS (5). However, PMP-22 expression is regulated only during periods of myelination in the PNS but not in the CNS, indicating that the PMP-22 protein is not incorporated into CNS myelin (5).

Mutations in several major myelin proteins lead to spontaneous neurological mouse mutants like shiverer (*shi*; deletion in MBP) or jimpy (*jp*; point mutation and splice defect in PLP; reviewed in ref. 8). Such mouse strains are powerful tools in developmental neurobiology, as they yield important insights into the function of the affected genes *in vivo*. Furthermore, neurological mouse mutants provide excellent model systems to study related diseases in animals and humans. It is crucial for such analyses to identify the primary genetic defects leading to particular phenotypes.

The characterization of PMP-22 as a PNS-specific myelin protein led to the search for possible spontaneous PMP-22 mutations in mice. The *Tr* (trembler) mutation (9) was singled out as a likely candidate based on its PNS-specific defect in myelination. Analysis of cDNA as well as genomic DNA from inbred *Tr* mice identified a point mutation in the putative fourth transmembrane domain of PMP-22 replacing a glycine residue at position 150 by an aspartic acid residue (10). This finding suggests a role for PMP-22 in peripheral neuropathies of animals and related diseases in humans [hereditary motor and sensory neuropathies (HMSN I and III; reviewed in refs. 10 and 11)].

Tr^J (trembler-J) is a spontaneous mutation that arose independently from *Tr* (12). *Tr* and *Tr^J* were suggested to be allelic based on linkage to vestigial tail (13), on the fundamentally similar phenotype of the two disorders, and on the abnormal phenotype of the double heterozygous animals (12, 13). Histologically, *Tr* and *Tr^J* are characterized by a marked decrease of axon myelination in the PNS and a concomitant increase in Schwann cell numbers. However, there are important differences between *Tr* and *Tr^J*. Expression of the *Tr* allele is truly dominant, whereas that of *Tr^J* is semidominant, showing a possible gene dosage effect. Importantly, the *Tr^J/Tr^J* genotype leads to the most severe known peripheral myelin deficiency, which may be responsible for the early death of these animals at postnatal day 17 or 18 (12). This finding is in marked contrast to the long-lived *Tr/Tr* mice

Abbreviations: cM, centimorgan; CNS, central nervous system; Csfm, granulocyte-macrophage colony-stimulating factor; HMSN, hereditary motor and sensory neuropathy; MAG, myelin-associated glycoprotein; MBP, myelin basic protein; Myhs, skeletal muscle myosin heavy chain isoform; P₀, protein zero; PLP, proteolipid protein; PMP-22, 22-kDa peripheral myelin protein; PNS, peripheral nervous system; RFLP, restriction fragment length polymorphism; *Tr*, trembler; *Tr^J*, trembler-J.

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(14). Second, *Tr* shows more severe progression of abnormalities with age than *Tr^J*. In particular, "onion bulb" structures, the pathological fingerprints of repeated cycles of demyelination and remyelination, are more prominent in aged *Tr*/+ than *Tr^J*/+ animals (12).

We report here the fine mapping of the *Pmp-22* gene into the close proximity of the *Tr* locus on mouse chromosome 11 and the identification of the putative primary defect in *Tr^J* mice as a point mutation in the *Pmp-22* gene. The mapping data in conjunction with the detection of two independent nonconservative mutations in the same myelin gene, resulting in a similar myelin deficiency, provide strong evidence to support the hypothesis that mutations involving the *Pmp-22* gene are involved in hereditary peripheral neuropathies. Furthermore, the nature of the two mutations provides clues about possible mechanisms leading to the specific types of myelin disorders observed in *Tr* and *Tr^J* animals.

MATERIALS AND METHODS

Animals. Mice of the AEJ/Gn-*a* *bp^H/a^e* + strain were obtained from The Jackson Laboratory. The *Mus spretus* mice were a gift from Eva Eicher (The Jackson Laboratory). The interspecies backcross [(AEJ/Gn × *M. spretus*) × AEJ/Gn] was performed at the National Cancer Institute-Frederick Cancer Research and Development Center.

The *Tr^J* animals, 5–7 weeks old, were heterozygotes (*Tr^J*/+) on the C57BL/6J background. The *Tr^J* mutation arose in the C57BL/6J mouse strain and has been maintained coisogenic with it for 39 generations. Genotypes of all *Tr^J* animals used in this study were confirmed by behavioral and histological criteria (12). Animals of the parental wild-type C57BL/6J mouse strain served as controls.

DNA Isolation and Southern Blot Analysis. High molecular weight genomic DNA was prepared from frozen mouse tissues (15). Restriction endonuclease digestion, agarose gel electrophoresis, and Southern blot transfer were performed as described (15). Southern blot hybridization was performed as described (16) using nick-translated or random primed ³²P-labeled probes.

RNA Isolation and Sequencing. *Tr^J*/+ and +/+ control mice were sacrificed and the sciatic nerves were removed and frozen for RNA extraction. Both nerves from each individual animal were pooled and total RNA was extracted (yields of 5–10 µg of total RNA per animal). Two micrograms of total RNA was reverse transcribed (Superscript, Bethesda Research Laboratories) and amplified by PCR (94°C/30 sec; 55°C/1 min; 72°C/1 min; 30 cycles) using two oligonucleotide primers spanning nucleotides 91–109 (5'-GAGTTGTGCGCTGAGGCTA-3') and 832–814 (5'-ACAGTATCC-CCCACTCAAC-3') in the corrected PMP-22/gas3 sequence (10, 17). The PCR product was subcloned using a TA cloning kit (Invitrogen, San Diego) and the nucleotide sequence of the coding region of two mutated and two wild-type clones was determined by dideoxynucleotide sequencing (Sequenase version 2; United States Biochemical). Alternatively, PCR products were purified on Tris/borate/EDTA/agarose gels, isolated with GeneClean (Bio 101, La Jolla, CA), and directly sequenced using synthetic oligonucleotide primers that were based on the PMP-22/gas3 cDNA sequence (10, 17).

Isolation of cDNA Probes. Total RNA was isolated from growth-arrested mouse NIH 3T3 fibroblasts and reverse transcribed; a PMP-22 cDNA probe was amplified by PCR (94°C/1 min; 55°C/30 sec; 72°C/3 min) using the two oligonucleotide primers: 5'-CCGCGGAAGCTTCCCAACTCCAGCCACC-3' and 5'-GAATTCAAGCTTGCTCAGAGCTAGACGG-3'. The amplification product was digested using the restriction endonuclease *Hind*III and subcloned into a Bluescript vector (KS⁺; Stratagene). The

resulting mouse PMP-22 cDNA probe, PMP-22c, contained the complete coding region as verified by DNA sequencing. The *Csfgm* (granulocyte-macrophage colony-stimulating factor; ref. 18) probe, pE1-11, is a 0.79-kilobase (kb) cDNA clone containing the entire coding sequence of *Csfgm*. The *Myhs* (skeletal muscle myosin heavy chain isoform; ref. 19) probe, p32, is a 1.25-kb cDNA fragment cloned into the *Pst* I site of pBR322.

Statistical Analysis. The results of the interspecific backcrosses were analyzed by calculating the maximum likelihood estimates of linkage parameters as described by Green (20).

RESULTS

Identification of Restriction Fragment Length Polymorphisms (RFLPs). Genomic DNAs from AEJ/Gn and *M. spretus* parental control animals were digested with several restriction endonucleases and analyzed by Southern blot hybridization using probes that identified the loci *Csfgm*, *Myhs*, and *Pmp-22* (Table 1). At least one RFLP was identified for each of the probes tested and the sizes of the genomic restriction fragments detected are listed in Table 1.

The segregation pattern of the *M. spretus* allele in 92 backcross animals was determined for *Csfgm* and *Myhs* by Southern blot hybridization. As expected, mice were either homozygous for the AEJ/Gn allele or heterozygous for the *M. spretus* and AEJ/Gn alleles. The frequency of AEJ/Gn and *M. spretus* alleles for the loci mapped in the N2 progeny did not significantly differ from the expected 1:1 ratio. The chromosomal locations of *Csfgm* and *Myhs* were previously established from an interspecific backcross involving C57BL/6J and *M. spretus* mice (21). The results obtained in the interspecific backcross involving AEJ/Gn and *M. spretus* mice revealed six recombinant chromosomes out of a total of 92 N2 offspring, indicating that *Myhs* lies 6.5 ± 2.6 centimorgans (cM) from *Csfgm* (Fig. 1A). These results are consistent with the previous mapping using the [(C57BL/6J × *M. spretus*) × C57BL/6J] interspecific backcross, which placed *Myhs* 5.6 ± 2.4 cM distal to *Csfgm* (21).

Localization of *Pmp-22* on Mouse Chromosome 11. *Pmp-22* was originally localized to mouse chromosome 11 by analysis of somatic cell hybrids and suggested to be responsible for the *Tr* phenotype (10). We were interested in refining the localization of *Pmp-22* on mouse chromosome 11 to determine if its location is consistent with the genetically defined *Tr* locus. Using the interspecific backcross generated between AEJ/Gn and *M. spretus* mice, we mapped the location of *Pmp-22*. The results identified four recombinants between *Csfgm* and *Pmp-22* and two recombinants between *Pmp-22* and *Myhs* out of 92 N2 offspring analyzed (Fig. 1A). No double recombinants were observed within the small interval analyzed. The results show that *Pmp-22* resides 4.4 ± 2.1 cM distal to *Csfgm* and 2.2 ± 1.5 cM proximal to *Myhs* (Fig. 1B).

Identification of the Putative *Tr^J* Mutation. The location of *Pmp-22* is consistent with its identity to the *Tr* locus. To determine whether the *Tr^J* mouse carries a mutation in the *Pmp-22* gene, total RNA was isolated from the sciatic nerves

Table 1. List of loci mapped in interspecific backcross animals

Locus	Probe name	Restriction enzyme	Restriction fragment size(s), kb	
			AEJ/Gn	<i>M. spretus</i> *
<i>Csfgm</i>	pE1-11	<i>Bam</i> HI	8.7	<u>6.7</u>
<i>Myhs</i>	p32	<i>Bam</i> HI	13.6, 4.7	13.6, <u>10.7</u>
<i>Pmp-22</i>	PMP-22c	<i>Taq</i> I	11.5, 4.5, 2.3	<u>5.3</u> , <u>3.8</u> , <u>1.9</u>

*Underlined restriction fragment size indicates the segregating allele(s) that was typed in the backcross.

FIG. 1. (A) Summary of the results of the interspecific backcross analysis. Genes mapped are listed on the left. Each column represents the chromosome identified in the N2 progeny inherited from the (AEJ/Gn $\times$ *M. spretus*)F₁ parent. The closed boxes represent the AEJ/Gn allele and the open boxes represent the *M. spretus* allele. The number of each type of chromosome identified in the backcross progeny is listed at the bottom. (B) Genetic localization of *Pmp-22*. The region of mouse chromosome 11 analyzed in the interspecific backcross is represented. The genes mapped are listed on the right of the chromosome with their corresponding location on human chromosomes, if known. The genetic map distance (in cM) between adjacent loci is listed on the left. The location of *Pmp-22* in between *Csfgm* and *Myhs* is consistent with the location of *Tr* on the composite linkage map (22).

of an affected *Tr*^J/+ animal. The RNA was reverse transcribed and PMP-22 cDNA was amplified by PCR using PMP-22-specific primers. The resulting cDNAs were subcloned and analyzed by DNA sequencing. A point mutation at nucleotide position 190 of the mouse PMP-22/gas3 sequence was found in two out of six clones analyzed (Fig. 2). The detected transition mutation from thymine (T) to cytosine (C) translates into a nonconservative amino acid exchange at amino acid position 16 (leucine to proline) of the PMP-22 polypeptide (Fig. 3). Possible PCR errors were excluded by repeating the PCR step and direct sequencing (without subcloning) of the amplified product. As expected, signals in the T as well as the C lanes of the corresponding sequencing gel were detected at position 190 of the PMP-22 sequence (10, 17), reflecting the heterozygosity of the animal with respect to this nucleotide position (Fig. 2). The results were confirmed by a similar analysis of an additional (sibling) *Tr*^J/+ animal as well as two wild-type (+/+) C57BL/6J mice. Whereas the second *Tr*^J/+ animal was again heterozygous at nucleotide position 190, both +/+ animals carried only the wild-type allele (Fig. 2). Since the *Tr*^J mutation has been maintained on a C57BL/6J background, we conclude that the mutation in *Pmp-22* described here is most likely responsible for the *Tr*^J phenotype.

DISCUSSION

This report describes the mapping of the *Pmp-22* gene in close proximity to the genetically defined *Tr* locus on mouse chromosome 11 and identifies a nonconservative point mutation in the *Pmp-22* gene of the *Tr*^J mouse. Furthermore, our

FIG. 2. Autoradiograms of sequencing gels showing the putative *Tr*^J mutation. The order of nucleotides on the sequencing gels is given at the top. The respective nucleotides at the mutated nucleotide position 190 are marked by arrows. (Upper Left) Sequence of the cloned wild-type allele. (Upper Right) Sequence of the cloned *Tr*^J allele. (Lower Left) Direct sequencing of the PMP-22-specific PCR products derived from a wild-type animal. (Lower Right) Direct sequencing of the PMP-22-specific PCR products derived from a heterozygous *Tr*^J/+ animal.

study supports the hypothesis that *Tr* and *Tr*^J are allelic variants with defects in the *Pmp-22* gene.

The results of the mapping studies determined that *Pmp-22* is localized 4.4 cM distal to *Csfgm* and 2.2 cM proximal to *Myhs*. The localization of *Pmp-22* between *Csfgm* and *Myhs* is consistent with its identity with *Tr*. Based on composite linkage information, *Tr* appears to be located $\approx$ 4 cM distal to *Csfgm* and $\approx$ 2 cM proximal to *Myhs* (22). Thus, *Pmp-22* maps to the region where the *Tr* mutation resides. When the PMP-22 cDNA was originally cloned, sequence analysis revealed that it was identical to gas3 (4, 10, 17). Recently, the *Gas-3* gene was localized to mouse chromosome 11 through a combination of interspecific backcross mapping and recombinant inbred strain analyses (24). Colombo et al. (24) localized *Gas-3* 7.0 ± 2.4 cM distal to *Il-3* (interleukin 3) and 4.4 ± 1.8 cM proximal to *Trp53* (transformation-related protein 53). These results are consistent with the data presented in this report.

The distal half of mouse chromosome 11 shares exclusive conserved synteny with human chromosome 17. Comparing the relative gene order between mouse and humans, it appears that the order of genes has been conserved between human chromosome 17p and their homologues on mouse chromosome 11 (22). *MYHS* has been localized to 17p12-p13 in humans (25). Since *Pmp-22* has been localized proximal to *Myhs* in the mouse, we would predict that if the human homologue of *Pmp-22* maps to human chromosome 17, it resides on 17p11-p12. This putative location, in conjunction with its involvement in *Tr* and *Tr*^J, identifies *PMP-22* as a likely candidate gene to be affected in the Charcot-Marie-Tooth disease type 1a disorder (10, 11).

Two independent nonconservative mutations in the *Pmp-22* gene have now been shown to be associated with the *Tr* or the *Tr*^J phenotype. Based on these findings, it seems almost certain that mutations affecting the *Pmp-22* gene are involved in hereditary peripheral neuropathies in mouse and, possibly, in human. Further support for this hypothesis can be expected from the construction of suitable transgenic mouse models. It can be anticipated that overexpression of the described mutated *Pmp-22* alleles may result in a *Tr* (*Tr*^J)-like phenotype in otherwise normal mice, given the dominant (semidominant) inheritance of these mutations. Alternatively, similar experimental strategies may be applied

FIG. 3. Hypothetical model of PMP-22 illustrating the position of the *Tr* and *Tr^J* mutations. The amino acid sequence for mouse PMP-22 is given in the one-letter code. The positions of charged amino acids (neutral pH) are indicated as "+" or "-". The locations of the putative *Tr* and *Tr^J* mutations are marked with arrows. The model is based on computer-generated hydrophobicity plots, surface probability, and secondary structure predictions (23). The putative transmembrane sequences I, II, IV are predicted to form β -sheets, whereas transmembrane domain III is α -helical.

to alleviate the abnormalities in *Tr* and *Tr^J* mice, as demonstrated by correcting the defect in shiverer mice using a MBP transgene (26).

Tr and *Tr^J* animals are characterized by two major histological hallmarks. (i) Severely reduced myelin sheaths are found exclusively in the PNS of affected animals. This feature is consistent with possible direct consequences of mutations in the *Pmp-22* gene, since the PMP-22 protein is expressed by Schwann cells and is localized mainly in compact PNS myelin. (ii) An increase in the number of Schwann cells in PNS nerves of *Tr* and *Tr^J* animals is observed due to an abnormal continuous Schwann cell proliferation throughout life (12). In normal mice, Schwann cells cease to proliferate soon after birth, concomitant with the onset of myelination. In this context, it may be of significance that high levels of PMP-22 (gas3) mRNA expression can be found *in vitro* in NIH 3T3 fibroblasts under culture conditions that favor growth arrest (serum starvation and cell-cell contact; ref. 17). Thus, it might be argued that PMP-22 is important in growth control of Schwann cells. However, proliferation of Schwann cells and myelination are intimately connected, and it remains to be determined whether myelination is the consequence of Schwann cell growth arrest or vice versa (27). This peculiarity in Schwann cell development makes it difficult to address the exact role of PMP-22 *in vivo*. Nevertheless, *in vitro* systems using cell cultures of appropriately transfected Schwann cells in the presence or absence of neurons and mitogens, as well as under myelinating and nonmyelinating culture conditions, will help to clarify the importance of PMP-22 in myelination and/or proliferation.

Several possible mechanisms can be proposed leading to the *Tr* and *Tr^J* phenotype based on the two mutations described in PMP-22. The positions of both mutations in putative transmembrane regions (Fig. 3) of the PMP-22 protein may favor a structural model where the mutated proteins cannot integrate correctly into the myelin membranes, thereby disrupting the development of compact myelin. Such a hypothesis would be compatible with the dominant (semidominant) inheritance of *Tr* (*Tr^J*). The mutations in PMP-22 may, alternatively, affect extra- or intracellular interaction of PMP-22 with itself or other molecules. Clearly, the problem of how specific mutations in PMP-22 lead to heterogeneous peripheral neuropathies (compare *Tr* to *Tr^J*) can only be solved by determining the normal functions of PMP-22. The role of the transmembrane of membrane-associated domains of PMP-22 seems to be of particular interest given the position of the identified mutations.

The fact that homozygous *Tr* animals are long-lived implies that a mere lack of peripheral myelin is not fatal (14). In contrast, homozygous *Tr^J* animals die early, suggesting a more general function of PMP-22 outside of the nervous system. Interestingly, PLP, the putative CNS counterpart to PMP-22, is affected by several mutations in animals and humans (reviewed in ref. 28). Thus, we speculate that PLP and PMP-22 may serve similar functions. PLP has been suggested to act as a nonspecific pore or channel protein (29, 30). A similar function for PMP-22 seems conceivable, given the position of the identified mutations in transmembrane domains and the recent discovery that a single point mutation in a Na⁺ channel can lead to a dominantly inherited disease in humans (31). Alternatively, PMP-22 could be involved in cell-cell communication analogous to a family of structurally related Gap-junction proteins (connexins) that were recently implicated in cellular growth (32).

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