An in-frame deletion in peripheral myelin protein-22 gene causes hypomyelination and cell death of the Schwann cells in the new Trembler mutant mice

ARTICLE *in* NEUROSCIENCE · SEPTEMBER 1997

Impact Factor: 3.36 · DOI: 10.1016/S0306-4522(96)00692-6 · Source: PubMed

8 AUTHORS, INCLUDING:

Kohichi Tanaka
Tokyo Medical and Dental University
168 PUBLICATIONS **8,334** CITATIONS
SEE PROFILE

Tateki Kikuchi
National Institute of Neuroscience, NCNP, T…
117 PUBLICATIONS **2,799** CITATIONS
SEE PROFILE

Pergamon

PII: S0306-4522(96)00692-6

Neuroscience Vol. 79, No. 3, pp. 735–744, 1997

0306–4522/97 $17.00+0.00

AN IN-FRAME DELETION IN PERIPHERAL MYELIN PROTEIN-22 GENE CAUSES HYPOMYELINATION AND CELL DEATH OF THE SCHWANN CELLS IN THE NEW *TREMBLER* MUTANT MICE

J.-G. SUH,*†‡ N. ICHIHARA,* K. SAIGOH,† O. NAKABAYASHI,*
T. YAMANISHI,† K. TANAKA,† K. WADA† and T. KIKUCHI*§

*Department of Animal Models for Human Disease, National Institute of Neuroscience, NCNP, Kodaira, Tokyo 187, Japan

†Department of Degenerative Neurological Diseases, National Institute of Neuroscience, NCNP, Kodaira, Tokyo 187, Japan

Abstract—Cloning and sequencing of the peripheral myelin protein-22 cDNA and genomic DNA from newly found *Trembler* mice revealed an in-frame deletion including exon IV which codes for the second (TM2) and a part of third (TM3) transmembrane domain of peripheral myelin protein-22. This mutation was distinct from those in both other allelic *Trembler* and *Trembler-J* mice, which carry point mutations within the putative transmembrane spanning regions of peripheral myelin protein-22. Inheritance was autosomal dominant. The affected mice revealed an abnormal gait, which appeared at 15–20 days of age, followed by motor and sensory ataxia, which remained throughout life. Most of the affected mice could survive more than one year. One of the most notable pathological phenotypes was a giant vacuolar formation in the sciatic nerve of homozygotes. They vary in size within the cytoplasm of Schwann cells, which failed to assemble myelin at any ages studied. Heterozygotes showed normal myelination during the early postnatal stages, followed by a segmental demyelination at an advanced stage. Vacuolar formation was not so frequent as in the homozygotes.

These results suggest that the missing of transmembrane spanning region (TM2 and TM3) of peripheral myelin protein-22 may disturb a dual biological function of peripheral myelin protein-22, leading to a dysmyelination of axons and to a vacuolar formation within the cytoplasm of the Schwann cells. The latter phenotype is discussed in conjunction with the disruption of an intracellular transport system and subsequent cell death. © 1997 IBRO. Published by Elsevier Science Ltd.

Key words: PMP22, *Trembler*, deletion, Schwann cell, CMT1A, apoptosis.

Hereditary peripheral neuropathies are a common heterogeneous group of diseases in humans.[4,27] Genetic linkage studies and subsequent molecular analyses have shown that some peripheral myelin proteins are involved in the pathogenesis of hereditary peripheral neuropathies.[6,30,31,35] Among them, the peripheral myelin protein (PMP22) gene has been found to be responsible for Charcot–Marie–Tooth disease type 1A (CMT1A), Dejerine–Sottas disease (DSS), and hereditary neuropathy with liability to pressure palsies (HNPP).[6,30,31,35] CMT1A results predominantly from the duplication of a 1.5 Mb segment which comprises the PMP22 gene.[18] Point mutations of the PMP22 gene, resulting in the substitution of a single amino acid in predicted PMP22 protein, have been reported to be associated with rare cases of CMT1A[18,20,21] and cases of DSS.[19] In contrast, a deletion of the 1.5 Mb segment or a frame-shift mutation of the PMP22 gene has been proposed to be responsible for HNPP.[2,17] In the mouse, point mutations in the PMP22 gene have been shown to cause CMT-like phenotypes in two mutant strains, *Trembler* (Tr) and *Trembler-J* (Tr-J) mice. [30,31,33,34,39]

We recently found a spontaneous neurological mutant which arose from a normal colony of an inbred strain of the gracile axonal dystrophy mouse (GAD/Ncnp) at our Institute.[10,32] The affected mouse had a gait abnormality that progressed slightly with age. In the present study, we endeavoured to characterize this mutant mouse both pathologically and molecular genetically.

‡Present address: Experimental Animal Center, College of Medicine, Hallym University, 1 Okchon-Dong, Chunchon, Kangwon-Do, 200-702, Korea.

§To whom correspondence should be addressed.

Abbreviations: bp, base pairs; CMT1A, Charcot–Marie–Tooth disease type 1A; DSS, Dejerine–Sottas disease; GAD, gracile axonal dystrophy; HNPP, hereditary neuropathy with liability to pressure palsies; PCR, polymerase chain reaction; PLP, proteolipid protein; PMP22, peripheral myelin protein-22; rER, rough endoplasmic reticulum; RT, reverse transcriptase; SDS, sodium dodecyl sulphate; TM, transmembrane domain; Tr, *Trembler* mouse; Tr-J, *Trembler*-J mouse.

EXPERIMENTAL PROCEDURES

Animals

The gene symbol of the Tr-Ncnp is $PMP22^{Tr\text{-}Ncnp}$ (registered in the Mouse Genome Database, 1995). This strain was used throughout the present study. These mice were discovered within a normal inbred strain (GAD/Ncnp)[10,32] which is not carrying a *gad* mutation. The mice were maintained in the Laboratory of the Animal Research Center, National Institute of Neuroscience, Tokyo, Japan under a specific pathogen-free condition. The total number of mice used in this study was 394. They were provided with a commercial diet and water *ad libitum* under controlled temperature, humidity and lighting conditions (23 ± 4°C, 55 ± 5% and a 12:12 light/dark cycle with lights on at 08.00). The animals were cared for and used according to humane standards and in compliance with the law and related standards for the animal welfare.

Reverse transcription-polymerase chain reaction and sequence analysis

The total RNA was extracted from the sciatic nerves of the Tr-Ncnp mice using an Isogen kit (Nippon Gene, Japan). An aliquot of the RNA was converted to cDNA by oligo (dT)-primed cDNA synthesis using a reverse transcriptase (SuperScript™ Preamplification System for First Strand cDNA Synthesis, Life Technologies). A pool of synthesized cDNAs was amplified by polymerase chain reaction (PCR) using a set of primers; PMPF: 5′-GAGTTTGTGCCTGAGGCTA-3′ and PMPR: 5′-ACAGCAATCCCCACTCAAC-3′. The primer sequences were designed from a reported mouse PMP22 cDNA sequence (The cDNA sequence for the mouse PMP22 was retrieved from GeneBank; M32240). A PCR reaction was performed in a Perkin-Elmer thermal cycle (GeneAmp PCR system 9600) with denaturation at 94°C for 30 s, annealing at 50°C for 1 min, and extension at 72°C for 1 min, followed by a final extension for 7 min at the end of 35 cycles. PCR-products were analysed on a 2% agarose gel. The size of the fragments was confirmed by DNA sequencing. PCR products were further subcloned into an adequate plasmid using a pCR-Script™ SK(+) Cloning kit (Stratagene) and sequenced using a Pharmacia LKB A.L.F. DNA sequencer.

Genotyping by Southern blot analysis

Restriction endonuclease digestion, agarose gel electrophoresis, and Southern blot transfer were performed as described.[23] 10μg of DNA was digested with Pst I, blotted onto a membrane and hybridized to a mouse PMP22 cDNA probe. A mouse PMP22 cDNA probe containing the complete coding region (nucleotide position 58–580, Fig. 2) was radiolabelled using Megaprime™ DNA labelling systems (Amersham Life Science) to a specific activity of at least 10^9 c.p.m./μg. Hybridization was carried out at 65°C in 0.25 M Na_2HPO_4 and 7% sodium dodecyl sulphate (SDS), and the membrane was washed under stringent conditions (20 mM Na_2HPO_4 and 5% SDS) at 65°C. The final wash was performed at 65°C in 20 mM Na_2HPO_4 and 1% SDS, and the hybridized membrane was exposed to Kodak X-ray film.

Isolation of intronic sequences by cassette ligation-mediated polymerase chain reaction and genomic polymerase chain reaction

A cassette ligation-mediated PCR was performed by a modification of the previously described method.[38] PCR amplification, subcloning and sequence analysis were achieved as described above. A fragment of genomic cDNA was amplified with primers (5′-GCAGGAATGAATTGTTTTCACCA-3′;5′-TTTGGTGAGAGTGAAGAGATGG-3′). Five mice for each genotype were used to determine results in this experiment.

Pathological study

According to the mutant's genotyping described above, three types (*Tr-Ncnp*/*Tr-Ncnp, Tr-Ncnp*/+, +/+) were selected for the pathological observations. The sciatic nerves removed from nine-day, one-month and five-month-old mice were fixed with 3.0% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) immediately after the muscles and connective tissues surrounding the nerves were removed. Three mice were used for each genotype. The nerve stumps were then taken from the proximal region of larger size and fixed overnight in the same fixative solution at 4°C. The stumps were postfixed with 1% osmium tetroxide in a buffer-sucrose medium for 2 h, dehydrated in graded alcohol and propylene oxides, and embedded in Epon 812. Cross sections of 1.0 μm thickness were stained for light microscopic observation. Ultrathin sections were contrasted with uranyl acetate and lead citrate and observed under a Hitachi H-7000 electron microscope.

Detection of apoftotic cells in the sciatic nerves of normal and Tr-Ncnp mice

Normal mice aged 12 months and homozygous mice aged 6 months were perfused transcardially with 4% paraformaldehyde in 0.1 M phopsphate buffer (pH 7.3) at 4°C. Two mice were used for each genotype. The sciatic nerves removed were postfixed for 3 h at 4°C, embedded in paraffin after dehydration through graded alcohols. The deparaffinized sections were digested with proteinase K (20 μg/ml) for 5 min at room temperature and stained with the Oncor (Gaithersburg MD) ApopTag™ kit, a variation of the TUNEL method, to detect apoptotic cells in normal and Tr-Ncnp sciatic nerve.[26]

RESULTS

Clinical and genetic observations

Using an outcrossed background, we could distinguish an affected mouse from normal littermates by 15–20 days of age. The affected mice had a gait abnormality that progressed slightly with age. However, there was no significant difference between the life spans of normal and affected mice. Most of the affected mice could survive more than one year. The segregation results of the phenotype were obtained from the test crosses between normal and affected mice. Since F_1 offsprings display symptoms of the disorder and are clinically indistinguishable from homozygotes, it was predicted that the inheritance mode of this mutation is attributed to an autosomal dominant gene (data not shown). The segregation patterns of the Tr allele in a pedigree were followed by the Southern blot analysis (Fig. 1A and C). These results indicate that this mutation could be explained by a simple autosomal dominant inheritance.

Molecular genetic results

To identify the molecular basis of the pathogenesis of the Tr-Ncnp mouse, PMP22 cDNA from the affected mouse (with a genotype unidentifiable by clinical symptoms) was cloned and sequenced using

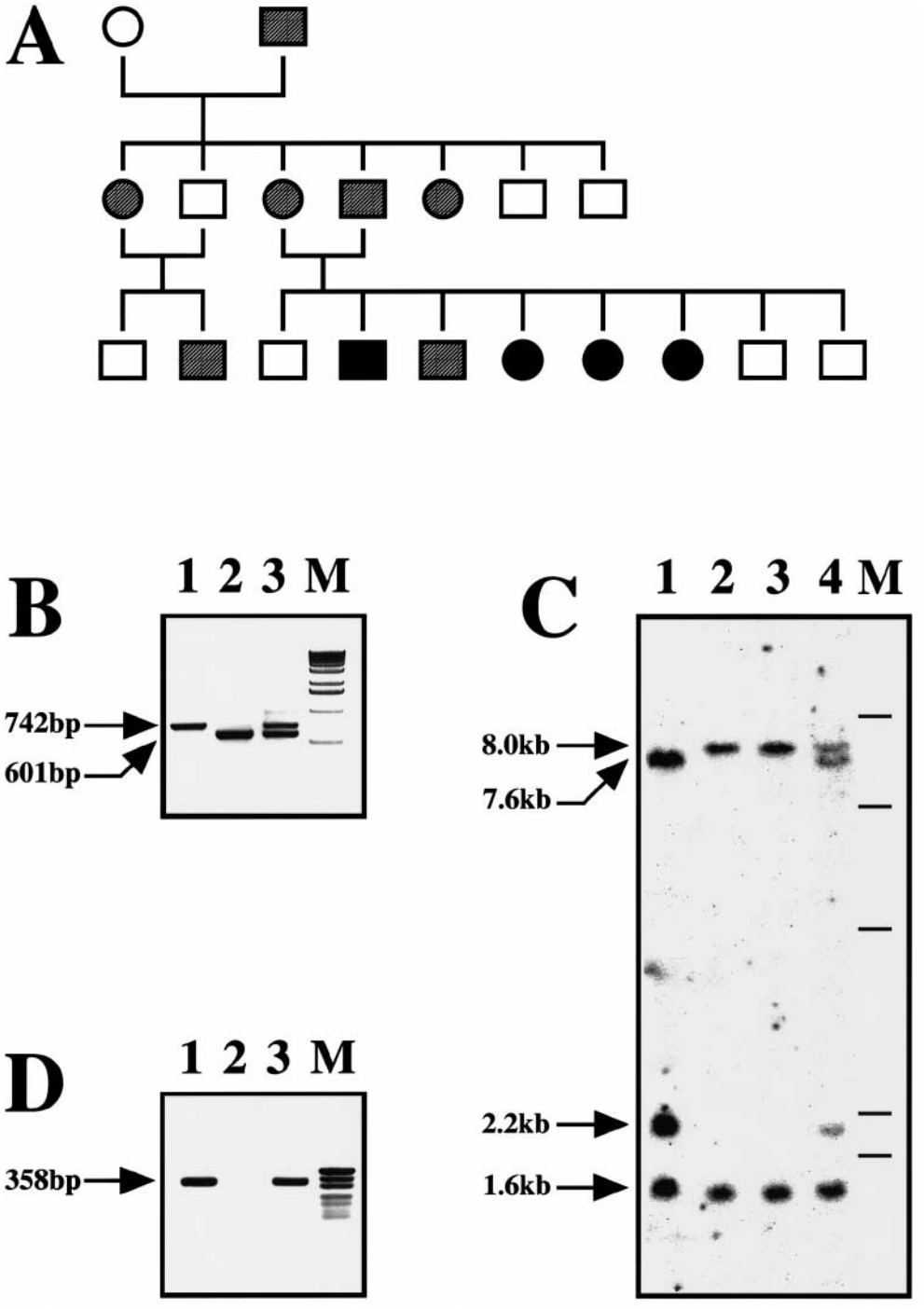

Fig. 1. Clinical and genetic characterization of Tr-Ncnp mouse. A) Genetic analysis of Tr-Ncnp allele in a pedigree. Open, hatched and filled symbols indicate normal, heterozygous and homozygous mice, respectively. Squares and circles represent males and females, respectively. The genetic pattern of affected animals could be explained by an autosomal dominant inheritance. B) RT-PCR analysis. Total RNA was isolated from the sciatic nerves of the normal (lane 1), homozygous (lane 2) and heterozygous (lane 3) mice. The 742 bp and 601 bp DNA fragments represent the wild-type and Tr-Ncnp allele, respectively. Size marker (M) is a 1 kb DNA ladder. C) Southern blot analysis. Tr-Ncnp mice have a deletion in the PMP22 gene. Genomic DNAs were isolated from normal (lane 1), homozygous (lanes 2 and 3) and heterozygous (lane 4) mice. In lane 1, fragments of 7.6, 2.2 and 1.6 kb were detected. At lanes 2 and 3, fragments of 8.0 and 1.6 kb were detected. At lane 4, fragments of 8.0, 7.6, 2.2 and 1.6 kb were detected. Size marker (M) is lambda DNA digested with Hind III. D) Genomic PCR analysis by cassette ligation-mediated PCR. No PCR products were obtained from the homozygotes (lane 2), while heterozygous (lane 3) and normal (lane 1) mice exhibited 358 bp PCR products. Size marker (M) is pUC19 DNA digested with Msp I.

```
GAGTTTGTGC CTGAGGCTAA TCTGCTCTGA GATAGCTGTC CCTTTGAACT GAAACAGGCA  60

CCGCTCCTCT GATCCCGAGC CCAACTCCCA GCCACCATGC TCCTACTCTT GTTGGGGATC 120
                                     M  L   L  L  L   G  I

CTGTTCCTGC ACATCGCGGT GCTAGTGTTG CTCTTCGTCT CCACCATCGT CAGCCAATGG 180
L  F  L  H   I  A  V   L  V  L   L  F  V  S   T  I  V   S  Q  W

CTCGTGGGTA ATGGACACAC GACTGATCTC TGGCAGAACT GTACCACATC CGCCTTGGGA 240
L  V  G  N   G  H  T   T  D  L   W  Q  N  C   T  T  S   A  L  G

GCCGTCCAAC ACTGCTACTC CTCATCAGTG AGCGAATGGC TGCAGTCTGT CCAGGCCACC 300
A  V  Q  H   C  Y  S   S  S  V   S  E  W  L   Q  S  V   Q  A  T

ATGATCCTGT CTGTCATCTT CAGCGTCCTG GCTCTGTTCC TGTTCTTCTG CCAGCTCTTC 360
M  I  L  S   V  I  F   S  V  L   A  L  F  L   F  F  C   Q  L  F

ACTCTCACCA AAGGCGGCCG GTTTTACATC ACTGGATTCT TCCAAATCCT TGCTGGTCTG 420
T  L  T  K   G  G  R   F  Y  I   T  G  F  F   Q  I  L   A  G  L

TGCGTGATGA GTGCAGCGGC CATCTACACA GTGAGGCACA GTGAGTGGCA TGTCAACACT 480
C  V  M  S   A  A  A   I  Y  T   V  R  H  S   E  W  H   V  N  T

GACTACTCCT ATGGCTTCGC CTACATCCTG GCCTGGGTGG CCTTTCCCCT AGCCCTCCTC 540
D  Y  S  Y   G  F  A   Y  I  L   A  W  V  A   F  P  L   A  L  L

AGTGGTATCA TCTATGTGAT CCTGCGGAAA CGCGAATGAG GCGCCCGACG ACGCACCGTC 600
S  G  I  I   Y  V  I   L  R  K   R  E

CGTCTAGGCT CTGAGCGCGC ATAGGGTCCA CAGGGAGGGA GGAAGGAAAC CAGAGAACAA 660

AACCAACCAA CCAAAAAAGA GCTAGCCCCA AACCCAAACG CAAGCCAAAC CAAACAGAAC 720

GCAGTTGAGT GGGGATTGCT GT                                           742
```

Fig. 2. Nucleotide sequence of the mouse PMP22 cDNA. The entire coding region was cloned using the underlined set of oligonucleotide primers. The regions of the cDNA deleted in the Tr-Ncnp allele correspond to the sequence of Exon IV in human PMP22 gene. These regions are indicated in the boxes.

the reverse transcriptase (RT)-PCR method. Two PCR products of different sizes (742 base pairs (bp) and 601 bp) were identified in the sciatic nerve of the pathologically characterized normal (lane 1) and affected (lanes 2 and 3) mice, respectively. Since both PCR products were detected in the lane 3 on gels, the genotype of this mouse was estimated to be heterozygote (Fig. 1B). The 742 bp and 601 bp DNA fragments were, therefore, likely to represent the wild-type and the Tr-Ncnp allele (Fig. 1B), respectively. The PCR amplifications were made in several independent mice, to eliminate possible PCR errors and differences among individuals. Sequence analysis of the PCR products showed that the smaller product lacks a 141 bp sequence within the PMP22 coding region.[40] The sequence deleted in the smaller products is indicated in the box within the whole sequence of the PMP22 cDNA shown in Fig. 2. Because the homologous region in human PMP22 is encoded by exon IV,[36] the RT-PCR result suggests that the genomic organization, including exon IV, is altered in the mutant allele.

To confirm the possibility raised by the RT-PCR study, Southern blot and PCR analysis were further performed on genomic DNA (Fig. 1C, D). Genomic DNA extracted from each genotype was digested by a Pst I restriction enzyme because exon IV of the PMP22 gene contains a site for Pst I. A cDNA probe (nucleotide position 58–580, Fig. 2) was hybridized with two fragments of 8.0 and 1.6 kb in the homozygote (lanes 2 and 3), and three fragments of 7.6, 2.2 and 1.6 kb in the normal mouse (lane 1). Four fragments of each size were detected in the heterozygote mouse (lane 4). The results of Southern hybridization explained each genotype of the mutant mouse and support the finding by RT-PCR in that the Tr-Ncnp allele has a deletion of a region containing the whole exon IV in the PMP22 gene. The length of the deleted sequence in the Tr-Ncnp allele is estimated about 1.8 kb by the length difference of fragments between normal (11.4 kb) and homozygous (9.6 kb) mice (Fig. 1C). For genomic PCR, intronic sequences flanking exon IV were partly determined using cassette ligation-mediated PCR (data not shown). A set of primers was designed from the obtained intronic sequence (5′-GCAGGAATGAATTGTTTCACCA-3′ and 5′-CATCCAATAGACCAATCACACAC-3′) and used for genomic PCR amplification to further prove the deletion of exon IV in the Tr-Ncnp allele. A PCR product of 358 bp in size was not detected in the homozygous (lane 2) mouse, while heterozygous and normal mice exhibited clear PCR products in lane 1 and 3 (Fig. 1D).

Pathological results

Hypomyelination was obvious in the peripheral nervous system of both the hind- and forelegs and the distal segments of the trigeminal nerves. Pathological

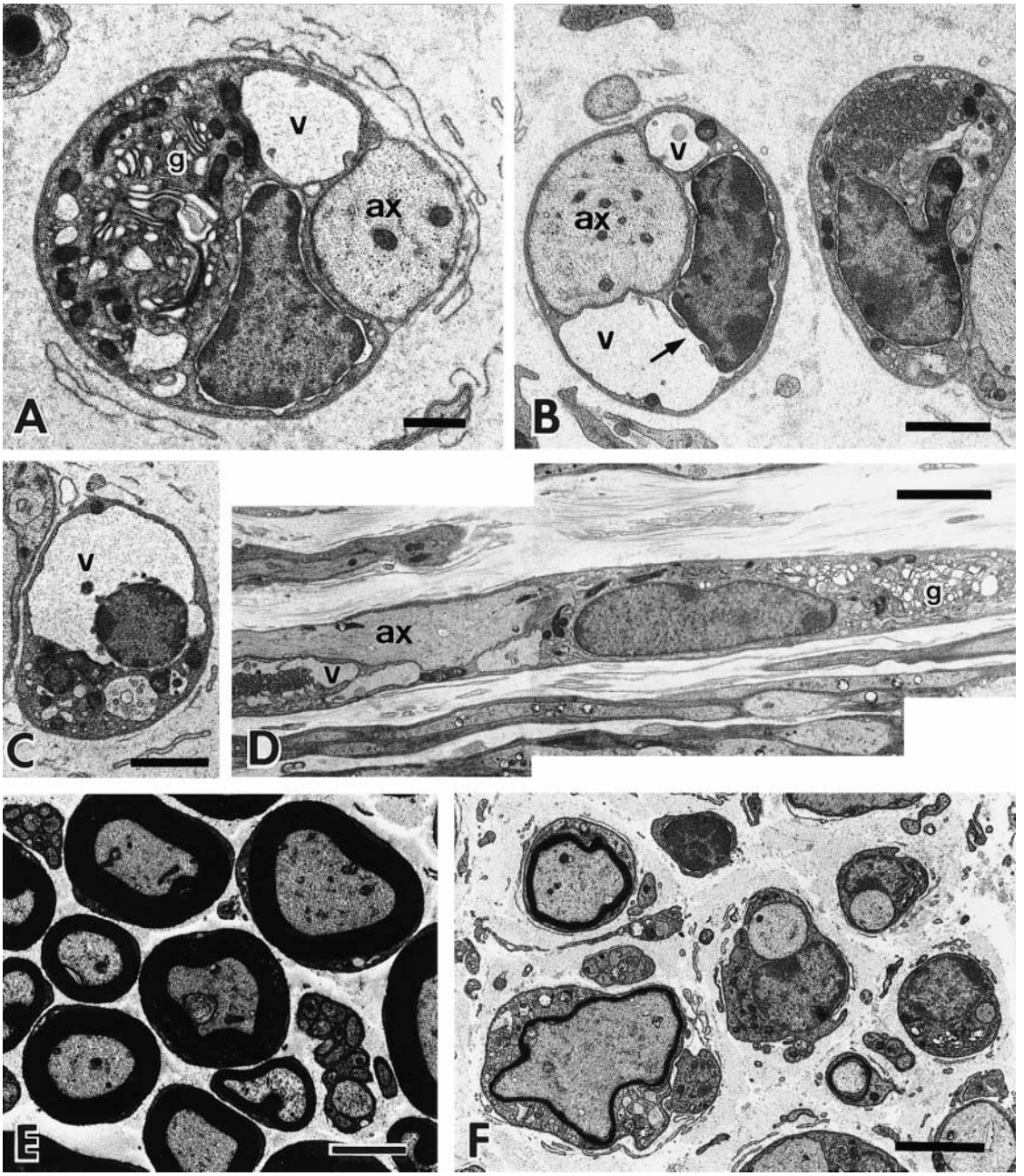

Fig. 3. Transverse sections of the sciatic nerve from five-month-old (A–C) homozygous, (F) heterozygous and (E) normal mice. A longitudinal section of homozygote is shown in (D). In the homozygous mouse, axons (ax) of the sciatic nerve have no myelin sheaths, while those of the heterozygote have incomplete and thin myelin sheaths. The Schwann cells in the homozygous mice had a heterochromatin-rich nucleus with a dark cytoplasm that contained a number of free polyribosomes, Golgi apparatuses (g) and mitochondria. Giant vacuoles (v) in the Schwann cells, were thought to be generated from rER, because they were frequently continuous with the limiting membranes of the rER near Golgi apparatuses and with the inner and outer nuclear membranes of nuclear envelope (arrow in B). A dark heterochromatin-rich pyknotic nucleus was not infrequently localized within a perinuclear space which extremely expanded (C). The amorphous substances and membranous complexes were found within some vacuoles (D). In the heterozygotes, the segmental demyelination and remyelination occurred along a single axon (F). Scale bars in A, B, C, D, E and F=1.0, 2.0, 2.0, 5.0, 5.0 and 5.0 μm, respectively.

alterations were not detected in the CNS. No myelination was found in the sciatic nerves in the homozygotes at postnatal day 9, one month and five months of age. Fig. 3A–C and Fig. 3E–F shows the ultramicroscopic pictures of transverse sections of the sciatic nerve from a five-month-old mouse.

In the homozygotes, the axonal development appeared to stay at the promyelin stage, with axons

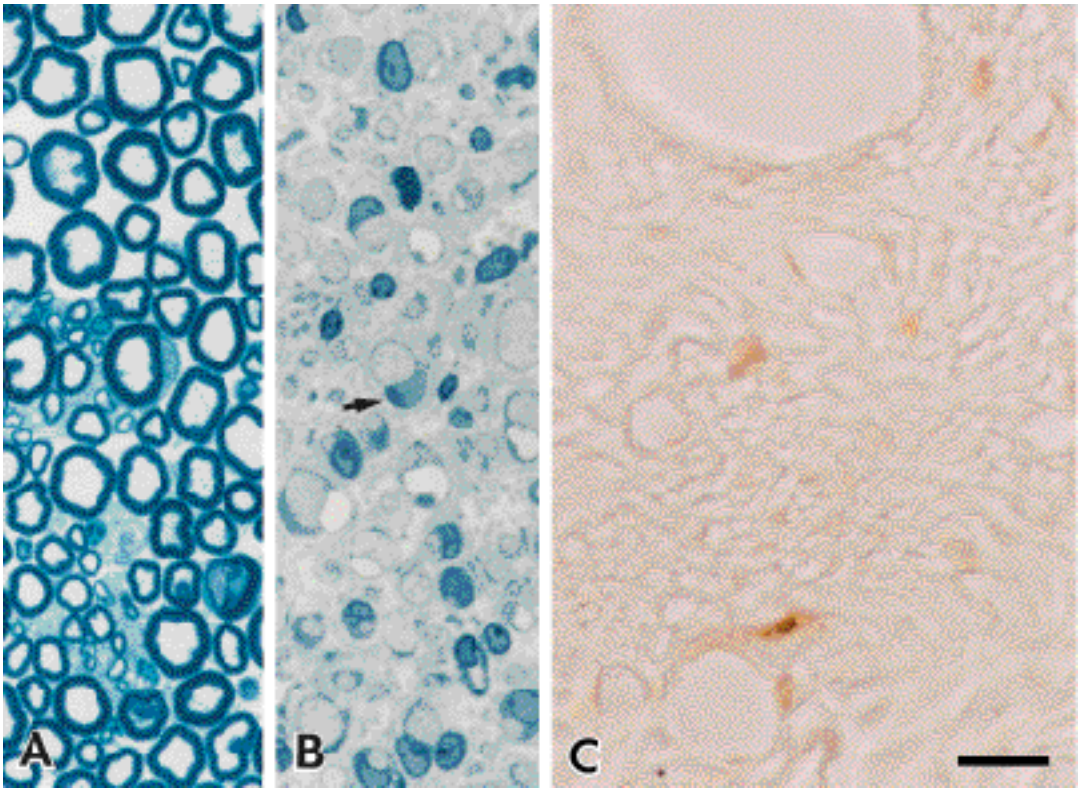

Fig. 4. The sciatic nerves from six-month-old homozygotes (B) contained a number of the Schwann cell nuclei which were pyknotic in nature and stained darker (arrow in B) than those from 12-month-old normal mice (A). Both pictures were from the Epoxy sections stained with Toluidine Blue. The *in situ* hybridization by the apoptosis detection kit demonstrated that peroxidase-positive cells in the sciatic nerve of homozygous Tr-Ncnp mouse were easily recognized from negatively-stained cells by their intense brown reaction product and the negative background staining. They were sparsely distributed across the sciatic nerve (C). Scale bar in C, indicating 10.0 μm, corresponds to A and B.

ensheathed but not myelinated by the Schwann cell projections. Occasionally, the Schwann cells with a dark nucleus and developed Golgi apparatuses had a large population of free ribosomes (Fig. 3A, B). Electron microscopic observations revealed the presence of many swollen vacuolar structures of various sizes which were localized near Golgi apparatus (Fig. 3A–D). They were often continuous with the limiting membranes of the rough endoplasmic reticulum (rER) near Golgi apparatuses. Large vacuoles were encircled with thin cytoplasmic processes of the Schwann cells, and were likely generated from the swelling of rER, since the limiting membrane of some vacuoles was continuous with the inner and outer nuclear membranes of nuclear envelope (Fig. 3B, C). A dark heterochromatin-rich nucleus was not infrequently localized within a perinuclear space which extremely expanded (Fig. 3C). The vacuoles were usually electron-lucent inside but occasionally contained closely packed membrane complexes and amorphous substances (Fig. 3D). Although many smaller vacuoles were also found near Golgi apparatuses, they tended not to have any contents of appreciable density (Fig. 3A, D). The collagen fibrils in the homozygotes were less abundant in the intercellular spaces compared with those in the heterozygotes (Fig. 3F).

In the heterozygote, the myelin deficit was variable along individual fibres (segmental demyelination), even within a single internode. The Schwann cells had a dark cytoplasm that contained a number of free polyribosomes, Golgi apparatuses and mitochondria. The compaction of myelin membrane was normal. The vacuoles in the Schwann cells were not so obvious as those in the homozygotes. The collagen fibrils in the intercellular spaces increased their density, and segmented basal lamella irregularly surrounded the axons in the heterozygote (Fig. 3F). In the sciatic nerve of the normal littermate, almost all axons were myelinated with regularly compacted myelin sheaths, except for smaller unmyelinated fibres, and the Schwann cells surrounded the axons with their fine projection (Fig. 3E).

The sciatic nerve of the homozygotes contained a number of the Schwann cell nuclei which were pyknotic in nature and stained darker than those in the normal mice (Fig. 4A, B). The *in situ* labelling by the apoptosis detection kit was applied only in adult

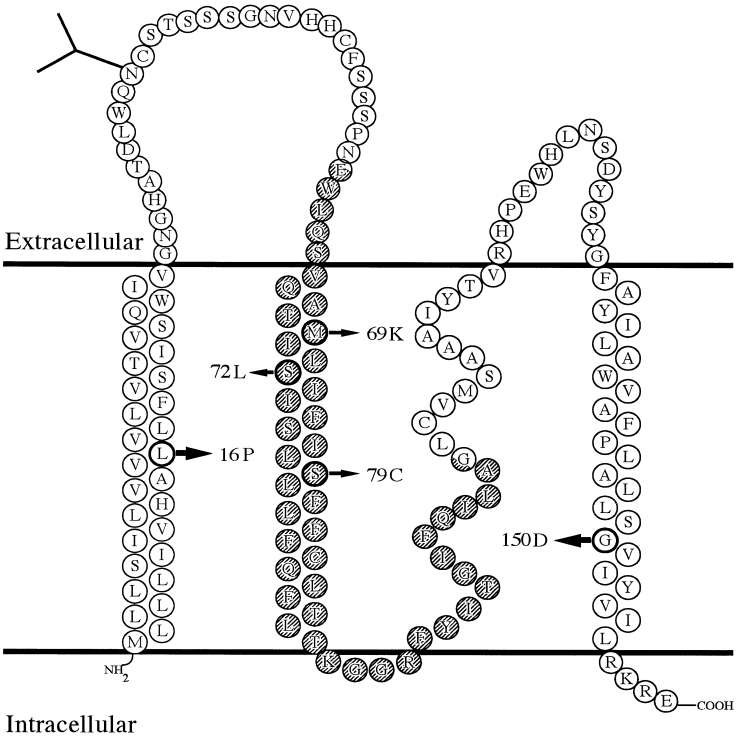

Fig. 5. Membrane topological model of PMP22 protein. A proposed membrane topology model for the PMP22 protein is illustrated,[34] with the potential glycosylation site (indicated as Y) and putative transmembrane domains. The deleted region in the gene is hatched. Thick arrows represent the substitution in *Trembler*-J (Leu to Pro at 16) and *Trembler* (Gly to Asp at 150) mutant mice. Thin arrows represent the reported mutations in humans. Substitutions of Met to Lys at 69 and Ser to Leu at 72 are associated with the DSS syndrome. Substitution of Ser to Cys at 79 is related to CMT1A. A point mutation identical to one found in *Trembler*-J is also reported in a case of CMT1A.

and homozygous mice, since it is known that the apoptotic glial cells increase to some extent in the CNS of the young normal mice,[26] and the chromatin condensation and vacuolar formation were not obvious in heterozygous sciatic nerve. Peroxidase-positive cells in the sciatic nerve of homozygous Tr-Ncnp mouse were easily recognized from negatively stained cells by their intense brown reaction product and the negative background staining. They were sparsely distributed across the sciatic nerve (Fig. 4C). The density of labelled nuclei in a cross section of the sciatic nerve was approximately 7.9%. No positive cells were observed in the normal sciatic nerve.

DISCUSSION

The Tr-Ncnp allele encodes a short PMP22 (113 amino acids in length) because of an in-frame deletion of the sequence coded by exon 1V. The short PMP22 appeared to be basically equivalent to mutated PMP22 produced by missense mutations in other Tr and Tr-J mice in that the genotype responsible for the trembler is inherited dominantly, and the myelin deficits in the sciatic nerve are obvious.[7,8,13] However, our mutant mice were distinct from the two mutant strains in some aspects. First, the Tr-Ncnp mouse had an exon IV deletion in PMP22 gene, whereas other two mutants have point mutations in their PMP22 gene. Second, the Schwann cells of Tr-Ncnp mice failed to myelinate their axons and occasionally contained a number of vacuolar structures of various sizes.

A proposed model for PMP22 has four evolutionary conserved putative transmembrane domains (TM1–TM4).[34,35] In Tr-Ncnp mice, the lack of the whole TM2 to lower half of the TM3 in PMP22 protein was due to the deletion of the PMP22 gene (Fig. 5). The substitutions Leu16Pro and Gly150Asp, which were predicted by the point mutations associated with Tr-J and Tr allele, occurred in distinct parts from a deletion site of PMP22 gene in Tr-Ncnp mice.[33,34] Although the identical mutation to that in Tr-Ncnp allele was not yet found in humans, a 5′ splice site mutation around exon IV of the PMP22 gene has recently been identified to cause severe CMT1A.[16]

The data from Tr-Ncnp mice suggest that abnormal PMP22, associated with the lack of TM2 and a part of TM3, is responsible not only for the myelin deficiency but also for the abnormal vacuolar formation, which might be caused by an excess production of aberrant PMP22 within the Schwann cells. The abnormal protein encoded by the Tr-Ncnp allele has a toxic gain-of function, because the truncated

protein may not be able to integrate correctly into the myelin membrane, or it may interfere with the interactions of PMP22 protein with itself or other myelin proteins. This speculation is reinforced by the fact that the normal PMP22 derived from a normal allele of heterozygous Schwann cells could not fully correct the abnormal trembler phenotypes, leading to the segmental demyelination and the active proliferation of the Schwann cells along a single axon. Furthermore, the knockout mice that completely lack PMP expression showed a delay in myelination and followed by focal hypermyelination and myelin degeneration.[1] Again, the morphological alterations characteristic of the Schwann cell death as was observed in Tr-Ncnp mice, have not been identified in the knockout mouse.

To date, it has generally been accepted that PMP22 plays a major role in maintaining the structural integrity of myelin and the cell adhesion.[28,29,30,36] The expression of PMP22 gene is observed outside the nervous tissue and controlled by alternative promoters driving the expression of different PMP22 mRNA species in tissue-specific manners, but most elevated mRNA levels are detectable in the Schwann cells of the adult PNS.[36] Although the precise pathophysiological function of PMP22 is not clear yet, recent studies have raised the possibility that the PMP22 gene is involved in apoptosis and acts as a negative modulator of the Schwann cell growth.[3,41] The PMP22 genes with point mutations for CMTA1 and Tr mouse, were overexpressed in cultured NIH-3T3 fibroblasts in which mutated genes behaved as dominant negative fashion with respect to the wild-type.[3] Those cells demonstrated apoptotic changes characterized by membrane blebbing, rounding up, and chromatin condensation.[3] However, an apoptotic cell death has not been confirmed by another study in which the PMP22 gene was overexpressed in the rats and cultured Schwann cells purified from three-day-old rats.[25] Although the pathophysiological mechanism has not been evaluated in transgenic animals carrying mutated PMP22 gene, we anticipate that the truncated PMP22 protein derived from the Tr-Ncnp allele with an in-frame deletion including exon IV of the PMP22 gene, alters the normal function of PMP22 in the Schwann cells, leading to the arrest of the Schwann cell maturation and subsequent cell death. We would further speculate that the aberrant PMP22 proteins derived from Tr and Tr-J mutant allele, were able to travel along the pathway transporting newly synthesized proteins from the ER via Golgi apparatus to the Schwann cell surface.

The biological functions of PMP22 can also be postulated by comparing the predicted structure of PMP22 to proteolipid protein (PLP) in the oligodendrocytes in the CNS.[28,30,35,37] The effects of various mutations in a PLP gene on myelin function have been investigated.[5] The *jimpy* mutant mouse has been characterized to be a defect in PLP mRNA by aberrant RNA processing. This mutation leads to an incorrectly spliced RNA transcript, which is the primary defect of this genetic disorder.[9,14,15] It has been reported that the vacuolar formation in the oligodendrocytes of *jimpy* mice is due to the overexpression of such aberrant PLP,[12] which cause abnormal swelling of rER, while the functional impairment of Golgi apparatus was not obvious in these mutant mice.[5,22,24] It was thought that oligodendrocytes in *jimpy* mice are unable to transport the protein beyond the rER compartment.[5,22]

The vacuolar structures in Tr-Ncnp mice were, however, larger in both size and number than those observed in *jimpy* mice and the myelin deficient rat.[11,22] They were generated mostly from the swelling of rER in the Schwann cells. The Golgi vesicles were not primarily involved in the vacuolar formation in the Schwann cells of Tr-Ncnp, because the vesicles localizing around the stacks of Golgi cisternae, were empty inside and not so larger than those derived from rER. Electron microscope clearly demonstrated the characteristic signs of cell death, showing condensed pyknotic nuclei with dark cytoplasm containing the swollen rER. Moreover, as in the case of *jimpy* mice, an immunocytochemical study in Tr-Ncnp mice using the TUNEL method detected a classical feature of apoptotic Schwann cells.[26] Taken together, the available genetic data indicates that PMP22 might not function only in compact myelin but also may play some important role in the normal function of the secretory transport mechanism within the cytoplasm of the Schwann cells.

CONCLUSION

The present study suggests that an exon IV deletion of the PMP22 gene is responsible for the lack of TM2 and a half of TM3 transmembrane domain in the PMP22 protein, leading to an arrest in the Schwann cell maturation and a vacuolar formation within the cytoplasm of the Schwann cells. Hypomyelination of the Schwann cells was obvious in the sciatic nerve of the homozygotes and, to a lesser degree, in the heterozygotes. The vacuolar structures of various sizes likely arise from the rER but not from Golgi apparatus. The apoptotic cell death occurs subsequently to these intracellular morphological changes in the Schwann cells. These results indicate that PMP22 has a dual biological function for both myelin formation and the secretory transport of PMP22 itself and other proteins.

Acknowledgements—The authors thank Dr Y. Takeda from Department of Physiology, Keio University School of Medicine, for helpful advice and discussion, and Mrs M. Shikama for the breeding and care of animals. This study was partially supported by grants from the Ministry of Health and Welfare of Japan, the Human Science Foundation, the Ministry of Education, Science and Culture of Japan, the Science and Technology Agency of Japan, and the Japan Foundation for Neuroscience and Mental Health.

REFERENCES

1. Adlkofer K., Martini R., Aguzzi A., Zielasek J., Toyka K. V. and Suter U. (1995) Hypermyelination and demyelinating peripheral neuropathy in Pmp22-deficient mice. *Nature Genet.* **11,** 274–280.
2. Chance P. F., Alderson M. K., Leppig K. A., Lensch M. W., Matsunarni M., Smith B., Swanson P. D., Odelberg S. J., Disteche C. M. and Bird T. M. (1993) DNA deletion associated with hereditary neuropathy with liability to pressure palsies. *Cell* **72,** 143–151.
3. Fabbretti E., Edomi P., Brancolini C. and Schneider C. (1995) Apoptotic phenotype induced by overexpression of wild-type gas3/PMP-22: its relation to the demyelination peripheral neuropathy CMT1A. *Gene Develop.* **9,** 1846–1856.
4. Gabreels-Festen A. and Gabreels F. (1993) Hereditary demyelinating motor and sensory neuropathy. *Brain Path.* **3,** 135–146.
5. Gow A., Friedrich V. L. Jr and Lazzarini R. A. (1994) Many naturally occurring mutations of myelin proteolipid protein impair its intracellular transport. *J. Neurosci. Res.* **37,** 574–583.
6. Harding A. E. (1995) From the syndrome of Charcot, Marie and Tooth to disorders of peripheral myelin proteins. *Brain* **118,** 809–818.
7. Henry E. W., Cowen J. S. and Sidman R. L. (1983) Comparison of trembler and tremblers phenotypes: varying severity of peripheral hypomyelination. *J. Neuropath. exp. Neurol.* **42,** 688–706.
8. Henry E. W. and Sidman R. L. (1988) Long lives for homozygous trembler mutant mice despite virtual absence of peripheral nerve myelin. *Science* **241,** 344–346.
9. Hudson L. D., Berndt J. A., Puckett C., Kozak C. A. and Lazzarini R. (1987) Aberrant splicing of proteolipid protein mRNA in the dysmyelinating *jimpy* mutant mouse. *Proc. natn. Acad. Sci. U.S.A.* **84,** 1454–1458.
10. Ichihara N., Wu J., Chui D., Yamazaki K., Wakabayashi T. and Kikuchi T. (1995) Axonal degeneration promotes abnormal accumulation of amyloid β protein in ascending gracile tract of gracile axonal dystrophy (GAD) mouse. *Brain Res.* **695,** 173–178.
11. Jackson K. F. and Duncan I. D. (1988) Cell kinetics and cell death in the optic nerve of the myelin deficient rat. *J. Neurocytol.* **17,** 657–670.
12. Kagawa T., Ikenaka K., Inoue Y., Kuriyama S., Tsujii T., Nakao J., Nakajima K., Aruga J., Okano H. and Mikoshiba K. (1994) Glial cell degeneration and hypomyelination caused by overexpression of myelin proteolipid protein gene. *Neuron* **13,** 427–442.
13. Low P. A. (1976) Hereditary hypertrophic neuropathy in the trembler mouse. *J. neurol. Sci.* **30,** 343–368.
14. Morello D., Dautigny A., Pham-Dinh D. and Jollès P. (1986) Myelin proteolipid protein (PLP and DM-20) transcripts are deleted in *jimpy* mutant mice. *Eur. molec. Biol. Org. J.* **5,** 3489–3493.
15. Nave K.-A., Lai C., Bloom F. E. and Milner R. J. (1986) *Jimpy* mutant mouse: a 74-base deletion in the mRNA for myelin proteolipid protein and evidence for a primary defect in RNA splicing. *Proc. natn. Acad. Sci. U.S.A.* **83,** 9264–9286.
16. Nelis E., Timmerman V., De Jonghe P. and Von Broeckhoven C. (1994) Identification of a 5′ splice site mutation in the PMP-22 gene in autosomal dominant Charcot–Marie–Tooth disease type l. *Human molec. Genet.* **3,** 515–516.
17. Nicholson G. A., Valentin L. J., Cherryson A. K., Kennerson M. L., Bragg T. L., DeKroon R. M., Rose D. A., Pollard J. D., McLeod J. G., Bolhuis P. A. and Bass A. (1994) A frame shift mutation in the PMP22 gene in hereditary neuropathy with liability to pressure palsies. *Nature Genet.* **6,** 263–266.
18. Patel P. I. (1993) Charcot–Marie–Tooth disease type 1A: mutational mechanisms and candidate gene. *Curr. Opin. Genet. Develop.* **3,** 438–444.
19. Roa B. B., Dyck P. J., Marks H. G., Chance P. F. and Lupski J. R. (1993) Dejerine–Sottas syndrome associated with point mutation in the peripheral myelin protein 22 (PMP22) gene. *Nature Genet.* **5,** 269–273.
20. Roa B. B., Garcia C. A., Pentao L., Killian J. M., Trask B. J., Suter U., Snipes G. J., Ortiz-Lopez R., Shooter E. M., Patel P. I. and Lupski J. R. (1993) Evidence for a recessive PMP22 point mutation in Charcot–Marie–Tooth disease type 1A. *Nature Genet.* **5,** 189–194.
21. Roa B. B., Garcia C. A., Suter U., Kulpa D. A., Wise C. A., Muller J., Welcher A. A., Snipes G. J., Shooter E. M., Patel P. I. and Lupski J. R. (1993) Charcot–Marie–Tooth disease type 1A; association with a spontaneous point mutation in the PMP-22 gene. *New Engl. J. Med.* **329,** 96–101.
22. Roussel G., Neskovic N. M., Trifilieff E., Artault J.-C. and Nussbaum J.-L. (1987) Arrest of proteolipid transport through the Golgi apparatus in *Jimpy* brain. *J. Neurocytol.* **16,** 195–204.
23. Sambrook J., Fritsch E. F. and Maniatis T. (1989) *Molecular Cloning: A Laboratory Manual*: 2nd edn, Cold Spring Harbor Lab., Cold Spring Harbor, New York.
24. Schneider A., Griffiths I. R., Readhead C. and Nave K.-A. (1995) Dominant-negative action of the *jimpy* mutation in mice complemented with an autosomal transgene for myelin proteolipid protein. *Proc. natn. Acad. Sci. U.S.A.* **92,** 4447–4451.
25. Sereda M., Griffiths I., Rühlhofer A., Stewart H., Rossner M. J., Zimmermann F., Magyar J. P., Schneider A., Hund E., Meinck R.-M., Suter U. and Nave K.-A. (1996) A transgenic rat model of Charcot–Marie–Tooth disease. *Neuron* **16,** 1049–1060.
26. Skoff R. P. (1995) Programmed cell death in the dysmyelinating mutants. *Brain Path.* **5,** 283–288.
27. Skre H. (1974) Genetic and clinical aspects of Charcot–Marie–Tooth's disease. *Clin. Genet.* **6,** 98–118.
28. Sidman R. L., Dickle M. M. and Appel S. H. (1964) Mutant mice (quaking and jimpy) with deficient myelination in the central nervous system. *Science* **144,** 309–311.
29. Snipes G. J., Suter U. and Shooter E. M. (1993) Human peripheral myelin protein-22 carries the L2/HNK-1 carbohydrate adhesion epitope. *J. Neurochem.* **61,** 1961–1964.
30. Snipes G. J. and Suter U. (1995) Molecular basis of common hereditary motor and sensory neuropathies in humans and in mouse models. *Brain Path.* **5,** 233–247.
31. Snipes G. J. and Suter U. (1995) Molecular anatomy and genetics of myelin protein in the peripheral nervous system. *J. Anat.* **186,** 483–494.
32. Suh J.-G., Yamanishi T., Matsui K., Tanaka K. and Wada K. (1995) Mapping of the gracile axonal dystrophy (gad) gene to a region between D5Mit197 and D5Mit113 on proximal mouse chromosome 5. *Genomics* **27,** 549–551.

33. Suter U., Moscow J. J., Welcher A. A., Snipes G. J., Kosaras B., Sidman R. L., Buchberg A. M. and Shooter E. M. (1992) A leucine-to-proline mutation in the putative first transmembrane domain of the 22 kDa peripheral myelin protein in the tremblers mouse. *Proc. natn. Acad. Sci. U.S.A.* **89,** 4382–4386.
34. Suter U., Welcher A. A., Ozcelik T., Snipes G. J., Kosaras B., Francke U., Billings G. S., Sidman R. L. and Shooter E. M. (1992) Trembler mouse carries a point mutation in a myelin gene. *Nature* **356,** 241–244.
35. Suter U., Welcher A. A. and Snipes G. J. (1993) Progress in the molecular understanding of hereditary peripheral neuropathies reveals new insights into the biology of the peripheral nervous system. *Trends neurol. Sci.* **16,** 50–56.
36. Suter U., Snipes G. J., Schoener-Scott R., Welcher A. A., Pareek S., Lupski J. R., Murphy R. A., Schooter E. M. and Patel P. I. (1994) Regulation of tissue-specific expression of alternative peripheral myelin protein-22 (PMP-22) gene transcripts by two promoters. *J. biol. Chem.* **269,** 25795–25808.
37. Suter U. and Snipes G. J. (1995) Peripheral myelin protein 22, facts and hypotheses. *J. Neurosci. Res.* **40,** 145–151.
38. Takayama Y., Takada F., Takahashi A. and Kawakami M. (1994) A 100 kDa protein in the C4-activating component of Ra-reactive factor is a new serine protease having module organization similar to C1r and C1s. *J. Immunol.* **152,** 2308–2316.
39. Valentijn L. J., Bass F., Wolterman R. A., Hoogendijk J. E., van den Bosch N. H. A., Zorn I., Gabreels-Festen A. A. W. M., de Visser M. and Bolhuis P. A. (1992) Identical point mutations of PMP-22 in Trembler-J mouse and Charcot–Marie–Tooth disease type 1A. *Nature Genet.* **2,** 288–291.
40. Welcher A. A., Suter U., De Leon M., Snipes G. J. and Shooter E. M. (1991) A myelin protein is encoded by the homologue of a growth-arrest specific gene. *Proc. natn. Acad. Sci. U.S.A.* **88,** 7195–7199.
41. Zoidl G., Blass-Kampmann S., D'Urso D., Schmalenbach C. and Muller H. W. (1995) Retroviral-mediated gene transfer of the peripheral myelin protein PMP22 in Schwann cells: modulation of cell growth. *Eur. molec. Biol. Org. J.* **14,** 1122–1128.

(*Accepted* 10 *December* 1996)