The gene for the peripheral myelin protein PMP-22 is a candidate for Charcot-Marie-Tooth disease type 1A

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Charcot-Marie-Tooth disease type 1A (CMT1A) is an autosomal dominant peripheral neuropathy associated with a large DNA duplication on the short arm of human chromosome 17. The *trembler* (*Tr*) mouse serves as a model for CMT1A because of phenotypic similarities and because the *Tr* locus maps to mouse chromosome 11 in a region of conserved synteny with human chromosome 17. Recently, the peripheral myelin gene *Pmp-22* was found to carry a point mutation in *Tr* mice. We have isolated cDNA and genomic clones for human *PMP-22*. The gene maps to human chromosome 17p11.2–17p12, is expressed at high levels in peripheral nervous tissue and is duplicated, but not disrupted, in CMT1A patients. Thus, we suggest that a gene dosage effect involving *PMP-22* is at least partially responsible for the demyelinating neuropathy seen in CMT1A.

Charcot-Marie-Tooth disease (CMT)[1–3] with a prevalence rate of 1 in 2,500[4] is the most common inherited peripheral neuropathy in humans involving both motor and sensory nerves. The common subtype, CMT type 1A (CMT1A), is characterized clinically by distal muscle atrophy and weakness, decreased nerve conduction velocity (NCV) and hypertrophic neuropathy[2]. Genetically, CMT1A is inherited in an autosomal dominant fashion and is associated with a submicroscopic duplication involving more than 1 megabase (Mb) of sequence on the short arm of chromosome 17(refs 3, 5–8). Electrophysiological examination of a patient with an interstitial duplication of most of 17p revealed the abnormal nerve conduction velocity associated with CMT, and molecular analysis showed that sequences duplicated in CMT1A patients are contained entirely within the large duplication present in this patient[5]. These data support a gene dosage model as a mechanism for CMT1A involving over-expression of one or more genes mapping within the duplicated region.

The allelic *trembler* (*Tr*)[9] and *trembler-J* (*Tr*^{J})[10] mouse mutations map to chromosome 11 in a region of conserved synteny with human chromosome 17p[11–13]. Recently, these mouse strains have been shown to carry point mutations in two distinct putative membrane-associated domains of a potentially growth-regulating 22 kD protein, peripheral myelin protein-22 (PMP-22)[14,15]. PMP-22 protein is expressed by Schwann cells and is localized mainly in compact peripheral nervous system (PNS) myelin[16]. Thus, as likely consequences of defects in PMP-22, *Tr* and *Tr*^{J} exhibit severe myelin deficiencies in the peripheral nervous system and continued Schwann cell proliferation throughout life[10,17,18]. Given the resemblance of these features to the neuropathology seen in CMT1A patients, as well as the conserved syntenic group of genes on mouse chromosome 11 and proximal short arm of human chromosome 17, we proposed that mutations affecting the *PMP-22* gene might be responsible for CMT1A and related peripheral neuropathies[14]. We describe in this report the cloning of a human *PMP-22* cDNA and genomic sequences spanning the *PMP-22* gene. We also demonstrate that the *PMP-22* gene maps within the duplication interval in CMT1A patients close to the marker D17S122 (VAW409) which had been previously associated with the CMT1A duplication[3,6].

Cloning of a human *PMP-22* cDNA

A human *PMP-22* cDNA was cloned and analysed by a combination of cDNA library screening and PCR technology (Fig. 1*a*). The longest open reading frame of the human *PMP-22* cDNA predicts a polypeptide of 160 amino acids, identical in length to the previously characterized mouse and rat PMP-22 proteins. Comparison of deduced amino acid sequences of human, rat, mouse and bovine PMP-22 proteins indicates a very high degree of evolutionary conservation (Fig. 1*b*). There is 87% identity between human and rat PMP-22 and 86% identity between human and mouse PMP-22. In particular, a consensus sequence for N-linked glycosylation (amino acid position 41), shown to be functional *in vivo*[19] and *in vitro*[20,21], is conserved in all four species. Strikingly, predicted membrane-associated regions of the PMP-22 protein are especially highly conserved, suggesting a possible functional role of these domains.

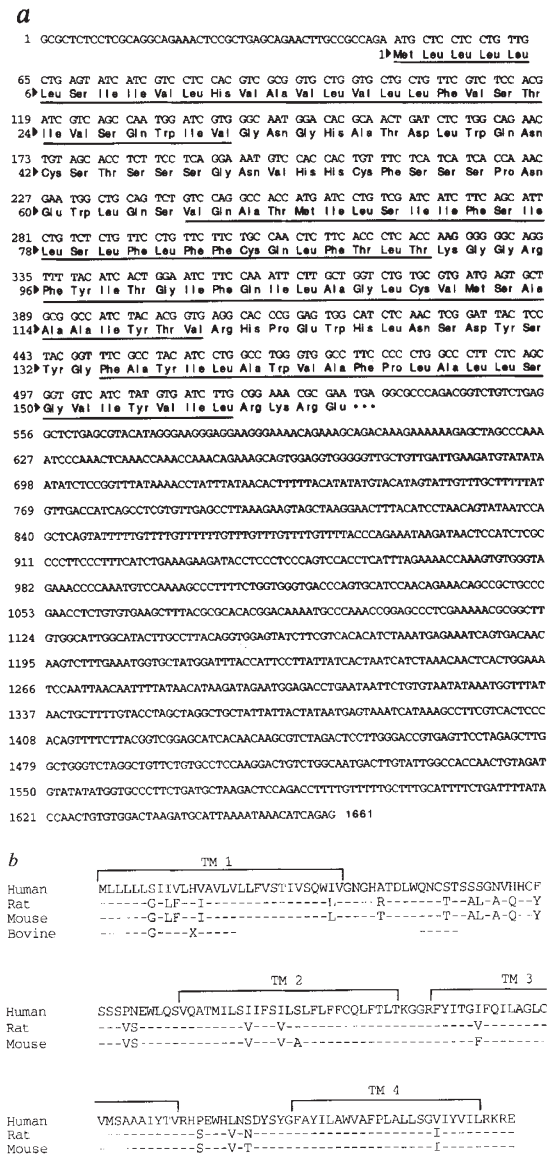

Fig. 1 Human *PMP-22* sequence and homology. *a*, Nucleotide sequence of a human *PMP-22* cDNA and predicted amino acid sequence of the human PMP-22 protein. Plain numbers refer to the nucleotide sequence, while numbers next to arrows refer to the amino acid sequence. Putative membrane-spanning domains are underlined. The cDNA sequence shown represents a consensus sequence of partial, overlapping clones isolated by screening of a human brain cDNA library (Stratagene) and RT-PCR carried out with human dorsal root ganglia (DRG) RNA using oligonucleotides based on the rat PMP-22/SR13 nucleotide sequence[21]. The DNA sequence of the entire human *PMP-22* cDNA coding region was confirmed by partial sequencing of the corresponding genomic *PMP-22* gene (see Fig. 4) as well as cDNA clones obtained by RT-PCR (DRG-derived RNA) using human specific *PMP-22* oligonucleotides specific for the 5' and 3' untranslated regions (GenBank accession number M94048). *b*, Comparison of the PMP-22 amino acid sequences of human, rat, mouse and bovine. The human, rat and mouse amino acid sequences are derived from cloned cDNA sequences[14,21], while the partial bovine sequence represents direct amino acid sequencing from two regions of the PASII protein[19,21]. Amino acid residues which differ from the human sequence are indicated. Identical amino acids are represented by hyphens. 'X' in the bovine sequence identifies an undetermined amino-acid residue. 'TM' refers to putative transmembrane domains.

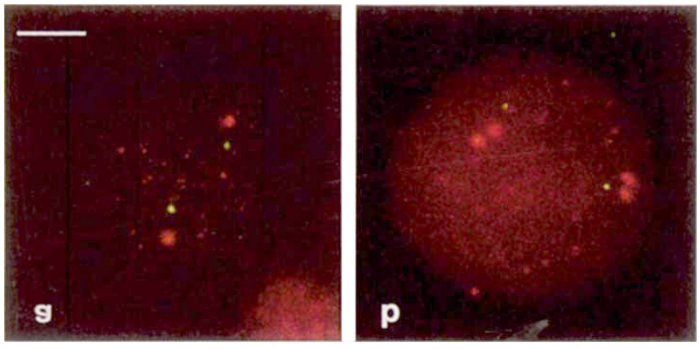

Fig. 5 Fluorescence *in situ* hybridization analysis of *PMP-22* in control and CMT1A individuals. Interphase nuclei were prepared from lymphoblastoid lines of a control individual, HOU76-289[3] (*a*) and from a CMT1A patient, HOU42-333 who is homozygous for the duplication mutation[44] (*b*). Nuclei were hybridized simultaneously with biotinylated cosmid c132-G8 representing a portion of the *PMP-22* gene and digoxigenin-labelled cosmid c1516, which maps to 17p11.2, as described previously[46]. The hybridization sites of c132-G8 and c1516 were labelled with Texas red and fluorescein, respectively and viewed together through a double band-pass filter. The hybridization pattern of c1516 was used as an internal assay for the replication status of the proximal 17p region. The nucleus in *b* was counterstained with propidium iodide. Scale bar, 5 μm.

Mapping *PMP-22* within the CMT1A duplication

To determine if *PMP-22* is a likely candidate for CMT1A, we determined its chromosomal location using somatic cell hybrid mapping panels. Preliminary PCR-based studies assigned *PMP-22* to chromosome 17, and specifically to the 17p11.2–17p12 region (data not shown). These results were confirmed by Southern analysis of *Hin*dIII-digested DNA from the somatic cell hybrid panel using the human *PMP-22* cDNA as a probe (Fig. 2). Two genomic *Hin*dIII fragments of 7.4 kilobase (kb) and 2.5 kb were detected in human DNA which were only present in somatic cell hybrids retaining the 17p11.2–17p12 region. In particular, the hybrids Hy254-1 and Hy357-2D(ref. 22) which have distal breakpoints in 17p11.2 and 17p12, respectively, played an important part in mapping *PMP-22* to the duplication region in CMT1A patients. The hybrid Hy357-2D is deleted for all of the markers duplicated in CMT1A patients[22] and did not retain human *PMP-22* sequences (lane 6, Fig. 2) whereas the hybrid Hy254-1, which contains the CMT1A region, retained these sequences (lane 5, Fig. 2).

Expression analysis of human PMP-22

The pattern of expression of the human *PMP-22* gene was determined by northern analysis of RNA from various human tissues using a cDNA probe, FBR1. Three transcripts of about 1.8, 1.3 and 0.8 kb were detected in the spinal cord and the femoral nerve (Fig. 3*a*). Trace amounts of the 1.8 kb transcript were found in the brain, and in the skeletal muscle and heart possibly due to innervation of the latter tissues. Hybridization of the same northern blot to the cDNA for the ubiquitously expressed glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene was used to confirm the integrity of the mRNA and to obtain a measure of the amounts of RNA loaded on the gel (Fig. 3*b*). This control experiment indicated underloading of the lane with the femoral nerve RNA sample and underlined the observation that *PMP-22* mRNA is abundantly expressed

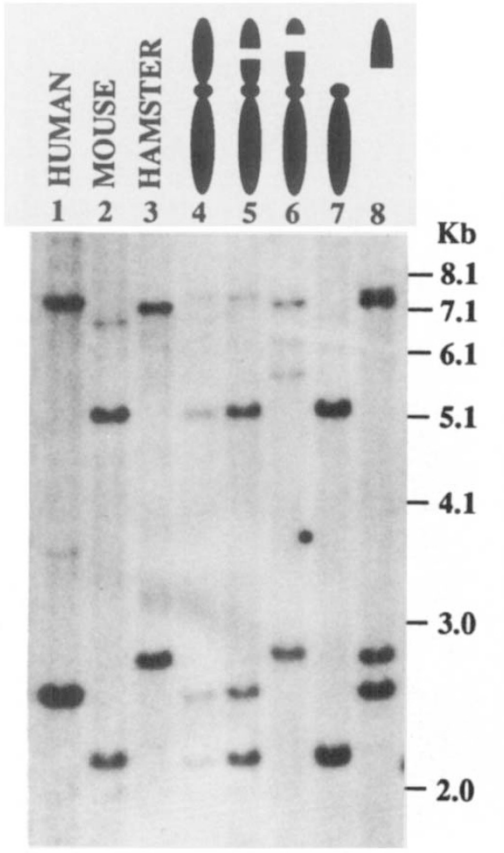

Fig. 2 Mapping of *PMP-22* to the 17p11.2 - 17p12 region. Genomic DNA from a human control individual, a mouse TK$^-$ cell line, Cl-1D, the hamster HPRT$^-$ cell line RJK88 and several somatic cell hybrids retaining portions of human chromosome 17 were digested with *Hin*dIII and the Southern blot hybridized to the cDNA probe phPMP22-1 as described previously[44]. Hybrid lanes are identified by schematic idiograms representing portions of chromosome 17 retained in the hybrids. Lane 1, 5 μg of human DNA; lanes 2 and 3, 10 μg of Cl-1D (mouse) and RJK88 (hamster) DNA, respectively; lanes 4–8, 10 μg of DNA from hybrids MH22-6, Hy254-1, Hy357-2D, LS-1 and 88H5, respectively. The migration of the molecular weight markers is shown on the right. Note the retention of the human *PMP-22* genomic *Hin*dIII fragments of 7.4 kb and 2.5 kb only in lanes 4, 5 and 8 but not in lanes 6 and 7.

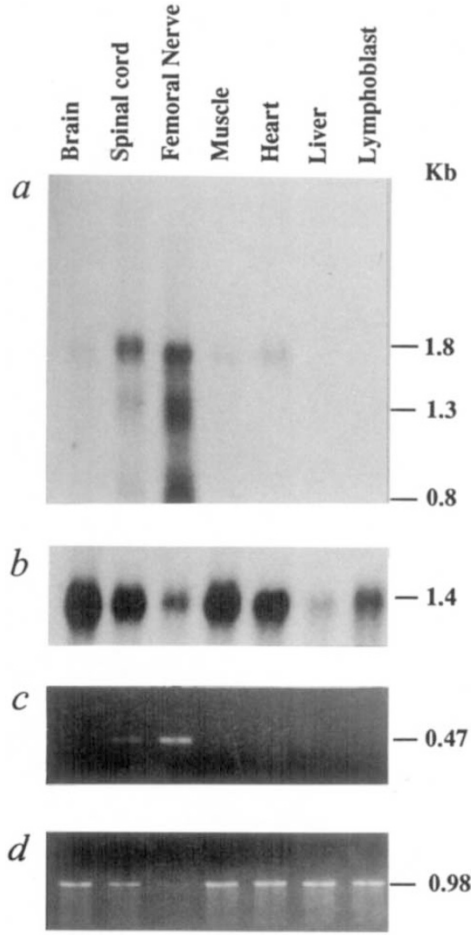

Fig. 3 Expression pattern of *PMP-22* in human tissues. *a*, Northern analysis of human RNAs with a *PMP-22* cDNA probe. Approximately 5 μg of total RNA from human brain, spinal cord, femoral nerve, skeletal muscle, heart, liver, and lymphoblasts was subjected to northern hybridization (see Methodology). *b*, The blot in *a* was stripped and probed with the cDNA for glyceraldehyde-3-phosphate dehydrogenase gene (G3PDH) as a control for the integrity and amounts of RNA in each lane. *c*, RT-PCR analysis of human RNAs using primers specific for the *PMP-22* cDNA. Approximately 0.5 μg of total RNA from human brain, spinal cord, femoral nerve, skeletal muscle, heart, liver and lymphoblasts was used for RT-PCR amplification (see Methodology). 20 μl out of 50 μl of the reaction products were electrophoresed on a 2% agarose gel. The expected RT-PCR product of 470 bp was detected using femoral nerve and spinal cord RNA as template. *d*, RT-PCR analysis was performed as noted in *c* using primers specific for the G3PDH cDNA. The expected RT-PCR product of 983 bp was noted in all tissues examined.

in this tissue. The expression patterns of *PMP-22* and *G3PDH* were further assayed by RT-PCR amplification of the *PMP-22* cDNA using the various RNAs as templates (Fig. 3*c* and 3*d*). These results confirm that *PMP-22* is expressed at high levels only in the peripheral nerve and spinal cord, identical to previous *PMP-22* expression studies in the rat[16,21].

PMP-22 genomic clones

To isolate genomic clones spanning *PMP-22*, a gridded cosmid library constructed from flow-sorted chromosome 17 was screened with several probes representing the *PMP-22* gene. Eight unique positively hybridizing cosmids were identified and used to establish an *Eco*RI restriction map of the *PMP-22* gene region (Fig. 4). Three *Eco*RI fragments of 11, 8.2 and 4.9 kb hybridized to the *PMP-22* cDNA. Parts of the 5' untranslated region of the gene were localized to the 11 kb *Eco*RI fragment by sequencing of the subclone p132-G8R1 containing this fragment (data not shown). A 4.5 kb *Eco*RI fragment immediately upstream of the 11 kb *Eco*RI fragment was also subcloned (p132-G8R5) and shown to retain a portion of the CpG-rich island associated with the *PMP-22* gene (see below).

Duplication of *PMP-22* in CMT1A

Fluorescence *in situ* hybridization (FISH) of interphase nuclei from CMT1A and control individuals was used

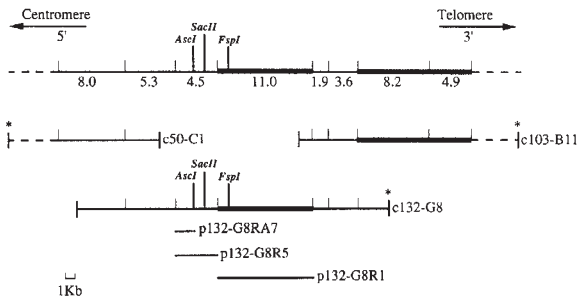

Fig. 4 Genomic clones for the *PMP-22* region. A cosmid contig was constructed for the *PMP-22* genomic region by identification of overlapping cosmids from a cosmid library constructed from chromosome 17. Fragments showing hybridization to the cDNA are indicated by bold lines. The thin vertical lines represent *Eco*RI sites and the asterisks indicate the position of the T3 polymerase promoter sequence in the vector. The names of the cosmids and subclones are indicated to the right of the line diagrams. The numbers below the uppermost line refer to *Eco*RI fragment sizes in kb. The dotted lines represent regions where the *Eco*RI restiction map is not available. The order of *Eco*RI restriction fragments at the 3' region of PMP-22 is tentative. The *Asc*I, *Sac*II and *Fsp*I sites indicated on the map represent the Cps island associated with the PMP-22 locus and are unmethylated in genomic DNA. The centromere-telomere orientations are based on the genetic map of chromosome 17(ref. 45).

previously to demonstrate the duplication in CMT1A patients[3]. The cosmid c132-G8 containing a portion of the 5' region of *PMP-22* and a control cosmid c1516 mapping outside the CMT1A duplication but within 17p11.2, were used for similar studies. Two-colour FISH analysis was performed on interphase nuclei from lymphoblasts of a control individual 76-289, and a severely affected CMT1A patient 42-333 previously shown to be homozygous for the duplication mutation[3]. The results show that the *PMP-22* sequences are present in one copy on each of the chromosomes 17 of the control individual (Fig. 5*a*) and in two copies on both chromosomes 17 of the homozygous CMT1A patient (Fig. 5*b*).

Dosage differences in *Msp*I restriction fragment length polymorphisms (RFLPs) associated with the markers VAW409R3 and VAW409R1 had been previously demonstrated in CMT1A patients[3,6,7]. To identify polymorphisms within *PMP-22* which might demonstrate similar dosage differences, DNA from 13 unrelated CMT1A patients and five control individuals were subjected to Southern analysis after digestion with *Ava*II, *Ban*I, *Ban*II, *Bcl*I, *Bgl*I, *Bgl*II, *Dra*I, *Eco*RV, *Hin*cII, *Mbo*I and *Rsa*I using the cosmid c132-G8 as a probe. A *Hin*cII RFLP was identified within the *PMP-22* gene. To ensure reliable discernment of dosage differences of the polymorphic alleles in CMT1A patients, double digests were performed with *Eco*RI and *Hin*cII to reduce the size of the polymorphic alleles. Polymorphic alleles of 11 kb and 9.6 kb were observed in *Eco*RI/*Hin*cII digested DNA hybridized to the p132-G8R1 fragment. Figure 6 shows a Southern analysis of *Eco*RI/*Hin*cII digested DNA from a CMT1A nuclear family with the probe p132-G8R1. Dosage differences of the polymorphic alleles are detected in CMT1A patients and not in unaffected individuals. Furthermore, the segregation of alleles demonstrates mendelian inheritance of a disease chromosome which carries both an A and a B allele in affected individuals of this particular family. This RFLP will be useful in conjunction with other RFLPs associated with markers within the duplication interval for the diagnosis of CMT1A patients with the duplication mutation.

Fine mapping of *PMP-22*

A novel *Sac*II fragment of about 500 kb was previously identified in CMT1A patients by pulsed field gel electrophoresis (PFGE), which was specific for the duplication and likely represents a junction fragment[3,7]. Two additional novel restriction fragments of 500 kb each are also detected by the restriction enzymes *Fsp*I[5] and *Asc*I (this study) only in CMT1A patients. The *Asc*I fragment is only evident in partially digested patient DNA. In order to determine the relative position of the *PMP-22* gene with respect to these novel restriction fragments observed in CMT1A patients, as well as the location of the gene within the physical map of the duplication interval, we performed PFGE on patient and control DNA (Fig. 7). The pattern of hybridization with the *PMP-22* third exon probe (Fig. 7*a*), as well as several other segments of *PMP-22* including a 10 kb *Fsp*I/*Eco*RI fragment from p132-G8R1 and the entire cosmid c103-B11 (data not shown) were similar and did not reveal any novel fragments in DNA from the CMT1A patient. However, when a 2.2 kb *Eco*RI/*Asc*I fragment (p132-G8RA7; Fig. 4) located immediately upstream of *PMP-22* was used as a probe, it detected the novel CMT1A-specific *Sac*II, *Fsp*I and *Asc*I fragments alluded to above (Fig. 7*b*). The same Southern blot was hybridized to the probe VAW409R3 (D17S122),

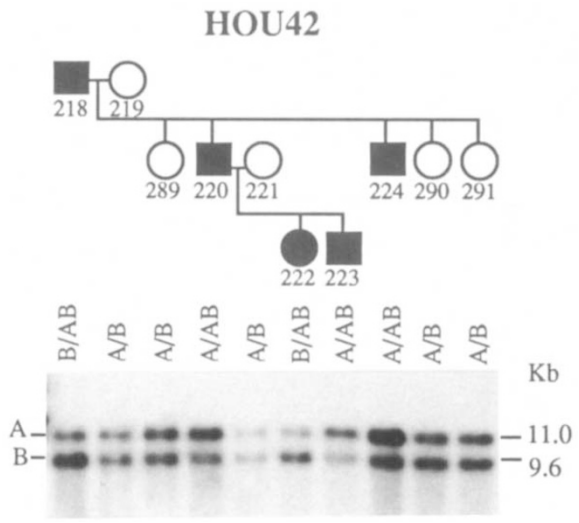

Fig. 6 Mendelian inheritance of dosage differences of polymorphic *PMP-22* alleles in a CMT1A nuclear family. Five µg of genomic DNA from individuals within a nuclear family (HOU42) of CMT1A patients[44] was digested with the enzymes *Hin*cII and *Eco*RI and Southern analysis performed with the probe p132-G8R1 shown in Fig. 4, after preassociation of repeats[44]. The polymorphic site is a *Hin*cII site. The members of the nuclear family are indicated above the autoradiograph. The genotypes are indicated below the pedigree, with the slash separating the pair of alleles segregating with CMT1A in the family. Note the difference in the relative intensity of alleles A and B in CMT1A patients 218, 220, 222, 223, and 224 versus unaffected individuals 219, 289, 221, 290 and 291.

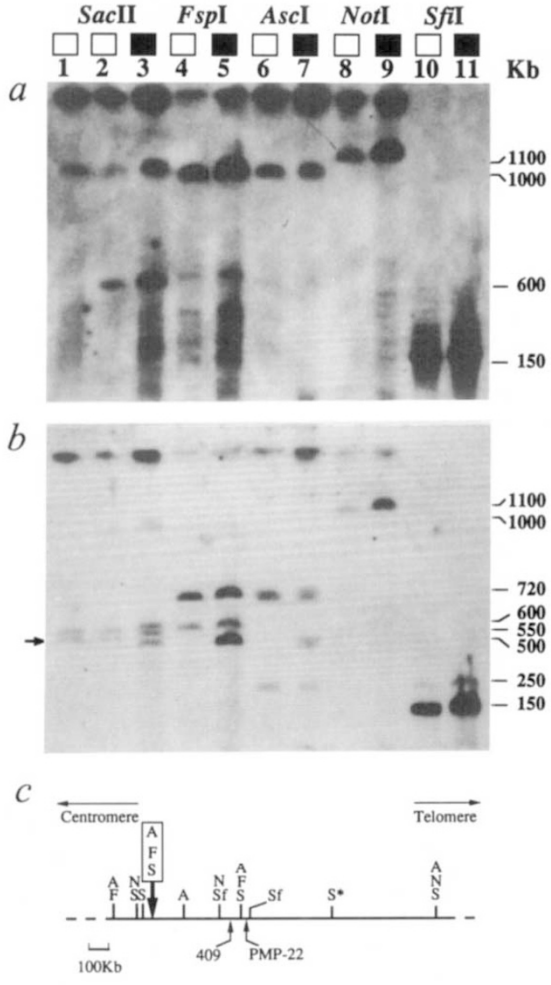

Fig. 7 Pulsed field analysis of control and CMT1A individuals maps *PMP-22* within the CMT1A duplication region. *a, b*, Pulsed field analysis was performed as described previously[3] on genomic DNA from lymphocytes of control and CMT1A individuals with the probe p103-B11RH4 (*a*) and with the probe p132G8-RA7 (*b*). p103-B11RH4 is a 3.7 kb *Eco*RI/*Hin*dIII fragment containing the third exon of *PMP-22* which was subcloned from within the 8.2 kb *Eco*RI fragment of c103-B11 and p132G8-RA7 is a 2.2 kb *Eco*RI/*Asc*I fragment from cosmid c132-G8 (see Fig. 4). Lane 1: control male SD; lanes 2, 4, 6, 8, 10 : control male MM; lanes 3, 5, 7, 9, 11 : CMT1A patient HOU1-3[3]. CMT1A-specific duplication junction fragments were identified by the enzymes *Sac*II, *Fsp*I and *Asc*I when hybridized with the probe in *b* (indicated by the arrow), but not that in *a*. *c*, The pulsed field map of the *PMP-22* and VAW409 region deduced from these hybridizations. A, *Asc*I; F, *Fsp*I; N, *Not*I; S, *Sac*II; Sf, *Sfi*I. Note that the map locations of only two of the *Sfi*I sites are indicated. Centromere-telomere orientation is based on the genetic map of chromosome 17[45]. The boxed *Asc*I, *Fsp*I and *Sac*II sites are those introduced by the duplication event in CMT1A patients and hence, the map on the centromeric side of these novel sites is different in CMT1A patients. The *Sac*II site marked with an asterisk is polymorphic; *b* shows that it is absent in individual SD (lane 1) but present in individuals MM (lane 2) and 1-3 (lane 3).

which had first been used to identify the duplication, and a pattern identical to that seen in Fig. 7*b* was observed (data not shown). A pulsed field map was constructed on the basis of these data and is shown schematically in Fig. 7*c*. The probe VAW409R3 and *PMP-22* gene-specific probes hybridize to the same 150 kb *Sfi*I fragment indicating the close physical proximity of these markers. These data are consistent with the *PMP-22* gene being located entirely within the CMT1A duplication.

Discussion

The *Tr* mouse has been considered an animal model for CMT1A based on its neuropathological phenotype[14,15,23] and its location on mouse chromosome 11 near the homologous region for proximal human chromosome 17p[12]. Mutations in the mouse peripheral myelin protein gene *Pmp-22* have been proposed to be responsible for the *Tr* and *Tr*^{J} phenotypes[14,15]. In support of this hypothesis, the mouse *Pmp-22* gene has been mapped to the genetically defined *Tr* locus[15]. We now report the mapping of the human *PMP-22* gene to human chromosome 17p11.2–17p12, thereby extending the conservation of genes on mouse chromosome 11 and human chromosome 17 and identifying *PMP-22* as a candidate gene for CMT1A.

The identification of the first coding exon of the *PMP-22* gene within the 11 kb *Eco*RI fragment in c132-G8 (data not shown) as well as the restriction mapping data argue strongly that the *PMP-22* gene is not interrupted in CMT1A patients. Furthermore, previous findings demonstrating duplication of markers VAW409R3 (D17S122) and VAW412R3 (D17S125) in CMT1A patients[3,6,7] which are located about 50 kb and about 1 Mb upstream and downstream, respectively of the PMP-22 gene (unpublished results) suggest that the *PMP-22* gene is located entirely within the duplication. The location of the marker VAW409, the *PMP-22* gene and the novel *Sac*II, *Asc*I and *Fsp*I sites within the CMT1A duplication interval provides a rough estimate of the duplication junction being >50 and <500 kb from the *PMP-22* locus. Northern analysis with the human *PMP-22* cDNA identified three transcripts in peripheral nerve and spinal cord. Previously, a single 1.8 kb transcript was identified in the sciatic nerve and spinal cord of the rat[16,21]. The identity of the smaller transcripts observed in the human PNS is unknown; mechanisms leading to their production could include alternate processing or the use of multiple polyadenylation sites.

The *Tr* and CMT1A phenotypes are strikingly similar. Both the *Tr* mutation and CMT1A show dominant inheritance and are manifested pathologically as a marked decrease in the degree of myelination and in the number of large calibre axons in the peripheral nervous system, resulting in severely reduced nerve conduction velocities[24–27]. Hypertrophic peripheral nerve changes in both disorders include increased numbers of Schwann cell nuclei, the formation of 'onion bulbs' consisting of supernumerary Schwann cell processes, and increased collagen deposition surrounding axons[10,25–27]. In *Tr* nerves, the primary defect apparently resides in myelinating Schwann cells[28] whereas in CMT1A, both Schwann cell and neuronal defects have been individually implicated[27]. The observation of small axon diameters with altered axon/myelin ratios that are suggestive of a primary axonopathy in CMT1A patients supports the hypothesis that CMT1A is primarily a neuronal defect[27,29,30]. Recent studies on hypomyelinating *Tr* mice, however, in which *Tr* nerve segments were grafted into normal mice, have shown that the expression of the mutated *Pmp-22* allele is sufficient to cause axonal

abnormalities[14,28], which include small axon diameters, increased neurofilament density, and alterations in the phosphorylation of neurofilament protein[31].

The CMT1A mutation involves a chromosomal duplication of about 1.5 Mb in the majority of patients (unpublished results). This result in conjunction with our previous findings of CMT in a patient with a cytogenetically visible duplication for 17p(ref. 5) and the demonstration of a new duplication mutation in a nuclear CMT1A family[6] supports a model of gene dosage as a mechanism for CMT1A. As *PMP-22* is contained entirely within the CMT1A duplication, dosage differences in PMP-22 expression may be partially or entirely responsible for CMT1A. Some CMT1 patients as well as patients with similar peripheral neuropathies do not show the CMT1A duplication (unpublished observations). It will be imperative to examine the *PMP-22* gene in these patients for point or other subtle mutations.

The question of whether both abnormal *PMP-22* expression and point mutations in the *PMP-22* gene may lead to similar phenotypes can be addressed in several ways. First, transgenic mice overexpressing the *PMP-22* gene can be created and examined for onset of peripheral neuropathy. Given our previous findings of homozygosity of the duplication mutation producing a very severe clinical CMT1A phenotype[3], we anticipate that transgenic mice overexpressing *PMP-22* may be useful for testing threshold and dosage effects as well as tolerance to increased *PMP-22* expression. In addition, strategies for correction of the disease phenotype by normalizing the amount of PMP-22 gene product can be evaluated[5].

The second key approach is to study the biology and function of PMP-22. The *PMP-22* gene was initially identified and cloned by differential screening of cDNA libraries from injured versus uninjured rat sciatic nerve[21,32,33]. Subsequent experiments in rats showed that the PMP-22 protein is expressed in the compact portion of essentially all myelinated fibres in the peripheral nervous system and is regulated similar to other myelin proteins during development and nerve regeneration[16]. *In situ* hybridization studies on rat tissue sections and individual nerve fibres have shown that *PMP*-22 mRNA is produced mainly by Schwann cells[16,33]. Structurally, PMP-22 is predicted to be an integral membrane protein and the four putative transmembrane domains are highly conserved through evolution. The location of both *Tr* mutations in these domains makes a functional role for these domains highly likely. We had proposed earlier that PMP-22 might constitute a channel or pore protein or play a role in cell–cell interaction[15]. These hypotheses could accommodate both the dominant inheritance of the *Tr* mutations in the mouse as well as dosage effects in CMT1A patients.

The mouse *Pmp-22* gene is identical to the *Gas*-3 gene, identified in growth-arrested fibroblasts, after correction of a sequencing error in the originally reported *Gas*-3 gene sequence[14,20]. Cessation of Schwann cell proliferation and myelination are tightly connected[34]. Thus, it remains to be determined whether PMP-22 is involved in growth control, myelin formation or both.

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In summary, we have identified the *PMP-22* gene as the first candidate gene possibly underlying an inherited peripheral neuropathy in humans. While our data suggest that an extra copy of *PMP-22* may cause the CMT1A phenotype, we cannot exclude other mutational mechanisms involving *PMP-22*. Although there may be additional gene(s) within the CMT1A duplication interval which contribute to the CMT1A phenotype, the strong similarities between *Tr* and CMT1A suggest that the *PMP-22* gene has a primary role in the phenotypic effect of DNA duplication associated with CMT1A.

Methodology

Cell lines. Hybrids used for initial chromosomal assignment have been described previously[35,36]. The panel used for regional assignment on chromosome 17 is as follows: hybrid MH22-6 contains a single chromosome 17 as the only human complement[37], Hy254-1 and Hy357-2D retain a del(17)(p11.2p11.2) and a del(17)(p11.2p12), respectively derived from patients with the Smith-Magenis syndrome[22,38], 88H5 contains 17pter–p11.2[37], and LS-1 contains 17cen–qter[39]. The rodent parent of hybrids MH22-6, Hy254-1, and LS-1 is the mouse TK cell line, Cl-1D, that of hybrid Hy357-2D is the hamster TK cell line a23 and that of 88H5 is the hamster HPRT cell line RJK88.

DNA probes and primers. The mouse cDNA probe PMP-22c contains 561 bp of the coding sequence[15]. phPMP22-1 contains the human *PMP-22* cDNA [position 147–820 (Fig. 1*a*)] in the vector TA-1000 (Invitrogen). FUS1 is a 521 bp fragment of the human *PMP-22* cDNA (position 800–1320) derived by PCR-amplification with human-specific primers. FBR1 is a 362 bp fragment of the human *PMP-22* cDNA from position 171–532 derived by PCR amplification. For the RT-PCR experiments, *PMP-22* specific primers were used to amplify a 470 bp region from position 171–640. Control primers that amplified a 983 bp region G3PDH mRNA were obtained from Clontech.

RNA analysis. Human brain, spinal cord and skeletal muscle samples were obtained 6 hours postmortem from an 8 month infant who had expired from sudden infant death syndrome. Femoral nerve was obtained 22 h postmortem from an 18 month female infant with congenital heart disease. Liver tissue was obtained from an infant undergoing a liver transplant procedure for ornithine transcarbamoylase deficiency . Heart tissue was obtained from a 10 year old female patient with dilated cardiomyopathy. Total RNA was extracted from brain, heart, liver and lymphoblasts by guanidinium chloride/caesium chloride centrifugation[40], and from spinal cord, femoral nerve and skeletal muscle by the guanidinium isothiocyanate-phenol-chloroform method[41]. Northern analysis was performed by electrophoresis of approximately 5 µg of total RNA in 1.5% agarose gels containing formaldehyde. RT-PCR was performed using 1 U each of AMV reverse transcriptase and *Taq* polymerase and 0.5 µg of total RNA in a reaction volume of 50 µl essentially as described previously[42].

Cosmid contig for the *PMP-22* region. Approximately 7,500 cosmids representing a library constructed from flow-sorted chromosome 17 in the vector sCos1 (Los Alamos National Laboratory) grown on gridded arrays of 384 on nylon filters (Genescreen Plus, NEN) were hybridized sequentially to the human *PMP-22* cDNA probes, FUS1 and phPMP22-1, and to a mouse *Pmp-22* cDNA probe PMP-22c. Eight hybridizing cosmids were identified. Partial *Eco*RI restriction digests of representative cosmids c103-B11 and c132-G8 were electrophoresed in pulsed field gels and a restriction map constructed by hybridization to T3 and T7 polymerase promoter specific oligonucleotides as described[43]. Total *Eco*RI digests of all cosmids as well as a *Bam*HI and *Bam*HI/*Eco*RI digest of cosmid c103-B11 were used to confirm the accuracy of the restriction map.

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