J. Anat. (1999) **195**, pp. 331–339, with 4 figures Printed in the United Kingdom

Development of early postnatal peripheral nerve abnormalities in Trembler-J and PMP22 transgenic mice

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(*Accepted 20 April 1999*)

ABSTRACT

Mutations in the gene for peripheral myelin protein 22 (*PMP22*) are associated with peripheral neuropathy in mice and humans. Although PMP22 is strongly expressed in peripheral nerves and is localised largely to the myelin sheath, a dual role has been suggested as 2 differentially expressed promoters have been found. In this study we compared the initial stages of postnatal development in transgenic mouse models which have, in addition to the murine *pmp22* gene, 7 (C22) and 4 (C61) copies of the human *PMP22* gene and in homozygous and heterozygous Trembler-J (Tr^J) mice, which have a point mutation in the *pmp22* gene. The number of axons that were singly ensheathed by Schwann cells was the same in all groups indicating that PMP22 does not function in the initial ensheathment and separation of axons. At both P4 and P12 all mutants had an increased proportion of fibres that were incompletely surrounded by Schwann cell cytoplasm indicating that this step is disrupted in PMP22 mutants. C22 and homozygous Tr^J animals could be distinguished by differences in the Schwann cell morphology at the initiation of myelination. In homozygous Tr^J animals the Schwann cell cytoplasm had failed to make a full turn around the axon whereas in the C22 strain most fibres had formed a mesaxon. It is concluded that PMP22 functions in the initiation of myelination and probably involves the ensheathment of the axon by the Schwann cell, and the extension of this cell along the axon. Abnormalities may result from a failure of differentiation but more probably from defective interactions between the axon and the Schwann cell.

Key words: Myelination; Schwann cells; Charcot-Marie-Tooth disease; hereditary motor and sensory neuropathy.

INTRODUCTION

Mutations in the gene for peripheral myelin protein 22 (PMP22) are associated with peripheral neuropathy in mice and humans (Lupski et al. 1991; Raeymaekers et al. 1991; Suter et al. 1992*a*). PMP22 is a 22 kDa glycoprotein which is localised mainly in the myelin sheath of peripheral nerve, although it is also found in plasma membranes of the Schwann cells that surround the axons of unmyelinated fibres (Snipes et al. 1992; Haney et al. 1996). Representing less than 5% of total myelin protein, PMP22 is unlikely to play a structural role in myelin but its function is not yet clear.

A dual role for PMP22 has been suggested on the basis that 2 differentially expressed tissue specific PMP22 promoters have been found. Promoter 1 is preferentially expressed in myelinating Schwann cells and promoter 2 in tissues that do not form myelin (Bosse et al. 1994; Suter et al. 1994). PMP22 is a member of a small family of proteins most of which are expressed in epithelial cells (CL20, EMP-1, EMP-2, EMP-3, MP20) (Kumar et al. 1993; Marvin et al. 1995; Taylor et al. 1995; Bolin et al. 1997). Observations on epithelial cell lines suggest that these proteins are involved in cell–cell interactions, including roles both in the switch from proliferation to differentiation and in the maintenance of the differentiated state (Taylor et al. 1995). EMP-3 (HNMP-1), which has the highest amino acid homology to PMP22 (44%), is transiently expressed by Schwann cells during sciatic nerve myelination. The EMP-3 protein itself is axon associated and is thought to play a role

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in axon-Schwann cell interactions (Bolin et al. 1997). It has been speculated that promoter 1 for *PMP22* has been acquired specifically during evolution to allow high level expression in myelinating Schwann cells. This additional function is likely to coexist alongside the retained original function (Naef & Suter 1998).

PMP22 mutations are associated with 3 different disease phenotypes in humans. The most common human disorder involving PMP22, Charcot-Marie-Tooth disease 1A (CMT1A) or hereditary motor and sensory neuropathy 1a (HMSN1a), is associated with a 1.5 Mb duplication on chromosome 17p11.2–12, leading to the presence of an extra copy of the *PMP22* gene (Lupski et al. 1991; Raeymaekers et al. 1991). This duplication produces a peripheral neuropathy which generally presents in the first decade of life and typically results in distally accentuated muscle wasting and weakness. Histologically it is characterised by hypermyelination (Gabreëls-Festen et al. 1995) followed by demyelination and myelinated nerve fibre loss, and the development of onion bulbs (indicative of repeated cycles of demyelination and remyelination). In older patients hypermyelination is succeeded by hypomyelination (Thomas et al. 1997). A deletion of this same 17p11.2–12 region causes hereditary neuropathy with liability to pressure palsies (HNPP) (Chance et al. 1993; Tyson et al. 1996). The most characteristic pathological feature of HNPP is the presence of tomacula (sausage-shaped enlargements in the myelin sheath) (Madrid & Bradley 1975). The reciprocal disorders HNPP and CMT1A are thought to be the result of unequal crossing over at meiosis. Point mutations in the *PMP22* gene are also associated with a Charcot-Marie-Tooth like phenotype but with a hypomyelinating/demyelinating neuropathy (Gabreëls-Festen et al. 1995) or with a more severe childhood onset neuropathy, Dejerine-Sottas disease (DSD). As most CMT1A patients have 3 copies of the *PMP22* gene and HNPP patients have only 1, a gene dosage effect has been proposed as a mechanism for both diseases (Schenone et al. 1997). This is supported by observations which have shown that PMP22 protein expression is increased in CMT1A patients and reduced in HNPP patients as compared with patients with normal *PMP22* gene copy number (Vallat et al. 1996; Gabriel et al 1997).

Mutations in the mouse *pmp22* gene are responsible for the peripheral nerve abnormalites in the Trembler murine mutants *Tr*, *Tr^J* and *Tr-Ncnp* (Suter et al. 1992*a*, *b*; Suh et al. 1997). A Dutch kindred has the same Leu16Pro mutation as is found in the *Tr^J* mouse (Valentijn et al. 1992). Pathologically the human phenotype is more severe than the murine, with the human mutation resulting in a DSD phenotype and a reduction in myelinated fibre density of 80% (Gabreëls-Festen et al. 1995). In the *Tr^J* mouse the reduction in the proportion of myelinated fibres is a more modest 20% (Robertson et al. 1997).

We have recently reported the generation of transgenic mice carrying increasing copy numbers of the human *PMP22* gene and expressing increasing levels of the transgene (Huxley et al. 1996, 1998). Increased expression of PMP22 does not result in a steady worsening of phenotype but instead there appears to be a threshold level of PMP22 expression. Below a level which equates to 2 additional copies of *PMP22* there was no detectable effect. At around 4 additional copies of *PMP22* a degree of hypomyelination was found in older mice with some electrophysiological deficit but no behavioural signs. Above 7 additional copies of *PMP22* the mice had a severe dysmyelinating neuropathy affecting more than 40% of fibres in adult mice. These mice had a severe phenotype with a progressive paralysis of the rear legs, resulting in abnormal gait with splaying of the hindlimbs. Although different numbers of fibres were affected in the mildly and severely affected groups, the type of lesion was similar. Both showed demyelination of large axons and uncompacted myelin, usually on the outer aspects of the myelin sheath.

In this study we have compared the initial stages of postnatal development of the sciatic nerves of transgenic mice with 7 (C22) and 4 (C61) copies of the human *PMP22* gene with both homozygous and heterozygous Trembler-J mice (*Tr^J*), which have a point mutation in the *pmp22* gene.

MATERIALS AND METHODS

Tr^J animals were originally obtained from the Jackson Laboratories, Bar Harbor, Maine, USA, and are now maintained in a colony at the Royal Free and University College Medical School, London. Transgenic animals were generated by pronuclear injection of YAC 49G7 DNA, which contains the 40 kb human *PMP22* gene. The determination of copy numbers and the level of expression of the human *PMP22* transgenes have previously been described (Huxley et al. 1998). The animals are maintained at Imperial College School of Medicine, London. Transgenic line C22 is now designated TgN (PMP22) C22C1h and line C61 is designated TgN (PMP22) C61C1h following the international guidelines for naming transgenics.

Animals were killed by cervical dislocation. The sciatic nerve was fixed in situ for 20 min (1%

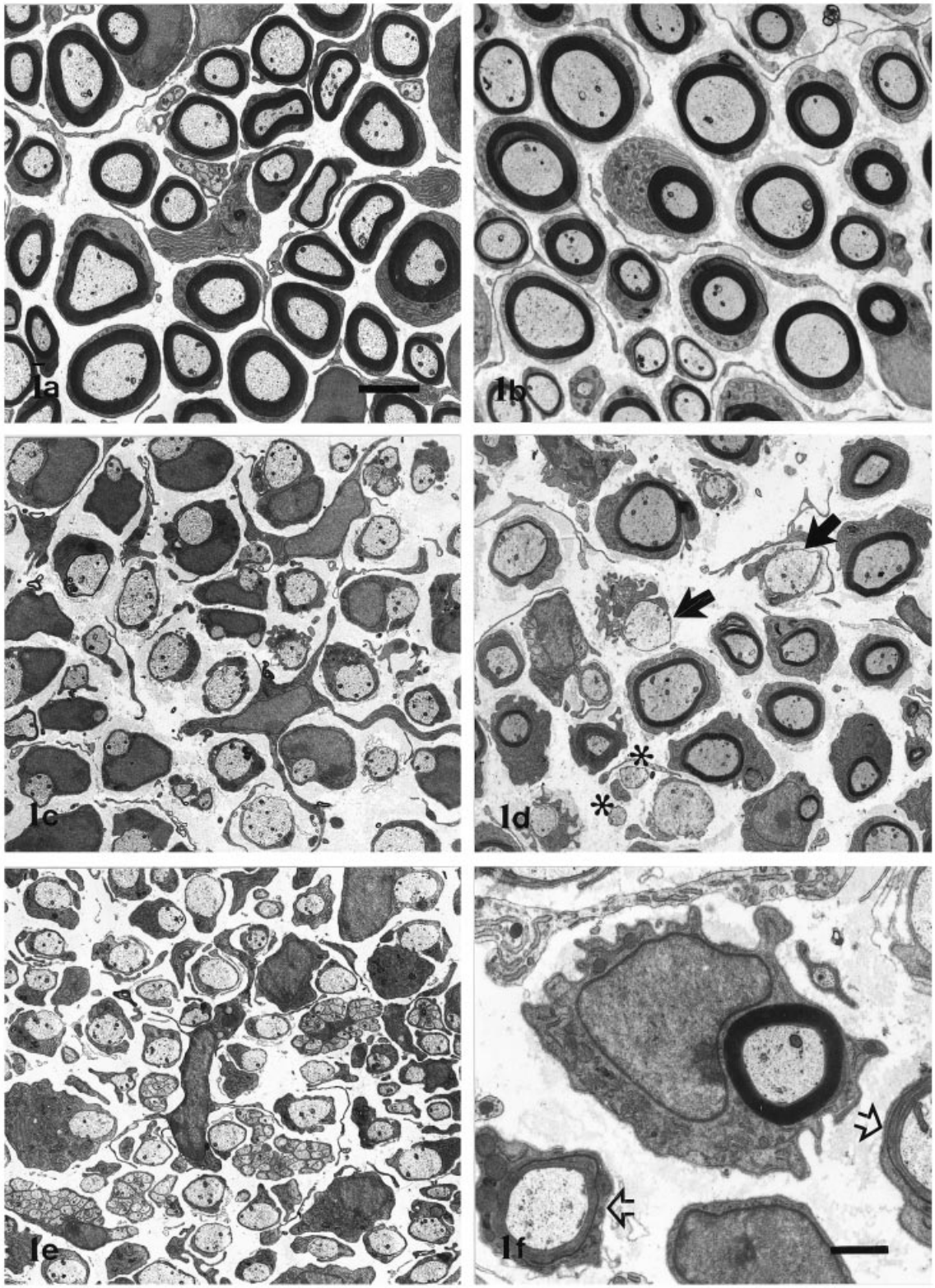

Fig. 1. Electron micrographs of the sciatic nerve of P10–12 animals. (*a*) Control; bar (and for *b–e*), 2.5 μm. (*b*) 4 copies of *PMP22* (C61); (*c*) 7 copies of *PMP22* (C22); (*d*) Tr^J heterozygote; incompletely surrounded axons (arrows); naked axons (asterisks). (*e*) Tr^J homozygote. (*f*) Tr^J heterozygote. Schwann cell cytoplasm with an irregular outline. Such irregular outlines as were commonly seen in heterozygotes and permitted them to be distinguished from C61 and control animals as early as P4.Uncompacted myelin (open arrows). Bar, 1 μm.

paraformaldehyde, 1% glutaraldehyde in 0.1 M PIPES buffer) and then removed and placed in fresh fixative overnight. Samples were processed for electron microscopy as previously described (Robertson et al. 1997). Sections examined were taken from the midthigh level of the sciatic nerve.

Table 1. *Total number of singly ensheathed fibres*

	Control	4 copies (C61)	7 copies (C22)	Tr^J het	Tr^J hom
P4	643 ± 20	629 ± 62	657 ± 25	538 ± 98	761 ± 68

Mean ± S.E.M. (n = 6).

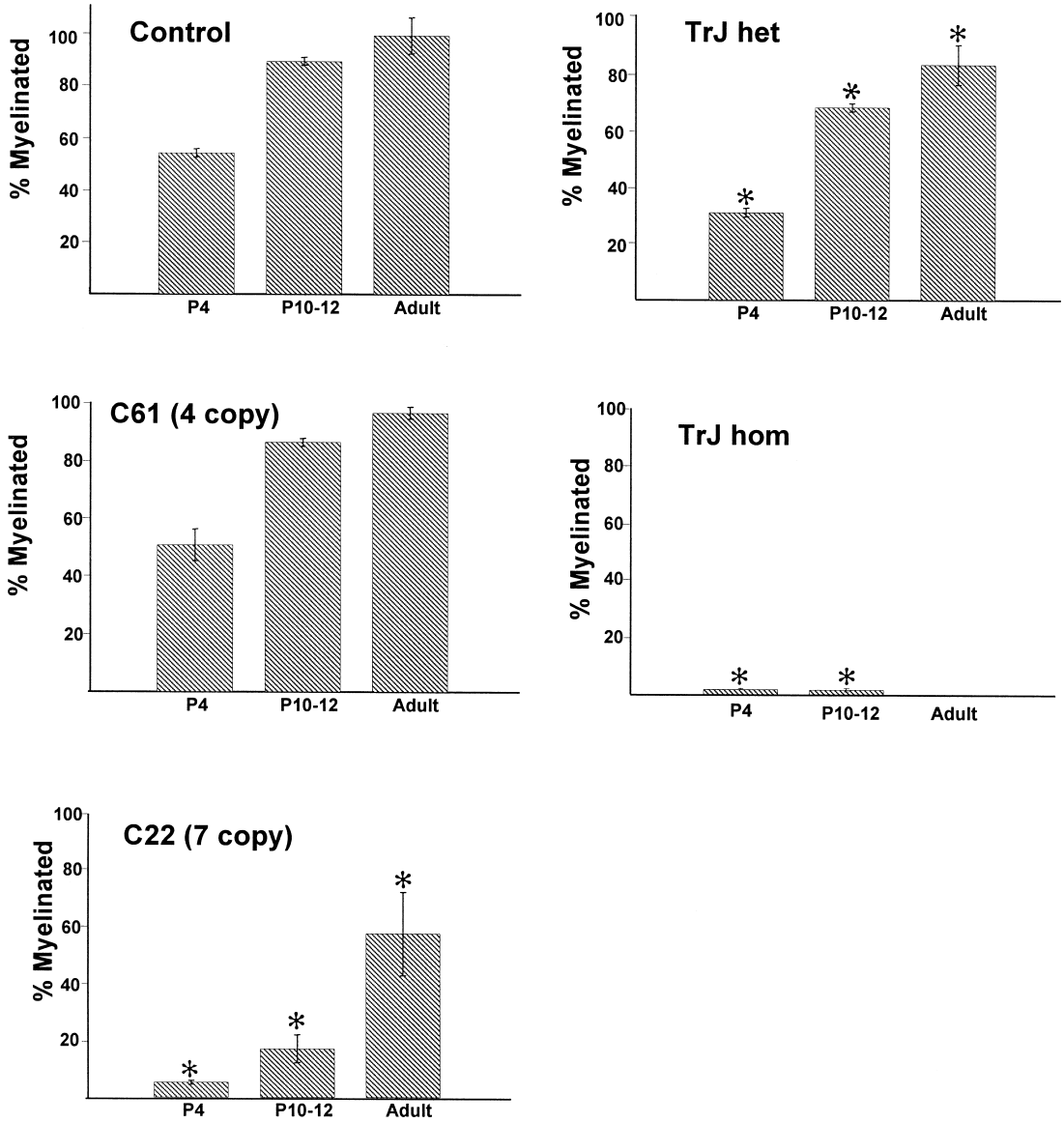

Fig. 2. Percentage of singly ensheathed fibres that were myelinated at the different ages examined. *Indicates significantly different from control at the same age.

In young animals at postnatal day 4 (P4) and P10–12, fibres were counted at a magnification of ×7000 using a Zeiss 902C electron microscope. Counts were taken from nonoverlapping fields; a total area of 0.63 mm² was analysed. Promyelin fibres were defined as those which had separated from axon bundles and were associated with a single Schwann cell. In adult animals the counts were performed on a Zeiss Axiophot microscope; an entire fascicle was counted. All statistics were calculated using the Mann-Whitney U test at a significance level of 5%.

RESULTS

On examination of semithin sections at P4 and P12, affected C22 (7 copies of *PMP22*) (Fig. 1*c*) and homozygous Tr^J animals (Fig. 1*e*) were easily

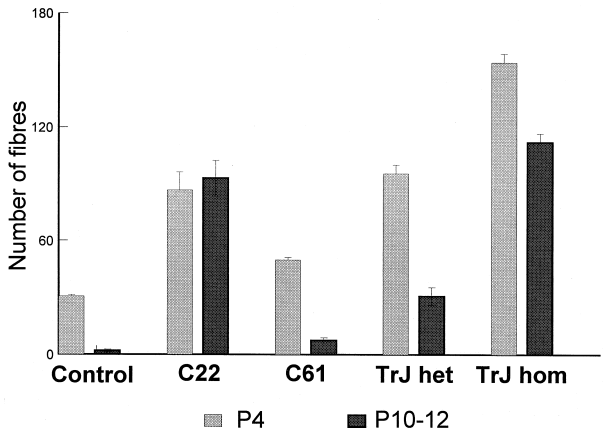

Fig. 3. Number of fibres incompletely surrounded by Schwann cell cytoplasm.

recognised as they had markedly decreased numbers of myelinated fibres. In heterozygous *Tr*^{J} animals (Fig. 1*d*), the decrease in myelinated fibres was more difficult to judge visually, particularly at P4, but they also could be distinguished from controls and C61 animals by the presence of many fibres with irregular contours of Schwann cell cytoplasm (Fig. 1*f*). The C61 strain (Fig. 1*b*) could not be distinguished morphologically from controls either at P4 or P12 although by 6 wk a small subgroup of the larger myelinated fibres had become hypomyelinated. (See Huxley et al. 1998).

In *Tr*^{J} mice and in both transgenic strains the process of axon separation from fetal bundles into a 1:1 relationship with individual Schwann cells appeared to proceed normally. At P4 there was no difference in the total number of singly ensheathed fibres in the different strains (Table 1). No abnormality was noted in unmyelinated fibre bundles.

The number of myelinated fibres was decreased at all ages in C22 (7 copies) and both heterozygous and homozygous *Tr*^{J}animals, while C61 (4 copies) were not significantly different from the controls (Fig. 2). Homozygous *Tr*^{J} animals were the most severely affected with less than 2% of the singly ensheathed axons being myelinated and these animals developed very little myelin before their death around weaning at P21 (Fig. 2). The proportion of myelinated fibres increased with age in control, C61 (4 copies), heterozygous *Tr*^{J} and to a lesser extent C22 animals. However, C22 (7 copies) mice remained very poorly myelinated, with fewer than 60% of axons being myelinated in adult animals.

Both at P4 and P12, heterozygous and homozygous *Tr*^{J} and the 2 transgenic strains had an increased number of fibres that were incompletely surrounded by Schwann cell cytoplasm. In C22 animals this number did not alter during development but in all the other strains the number decreased with age (Fig. 3). It is interesting to note that the only feature in which C61 mice differed from control animals at these ages was the inability of the Schwann cell to surround a fibre completely.

An increased number of Schwann cells is a feature of most neuropathies and this was also found in the mouse models (Table 2). At P4 the C22 (7 copies) mutants already had a significantly higher number of Schwann cells per 100 axons than controls. By P12 *Tr*^{J} heterozygotes and *Tr*^{J} homozygotes also had significantly increased numbers of Schwann cells. Between P4 and P12 the number of Schwann cells per 100 axons decreased in control and the mildly affected C61 strains as myelination progressed. In the more severe mutants the number of Schwann cells increased significantly (Table 2). When comparing early postnatal (P12) and adult values the number of Schwann cells decreased by half in control and C61 in which almost all the singly ensheathed fibres were myelinated. Schwann cells decreased to a much lesser extent in the poorly myelinated C22 animals and in *Tr*^{J} heterozygote nerves the number of Schwann cells did not alter from early postnatal values.

The 2 most affected strains (*Tr*^{J}/*Tr*^{J} and C22) could be distinguished morphologically from each other by P10–12. Schwann cell cytoplasm in the promyelin fibres of *Tr*^{J}/*Tr*^{J} animals failed to complete

Table 2. *Number of Schwann cell nuclei per 100 axons*

	Strain				
	Control	4 copies (C61)	7 copies (C22)	*Tr*^{J} het	*Tr*^{J} hom
P4	10.5± 0.5	10.7±1.1	13.3±0.6†	12.0± 0.6	11.6±0.5
P12	7.4±0.5*	8.2±0.4*	19.5±1.8*†	15.7±0.6*†	13.3±0.5*
Adult	4.4±0.4ψ	5.4±0.5ψ	12.3±0.9ψ†	13.9±0.8†	

Mean±S.E.M.. (n = 6) *Significantly different from P4 , ψSignificantly different from P12.
† Significantly different from control at the same age.

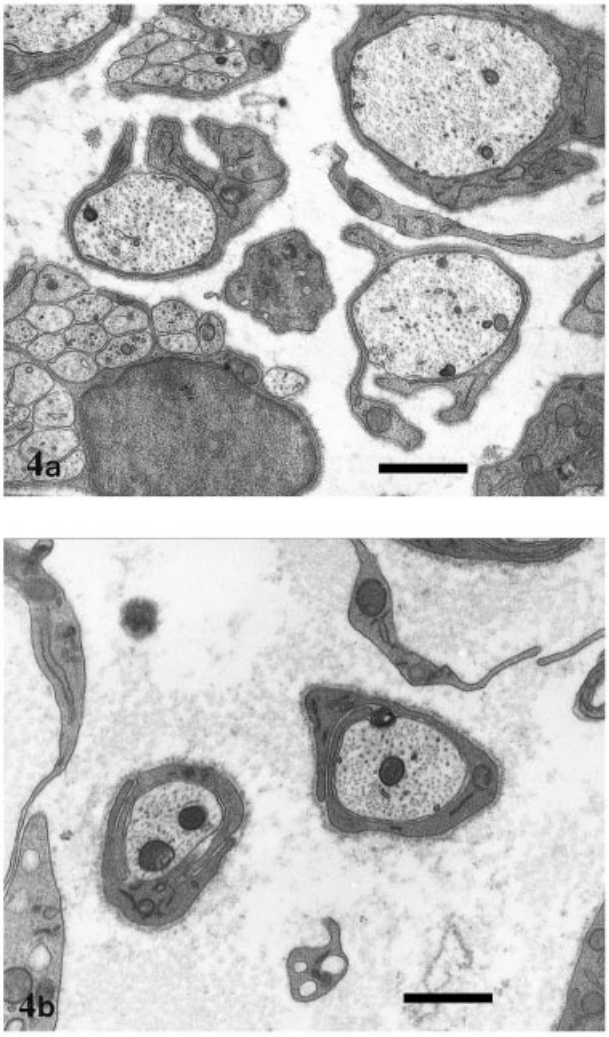

Fig. 4. (*a*) Fibres from a Tr^J homozygote animal in which the Schwann cell cytoplasm fails to complete a full turn around the axon.P10–12. (*b*) Fibres from a 7 copy (C22) animal in which the Schwann cell cytoplasm has completed a full turn around the axon.P10–12 Bar, 1 μm.

a full turn around the axon (Fig. 4*a*), whereas in C22 animals many had progressed so that a short mesaxon had been formed (Fig. 4*b*).

DISCUSSION

This study shows that the axons initially separate normally into a 1:1 relationship with individual Schwann cells both in mice which overexpress the *PMP22* gene and in those with a point mutation in the *pmp*22 gene. In all the mutants analysed there is a high proportion of fibres that are incompletely surrounded by Schwann cell cytoplasm. This suggests that the first abnormality and, by implication, the first function of PMP22 predates myelin formation and involves the attachment and extension of the Schwann cell around the axon. This is related specifically to axons that are destined to myelinate and is not a general feature of mutant Schwann cells. There was no evidence of abnormalities in the relationship of unmyelinated axons and Schwann cells.

A similar scenario of delayed myelination, following the establishment of normal 1:1 ratios of Schwann cells to axons, has been observed in 10 d old mice which overexpress the mouse pmp22 protein (Magyar et al. 1996) and in 5 wk rats overexpressing the mouse pmp22 protein (Serada et al 1996). Similarly, in 4 d mice lacking all pmp22 expression, the nonmyelinated axons were associated with prospective myelinating Schwann cells in a 1:1 ratio, but only 19%, in comparison to 60% in wild type rats, of the larger calibre axons were ensheathed by compact myelin. This is in contrast to in vitro data from neurons cocultured with Schwann cells which had been genetically modified with retroviruses to over or under-express PMP22 (D'Urso et al. 1997). Here, over and under expression of PMP22 was found to have virtually no effect on the early stages of myelination and membrane compaction. Despite this, in view of the consistent observations in all mouse and rat models with over and under expression of *pmp*22 and expression of mutant *pmp*22, it seems that *pmp22* has an important role in the initiation of myelination in vivo after the association of Schwann cells with axons.

In addition the 2 most severely affected strains (C22 transgenic and Tr^J homozygotes) could be distinguished from each other by differences in the initial ensheathment of axons. In homozygous Tr^J animals the 2 apposing edges of Schwann cell cytoplasm failed to overlap but in the C22 strain there was usually a small overlap of Schwann cell membranes and initial mesaxon formation. This suggests that PMP22 is involved either in mesaxon formation, i.e. the contact between the apposing edges of the Schwann cell surrounding the axon, or in the guidance/recognition processes involved in the spiralling of Schwann cell membranes to form the myelin sheath.

Two features of this early developmental morphology can be produced by interrupting Schwann cell differentiation with the thymidine analogue, 5-bromodeoxyuridine (BrdU). Following treatment of demyelinated nerve with BrdU, Hall & Gregson (1977) reported an increased number of promyelinated fibres with cytoplasm that was irregular in outline. These are morphologically very similar to the irregular fibres characteristic of Tr^J heterozygote animals. Additionally many cells possessed pseudopodial processes, a feature also commonly found in both heterozygous and homozygous Tr^J nerves.

These results raise 2 possible interpretations, firstly that the mutant phenotype results from a failure of the differentiation process or secondly that it results from disrupted Schwann cell/ Schwann cell or Schwann cell/axon interactions. If the Schwann cells fail to differentiate fully into their myelinating phenotype, they are unlikely to be capable of responding appropriately to axonal signals to myelinate. During the differentiation process Schwann cells cease proliferation and grow both longitudinally (internode length) and radially (myelin sheath thickness). An inability of mutant Schwann cells to surround axons and subsequently to produce a spiral ensheathment around them to form myelin may be seen as a manifestation of failed differentiation. Alternatively, if PMP22 functions in mediating the interaction between cell types, it is possible that differentiation failure itself is secondary to defective cell interactions. In a previous study we proposed that adult Tr^J animals exhibited abnormalities that were consistent either with defective Schwann cell/axon or Schwann cell/extracellular matrix interactions (Robertson et al. 1997). Axonal contact is known to be a prerequisite for the initiation of myelination. If the Schwann cell only partially surrounds the axon, the signal may be insufficient to produce differentiation and myelination. Similarly, Schwann cells in culture only cease to divide and commence myelin formation when they are in contact with the extracellular matrix (De Vries, 1993). In this study the only feature that distinguished the C61 (4 copy) strain from controls was the increased number of fibres that were not surrounded by Schwann cell cytoplasm. This leads us to conclude that the axon/Schwann cell interaction is more likely to be the site of abnormality than Schwann cell/extracellular matrix interaction. In either of these 2 scenarios the lack of myelination in these PMP22 related disorders can be seen as a secondary feature. This is largely overcome with time in most of the models, shown by improvement in the degree of myelination from P10–12 to adult.

Comparative observations on the severity of associated axonal loss will be of interest in the C61 strain, which is relatively unaffected during the early stages of development. A small proportion of larger fibres (7%) become hypomyelinated in adult mice suggesting that a moderate overexpression of PMP22 leads to myelin instability later in life (data not shown).

The early growth response 2 gene (EGR2), also known as Krox-20, may be involved in the regulation of cell proliferation. It is part of a multigene family encoding Cys2-His2 type zinc finger proteins (Joseph et al. 1988; Ragnekar et al. 1990). It is expressed in Schwann cells before the onset of myelination (Topilko et al. 1994) and *egr2* knock-out mice exhibit a failure of myelination in the peripheral nerves. The Schwann cells wrap their cytoplasmic processes only one and a half turns around the axon and fail to lay down myelin (Topilko et al. 1994). The Schwann cells express the early myelin marker, myelin associated glycoprotein (MAG), but not later myelin gene products including myelin protein zero (MPZ). *Egr2* is therefore likely to control a set of genes necessary for the completion of myelination. Its loss produces a more profound structural deficit than the PMP22 related disorders investigated in this present study. However the stage of arrest with the margins of the Schwann cell just overlapping around the axons is almost identical to that observed in the C22 mice with overexpression of PMP22. This suggests that egr2 and PMP22 are involved in a very similar stage of Schwann cell differentiation.

From the present results, the increased Schwann cell numbers found in adult Tr^J heterozygote and C22 animals appear to arise mainly from a failure in the normal maturational decrease in density per unit length (or cross sectional area) of nerve related to the cessation of mitosis after myelination and the progressive increase in Schwann cell length during development. This differs from the situation seen in the *Tr* mutant where Schwann cell numbers increase steadily with age and are associated with ongoing demyelination (Perkins et al. 1981). The absence of continuous Schwann cell proliferation suggests that C22 and Tr^J mutants are primarily dysmyelinating and not demyelinating. If demyelination subsequently takes place in the animals we have studied this would be expected to result in Schwann cell proliferation. Schwann cell proliferation usually precedes the onset of myelination and the protracted length of the myelination period, during which elongation of nerves related to increase in limb length will have occurred, may result in the increase in Schwann cell number seen in Tr^J heterozygote and C22 transgenic animals between P4 and P12. An increase in Schwann cell number was also seen in the Krox-20 mutant mice, again showing the similarities of these 2 models.

The transgenic PMP22 mice were developed in a endeavour to replicate the human disease, CMT1A, which is most commonly the result of the possession of an extra copy of the *PMP22* gene because of a segmental duplication on chromosome 17p11.2. It is evident from the present study and our previous observations (Huxley et al. 1998) that the morphological changes that are found resemble more

closely those in the *Tr*^{J} mouse than the human disorder. As already discussed, CMT1A related to a chromosome 17p11.2 duplication is probably the result of overexpression of the *PMP22* gene (Vallat et al. 1996). The transgenes may however be exerting a dominant negative effect, possibly because they are of human origin. The effect of introducing additional copies of the murine *pmp22* gene into the genome will therefore be of interest.

ACKNOWLEDGEMENTS

The authors are grateful to Action Research for financial support and to the Wellcome trust for a grant to enable the establishment of the Trembler-J mouse colony.

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