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Induced myelination and demyelination in a conditional mouse model of Charcot–Marie–Tooth disease type 1A

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Charcot–Marie–Tooth disease type 1A, a hereditary demyelinating neuropathy, is usually caused by overexpressing peripheral myelin protein 22 (PMP22) due to a genomic duplication. We have generated a transgenic mouse model in which mouse pmp22 overexpression can be regulated. In this mouse model, overexpression of pmp22 occurs specifically in Schwann cells of the peripheral nerve and is switched off when the mice are fed tetracycline. Overexpression of pmp22 throughout life (in the absence of tetracycline) causes demyelination. In contrast, myelination is nearly normal when pmp22 overexpression is switched off throughout life by feeding the mice tetracycline. When overexpression of pmp22 is switched off in adult mice, correction begins within 1 week and myelination is well advanced by 3 months (although the myelin sheaths are still thinner than normal), indicating that the Schwann cells are poised to start myelination. Upregulation of the gene in adult mice (which had previously had normal pmp22 expression) is followed by active demyelination within 1 week, which had plateaued by 8 weeks. This indicates that Schwann cells with mature myelin are sensitive to increased amounts of pmp22 such that they rapidly demyelinate. Thus, demyelination can largely be corrected within a few months, but the correction will be sensitive to subsequent upregulation of pmp22.

INTRODUCTION

Charcot–Marie–Tooth disease type 1A (CMT1A) usually results from a 1.5 Mb duplication on chromosome 17p11.2 (1,2). This region contains the peripheral myelin protein 22 (PMP22) gene, the overexpression of which results in a progressive demyelinating neuropathy (3–5). A more severe phenotype is seen in patients with point mutations in one copy of the PMP22 gene indicating that either overexpression or expression of a mutant form of the protein causes dominant demyelination (6).

PMP22 is a minor myelin protein making up 2–5% of the total myelin proteins (7) and it is not clear how the overexpression and point mutations act to cause dominant demyelination. Disruption of myelination could be due to altered stoichiometry of the myelin proteins, in particular by a disturbance of the PMP22/P0 complex formation. This complex has been proposed to stabilize myelin by holding Schwann cell membranes together (8). PMP22 may also have a role in Schwann cell differentiation and division (9,10). Finally, sequestration of mutant PMP22 in the endoplasmic reticulum may alter the amount of protein available at the cell surface (11–13).

Various mouse and rat models have been made in an attempt to replicate the effects of the overexpression of PMP22 seen in CMT1A (14–17). These have shown that overexpression of PMP22 causes dysmyelination in mice and that the severity is proportional to the level of expression of transgenic PMP22.

Potential therapeutic approaches to treating the overexpression of PMP22 could be aimed at reduction of PMP22 expression either by drugs or by antisense gene therapy. For such approaches to be feasible, it will be necessary for the myelin to be able to correct once the initial demyelination has occurred. It would also be advantageous if the myelin were stably corrected when therapy is withdrawn. Over-correction must also be avoided as PMP22 haploinsufficiency effects result in a clinically distinct demyelinating neuropathy known as hereditary neuropathy with liability to pressure palsies (HNPP) or tomaculous neuropathy.

In order to determine whether the phenotype is reversible, and how resistant mature myelin is to de novo overexpression
of PMP22, we have made a conditional mouse model using the tetracycline-responsive system developed by Herman Bujard et al. (18,19). This system uses a modified version of the tetracycline repressor protein from *Escherichia coli* fused to the transactivator region from VP16 (called tTA) to control gene expression. The gene to be expressed is placed under the control of a minimal cytomegalovirus (CMV) promoter with seven copies of the tetracycline operator sequence attached (called P<sub>CMV*-1</sub>). This system has been shown to give induction of gene expression over several orders of magnitude, works well in transgenic mice and can be made tissue-specific by expressing the tTA protein tissue specifically.

**RESULTS**

**DNA constructs used to produce tetracycline-conditional and tissue-specific pmp22 overexpression**

Two separate lines of transgenic mice were made, one with a construct that gives Schwann cell-specific expression of tTA and another with the mouse *pmp22* cDNA under control of the P<sub>CMV*-1</sub> promoter. The mice were then crossed to get double transgenic mice in which *pmp22* overexpression is dependent on the absence of tetracycline.

The first construct is based on the 560 kb yeast artificial chromosome (YAC), 49G7, which carries the intact human PMP22 gene. We have previously used the unmodified YAC to make transgenic mice and the resulting PMP22 expression was found to be independent of integration site, dependent on the copy number and tissue-specific (14,17). We therefore decided to use this YAC as the basis for achieving tissue-specific expression of tTA. The tTA open reading frame (ORF) was introduced into the second exon of the *PMP22* gene on the YAC by homologous recombination as shown in Figure 1A such that the start of translation of the *PMP22* gene is now used to start the tTA ORF. A new poly(A) addition site has been added 3' of the tTA ORF along with a HIS5 gene that was used for selection of homologous recombinants in the yeast host. None of the *PMP22* gene has been deleted from the YAC so that all the promoter, enhancer and long-range elements of the gene are still intact and should now control expression of the tTA mRNA. The *PMP22* gene on the YAC has, however, been disrupted and should not be expressed from this YAC construct. The transgenic line JY13 was made with this modified YAC.

The second construct was made by inserting the ORF of the mouse *pmp22* cDNA under the control of the P<sub>CMV*-1</sub> promoter (plasmid pJP7m; Fig. 1B). The transgenic line JP18 was made with this plasmid. The plasmid pUHG16.3 (19) was used to make a control transgenic line (JU2) in which the *lacZ* gene was under the control of the conditional promoter P<sub>CMV*-1</sub>, so that the efficacy of the tTA transgene could be evaluated.

**tTA-induced gene expression is specific to Schwann cells and can be switched on and off with tetracycline**

To check the tissue specificity and efficacy of the tTA transgene, the JY13 line with the tissue-specific tTA transgene was crossed with the JU2 *lacZ* reporter line of transgenic mice. Tissues from transgenic mice were stained for β-galactosidase (LacZ). Transgenic mice carrying just the *lacZ* transgene gave no detectable LacZ staining, showing that there is very low expression in the absence of the TTA activator (Fig. 2A). In contrast, there was strong expression in the Schwann cells of mice carrying both the tTA and the *lacZ* transgenes (Fig. 2A). The blue staining appears as doughnut shapes in cross-sections (Fig. 2B) and long sausage shapes in longitudinal sections (Fig. 2A) indicating that the LacZ protein is actually incorporated into the myelin sheath of individual Schwann cells. Clearly, the tTA transgene is being expressed, and is able to induce LacZ expression, specifically in the Schwann cells of transgenic mice. However, not every Schwann cell expresses LacZ and staining is patchy along the length of individual fibres (Fig. 2A, arrow).

In mice carrying both the tTA and the *lacZ* transgenes, staining was highly tissue-specific. There was strong staining in the Schwann cells of the sciatic nerve but no staining in tongue, muscle, skin, gut or lung, except in associated nerves (Fig. 2B). In addition, no staining was observed in any neuronal cell bodies. In the dorsal root ganglia, there was no staining of cell bodies, although there was clear staining of the nerve fibres running through them (Fig. 2C). In the cervical spinal cord, there was no staining except in associated dorsal...
Figure 2. LacZ staining on tissue sections. (A) LacZ staining on longitudinal sections of sciatic nerve. The top two panels show a double transgenic mouse (lacZ + tTA transgenes) and a mouse carrying the lacZ transgene alone, showing that there is no LacZ expression in the absence of tTA expression. The arrow indicates where LacZ staining is discontinuous along a fibre. The bottom four panels show switching off and on of LacZ expression. Double transgenic mice were untreated to ~2 months of age. They were then treated in the following ways: with no tetracycline, with tetracycline for 4 weeks, with tetracycline for 4 weeks and then not for 7 days, or with tetracycline for 4 weeks and then not for 12 days. Tetracycline was administered at 2 g/l in the drinking water. (B) LacZ staining on sections of sciatic nerve, tongue, muscle, skin, gut and lung from double transgenic mice carrying the tTA and lacZ transgenes. The sciatic nerve is a cross-section to show the doughnut-shaped staining of the myelin sheaths. The blue staining is observed only in the Schwann cells of the sciatic nerve and also in a nerve which was sectioned in the tongue sample (verified using neurofilament staining). (C) LacZ staining of a section through a dorsal root ganglion. The large cell bodies (arrow) show no staining whereas fibres traversing the ganglion stain blue. (D) LacZ staining of a section through the cervical spinal cord. Dorsal (arrow) and ventral roots stain blue whereas there is no staining in the white or gray matter of the spinal cord.
and ventral roots (Fig. 2D). Finally, no staining was evident in serial sections through the brainstem taken at 50 µm intervals (data not shown).

When double transgenic mice were fed tetracycline for an extended length of time, LacZ staining diminished slowly, over a 3-week period, to undetectable levels (Fig. 2A). When tetracycline was withdrawn from double transgenic mice that had previously been fed tetracycline for at least 1 month, LacZ staining became visible again after 7–12 days (Fig. 2A). Thus the system is reversible, and the lacZ gene can be switched both on and off over a period of time with very little expression during feeding with tetracycline.

**Conditional pmp22 mRNA expression can be switched from below detectable to high levels**

RT–PCR was used to investigate the level of induced pmp22 mRNA in the transgenic mice. The cDNA in the conditional transgene had been modified to carry an SstI site instead of a BssHII site which is present in the 3′-untranslated region (UTR) of the endogenous pmp22 gene (Fig. 1B). This change should not affect gene expression but allows the level of the transgene to be compared with the endogenous gene in a semi-quantitative manner by RT–PCR. Both the endogenous and the transgenic mRNA are converted to cDNA and then amplified with a single pair of primers. The RT–PCR product is cut separately with the two enzymes, resolved on a gel and the amount of product cut with each enzyme compared (Fig. 3A). As the two products are almost identical one can assume that both products will amplify at the same rate even when the PCR reaction has reached saturation, and the results are thus semi-quantitative.

Messenger RNA was extracted from the sciatic nerves of single and double transgenic mice, and the amount of transgene expression determined by RT–PCR as described above (Fig. 3). In mice carrying only the pmp22 cDNA transgene, there is no detectable product from the transgene in the RT–PCR reaction (product cuts with BssHII but not with SstI; Fig. 3B), indicating that there is very little background expression from this transgene and also that there is no DNA contamination of the mRNA preparations. However, in the double transgenic mice, there is significantly more expression of transgenic than endogenous product (more product cut with SstI than BssHII; Fig. 3B) indicating that the transgene is induced to high levels in this system. There is also undigested DNA when the PCR product is cut with both enzymes and this probably represents heteroduplex DNA made up of the two types of cDNA present, as controls of each product separately cut to completion.

The time course for switching off the pmp22 transgene, in the presence of tetracycline, was also investigated. Doubly transgenic mice were fed tetracycline for 1 or more days and the level of transgene investigated. Transgene expression was completely switched off within 1 day (no digestion with SstI, and almost no heteroduplex; Fig. 3C). This is much faster than the loss of LacZ staining which was found to take place over a 3-week period. However, in LacZ staining we were measuring the mature protein which is probably quite stable when incorporated into the myelin sheath. For the pmp22 gene we are measuring loss of the mRNA, which has decayed within 1 day, but loss of the pmp22 protein will probably be much slower, as for the LacZ protein.

To investigate switching on of pmp22, the mice were fed tetracycline from before birth to at least 2 months of age before withdrawal of tetracycline for different periods of time (Fig. 3D). After 2 weeks, the pmp22 mRNA is just detectable (data not shown). It is still very low at 3 weeks (very little cut with SstI, but considerable amounts of heteroduplex present), nearly equal to endogenous levels at 5 weeks and fully on at 6 weeks. This is slower than the timecourse for switch-on of LacZ. However, the mice were fed tetracycline for only 1 month for the LacZ studies, as opposed to for life to at least 2 months of age for the pmp22 mRNA studies. It is probable that the tetracycline builds up in the mice over time and hence takes longer to clear from the animals if they have been fed for longer.
Overexpression of pmp22 causes dysmyelination

We investigated the phenotype of mice carrying the tTA transgene (YJ13) and the conditional pmp22 cDNA transgene (JP18). The double transgenic mice had a slightly abnormal gait. Motor nerve conduction velocity (MNCV) in the sciatic/tibial nerves was found to be slightly reduced (36, 36.9, 21.6, 37, 41, average 34.5 m/s) compared with non-transgenic littermates (53, 41, 41.6, average 44.15 m/s). Electromyography of calf muscles and small foot muscles was normal in all mice except in the mouse with the lowest conduction velocity (21.6 m/s) where there was sustained fibrillation and intermittent high-frequency discharges indicative of denervation.

We next examined the histology of the double transgenic mice. In 16-week-old mice carrying both transgenes a proportion of the axons (in particular, medium sized and smaller axons) possessed very thin or no myelin (Figs 4D and 5A). In counts taken from cross-sections of sciatic nerve from these double transgenic animals, the percentage of fibres with very thin or absent myelin was 26%, whereas in wild-type mice it was 1% (Fig. 6A). The number of Schwann cell nuclei was also counted as an indicator of disturbed myelination. In affected animals there were on average 88 Schwann cell nuclei (SCN)/1000 axons, which is over three times the normal ratio of 25/1000 (Fig. 6B). Signs of axonal atrophy were not observed and the total axon numbers and the density of fibres in sciatic nerves were not appreciably different between the affected and wild-type mice (data not shown). g ratios (axon diameter/total fibre diameter) were not determined as the small size and very thin myelin on the affected fibres makes the measurements very inaccurate at the light microscope level.

Double transgenic mice are thus clearly distinguishable from the wild-type in having 26% of axons lacking myelin though their gait and MNCV are only mildly affected.

Dysmyelination is reversible by feeding the mice with tetracycline

To determine whether the histology of the doubly transgenic mice is normal when the pmp22 overexpression is switched off throughout development, double transgenic mice were administered tetracycline from at least a week before birth. In adult mice at 16 weeks of age the thickness of the myelin appears nearly normal (Fig. 4E) and the percentage of fibres with very thin or absent myelin was 26%, whereas in wild-type mice it was 1% (Fig. 6A). The number of Schwann cell nuclei was also counted as an indicator of disturbed myelination. In affected animals there were on average 88 Schwann cell nuclei (SCN)/1000 axons, which is over three times the normal ratio of 25/1000 (Fig. 6B). Signs of axonal atrophy were not observed and the total axon numbers and the density of fibres in sciatic nerves were not appreciably different between the affected and wild-type mice (data not shown). g ratios (axon diameter/total fibre diameter) were not determined as the small size and very thin myelin on the affected fibres makes the measurements very inaccurate at the light microscope level.

Double transgenic mice are thus clearly distinguishable from the wild-type in having 26% of axons lacking myelin though their gait and MNCV are only mildly affected.

Correction of the phenotype starts within 1 week of switching off pmp22 overexpression

Eight-week-old double transgenic mice, which would be moderately dysmyelinated due to pmp22 overexpression, were fed tetracycline for different periods of time (Figs 5B and 6). From the RT–PCR results, the transgenic mRNA will have disappeared by 1 day though the protein may be more stable. Myelination had begun in many previously non-myelinated fibres within 1 week of starting tetracycline feeding (the shortest time point investigated; appearances not shown). By 2 weeks almost all the fibres had some myelin (Fig. 5B) but the myelin sheaths of these fibres were still very thin and were included in the ‘abnormally thin’ category so the level of improvement is not reflected in the fibre counts at 2 weeks (Fig. 6A). By 4 weeks (total age of 12 weeks) the percentage of very thinly myelinated fibres had fallen dramatically to ~2%. After 12 weeks of treatment, the percentage was still ~2%, which is only marginally higher than the 1% in mice which had been fed tetracycline from before birth (corrected), but the myelin had still not reached normal thickness.

The number of SCN/1000 axons in correcting animals had reduced to an average of 54/1000, from 88/1000 in the untreated animals, within 2 weeks. Thereafter it stayed fairly uniform (average 52/1000) and slightly higher than the value for animals fed tetracycline for life (corrected, average 45/1000) (Fig. 6B). This is consistent with the shortened internodes (Schwann cell territories) found in remyelinated adult nerve in comparison with normal internodes (25).

Demyelination occurs on upregulation of pmp22

Double transgenic mice were fed tetracycline from before birth to 8 weeks of age; at this point they had developed nearly normal mature myelin due to the pmp22 transgene being switched off. Tetracycline was then withdrawn and the nerves analysed after different lengths of time (Figs 4F, 5C and 6). From the RT–PCR results, mRNA is detectable at 2 weeks (the earliest time-point analysed by RT–PCR), but is not on fully until 6 weeks. One week after stopping tetracycline, and switching on the pmp22 gene (the earliest time-point analysed
by electron microscopy but not counted), the myelin was still largely of normal thickness but a few fibres were undergoing active demyelination and macrophages containing myelin debris were seen (Fig. 5C). After 8 weeks of pmp22 overexpression...
(total age of 16 weeks), a modest proportion (9%) of fibres of all thicknesses had demyelinated, whereas the other axons had nearly normal myelin (Figs 4F and 6A). There appeared to be little further change at 16 weeks. The ratio of SCN/axon did not seem to change with time and was not significantly different from that seen in corrected animals which had been fed tetracycline for life (Fig. 6B). However, as only 9% of fibres are demyelinated, an associated increase in SCN may not be noticeable.

**DISCUSSION**

In order to investigate whether the phenotype of demyelination caused by overexpression of PMP22 is reversible, we have made a conditional mouse model using the tetracycline system described by H. Bujard et al. (18,19). To achieve tissue-specific as well as conditional expression of the pmp22 transgene, we expressed the tTA inducer of the PMP22 gene present in a 560 kb YAC, which we have previously shown can drive full levels of tissue-specific expression in transgenic mice.

The induction of gene expression by the tTA protein was found to be tissue-specific for peripheral nerve, and for Schwann cells in particular, using the lacZ reporter gene. There was no detectable LacZ expression in the tongue, muscle, skin, gut or lung, except where a nerve was present. Detailed analysis of neuronal cell bodies showed no LacZ staining in dorsal root ganglia, cervical spinal cord or brainstem. Previous work using immunohistochemistry onto rodent tissues has indicated the presence of PMP22 in neuronal cell bodies of the spinal cord (26,27), in satellite cells and neuronal cell bodies in dorsal root ganglia (26) and in neuronal cell bodies in brainstem (26). In addition, Pmp22 is normally expressed in all tissues of the body but at much lower levels than found in peripheral nerves (28,29). Most of the non-Schwann cell expression is driven by the promoter upstream of exon 1B while the Schwann cell-specific promoter is upstream of exon 1A. The YAC construct contains both promoters and we might have expected to see weak LacZ staining in a large number of tissues including neuronal cell bodies. However, the level of induced LacZ expression in the double transgenic mice in tissues other than Schwann cells is clearly very low.

Expression of LacZ was strong, but did not occur in all Schwann cells, which is typical of the variegated expression often seen with transgenes. Such variegated expression of transgenes has been shown to be due to high copy numbers of tandem repeats or to a heterochromatic integration site (30). The variegated expression could be acting either on the tTA or the lacZ transgenes. In addition, as we have not studied the transgenic pmp22 mRNA or protein at the cellular level (which would be difficult as it is a mouse pmp22 transgene), we do not know whether the pmp22 expression is similarly variegated. However, the relative number and distribution of fibres with very thin or absent myelin in the affected mice (Fig. 4D) is markedly similar to that of the LacZ staining, suggesting that there is variegated expression of the pmp22 protein as well. Conversely, the patchy distribution of dysmyelination is probably due to variegated expression of the pmp22 protein in some Schwann cells rather than some classes of fibre being more prone than others to dysmyelination.

In the absence of tetracycline there is higher expression of the transgenic pmp22 mRNA than of endogenous mRNA (by
at least 5-fold) in the double transgenic mice, though it is probably unevenly distributed between Schwann cells. This leads to ∼26% of axons possessing very thin or no myelin. The gait and stance of the mice is only slightly affected and MNCV was moderately reduced in two mice, more severely reduced into the demyelinating range in one mouse (21.6 m/s) and normal in one mouse. No evidence of muscle denervation was detected except in the mouse with most severely reduced nerve conduction velocity, where it was found in the small foot but not in the calf muscles. The phenotype is thus less severe than in the transgenic C22 mice, which express about twice as much human as mouse pmp22 mRNA and have a clearly abnormal gait and an average MNCV of 3.7 m/s (17). The milder phenotype is probably due to the uneven distribution of the overexpressed pmp22 mRNA in the present model leaving a majority of fibres unaffected.
In contrast, double transgenic mice fed tetracycline for life have nearly normal myelin in the sciatic nerves, although there are increased numbers of Schwann cells and occasional tomacula. This corresponds to the absence of LacZ staining when the mice are fed tetracycline, and transgenic pmp22 mRNA being undetectable within 24 h of starting feeding the mice tetracycline. Therefore, the mice allow one to switch between a nearly normal phenotype and overexpression of pmp22 causing a phenotype similar to CMT1A.

When overexpression of pmp22 was stopped by administration of tetracycline to 8-week-old mice (26% of axons with very thin or absent myelin), rapid initiation of myelination in the population of previously non-myelinated fibres occurred, with new myelin visible within 1 week and nearly normal myelin after 12 weeks. This is consistent with the timescale of remyelination following artificial demyelination, which usually begins within a few days of the myelin having completely degenerated (31,32). The rapid myelination indicates that overexpression of pmp22 has not caused any long-term damage to the Schwann cells which are poised to commence myelination almost as soon as the pmp22 overexpression is stopped. This is consistent with the observation that overexpression of pmp22 in a transgenic rat model prevents myelination but does not prevent the underlying program of Schwann cell differentiation as indicated by expression of myelin genes (33). However, it does not elucidate the mechanism by which overexpression of pmp22 acts to disrupt normal myelination.

There was clear evidence of rapid demyelination on upregulation of pmp22. Even at 1 week, when the mRNA was still at a very low level, occasional actively demyelinating fibres were observed with associated macrophages containing myelin debris. By 6 weeks, when the mRNA had just reached maximum levels, the percentage of demyelinated axons had clearly risen to ~5% and this had risen to 9% by 8 weeks. Thus demyelination of ~9% of fibres occurs rapidly with the other fibres remaining fully myelinated. The percentage of axons affected may largely reflect the variegated expression of the fibres remaining fully myelinated. The percentage of axons affected may largely reflect the variegated expression of the fibres remaining fully myelinated. The percentage of axons affected may largely reflect the variegated expression of the fibres remaining fully myelinated.

The pathology in the demyelinating nerve also differs from that seen in animals that have been affected from birth. The demyelination following switch-on of pmp22 affects fibres of all sizes (Fig. 4F), whereas in animals overexpressing from birth the large fibre population appears relatively unaffected all sizes (Fig. 4F), whereas in animals overexpressing from birth the large fibre population appears relatively unaffected throughout life may limit the maximum size that can be attained by the axon in the region of affected Schwann cells (34), thus accounting for the small axons which are affected in these mice. In mice in which pmp22 is upregulated, de novo demyelination takes place on fibres of all sizes, including large ones, indicating that mature myelin, even on large axons, is sensitive to the increased expression of pmp22 which causes rapid and complete demyelination. Additionally, in demyelinating animals the abnormally myelinated fibres are frequently surrounded by redundant Schwann cell processes, which are perhaps the initial stages of onion bulb formation, reminiscent of CMT1A patients.

The mechanism of the demyelination is uncertain. Active stripping of myelin by macrophages was not observed but more extensive sampling is needed to establish whether this occurs or whether the myelin breaks down because of structural instability. Radioactive labelling studies have suggested that myelin proteins are extremely stable, up to 250 days (35), and Po, the major myelin protein, was found to be stable up to 72 days, which was the longest time period examined (36). Considering the slow turnover of proteins in mature myelin, the rapid demyelination observed suggests that the increased amount of pmp22 causes active demyelination either by affecting the differentiation state of the Schwann cells or by the production of abnormal myelin which then affects the whole myelin sheath.

CMT1A is a dominantly inherited genetic disorder caused by overexpression of PMP22. The best hope for therapeutic intervention seems to be downregulation of the PMP22 gene which could potentially be achieved by drug or antisense gene therapy. In either case, the rapid remyelination following switching off the overexpression is encouraging. However, treatment of patients will need to be done before significant axonal loss occurs, as it is most likely irreversible, and de novo demyelination is expected when the treatment ceases to work.

MATERIALS AND METHODS

Construction of the tissue-specific tTA construct

To achieve tissue-specific expression of the tTA gene, the tTA ORF was introduced into the PMP22 gene on the YAC 49G7 (14) which is ~560 kb in size and carries the ~40 kb human PMP22 gene flanked by ~100 kb of upstream and ~300 kb of downstream sequences. The tTA ORF was introduced into the YAC by homologous recombination in the yeast host such that it is driven from the AUG initiation codon in exon 2 of the PMP22 gene. The plasmid used to retrofit the tTA gene into the YAC was made as follows. (i) Two halves of PMP22 exon 2 were amplified from genomic mouse DNA and brought together in reversed order by PCR. A first PCR was made with the primers 5′-GGCCTTCTTGCAGGCAAGGCG-3′ and 5′-GATCTGTAAGACCTTGTCGCGG-3′. A second PCR was made with the primers 5′-TTTGAGCTTTGAGGATACAGACGGGTAATTGTTGCTGAGTATCATCGTC-3′ and 5′-GGCTCTCCTCCTGCACGAGCCTGACGATCGTGGAGAC-3′. The above plasmid was linearized with SalI and PstI and the tTA ORF was introduced into the PMP22 gene between the XbaI and NotI sites. (ii) A second fragment was cloned into the SacI-XbaI sites of pBluescript SK+ (Stratagene). (iii) This plasmid was cut with XbaI and HindIII and the 1472 bp XbaI-HindIII fragment of pUHD15-1 (18) was inserted so that the tTA ORF is fused in frame with the PMP22 AUG codon. (iv) Finally, a 2.1 kb SalI fragment from pCH45 carrying the intact yeast HIS5 gene (37) was cloned into the XhoI site in order to allow selection for integration into the YAC in the yeast host.

The above plasmid was linearized with PstI and introduced into yeast carrying the YAC 49G7 by spheroplast transformation (38). His+ positive clones were analysed with one primer in exon 1B and another in the tTA ORF to confirm that the correct insertion event had taken place. The resulting YAC was shown to be of the correct size by pulsed-field-gel electrophoresis.

The transgenic mouse line carrying this construct is called Tgn(Pmp2TA)/JY13Clh and is referred to as JY13.
The pmp22 cDNA and lacZ responder plasmids

A fragment of the mouse pmp22 cDNA was amplified with primers to introduce an EcoRI site at the 3′ end, an XbaI site at the 2′ end and to modify the BssHII site in the 3′–UTR to an Ssfl site by changing a G to a T at position 707 (relative to GenBank accession no. NM_008885). The distal primers used were 5′-GTAGAATTCGAGATACGTTCCCTTTG-3′ and 5′-GTATCTAGTCCATCTCTAGCTGCTCCCTTTG-3′ and PCPF (5′-GTATCT-AGATCTGAGATACGTTCCCTTTG-3′). The lacZ construct was assayed for with the primers, BGALU (5′-ACTATCCCGACCCTTACT-3′) and BGALL (5′-TACCGGCTTCAAGCCACT-3′). The PCR reactions contained 50 mM Tris pH 8.8 (9.2 for the lacZ assay), 375 mM KCl, 6.5 mM MgCl2, 0.5 µM each oligo, Taq polymerase and 125 µM dNTPs. The PCR reaction was initiated with 94°C for 3 min, followed by 30 cycles of 94°C for 40 s, 59°C for 30 s and 72°C for 30 s. This was followed by 72°C for 10 min and then 4°C.

RNA analysis

Samples of sciatic nerve were collected and frozen immediately in liquid nitrogen. mRNA was isolated using the MicroFast-Track kit (Invitrogen) according to the manufacturer’s instructions, and the yield of mRNA from one nerve was usually 1–2 µg. To carry out the first strand cDNA synthesis the Cycle kit (Invitrogen) was used with the conditions recommended by the manufacturer. For a 20 µl reaction 1/4 of the mRNA prep was used.

The cDNA was then used to perform semi-quantitative RT–PCR with a pair of primers which amplify across the BssHII/SsrI site in the pmp22 mRNA. The primers were CPNM1 (5′-GAACTGTACACATCCGCCT-3′) and CB22 (5′-ATCCTCAATCAGACGAATCC-3′). For one 25 µl PCR reaction, 5 µl of cDNA (1/4 of the cDNA reaction) was used. The PCR reaction contained 10 mM Tris–HCl pH 8.3, 50 mM KCl, 2 mM MgCl2, 0.25 mM dNTPs, and 0.5 µM of each primer. Cycling conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 40 s, 55°C for 40 s, 72°C for 30 s and finally 72°C for 10 min. The 540 bp PCR product cuts once with BssHII (in the wild-type) or SsrI (in the transgene) leading to 400 and 140 bp fragments which were resolved on a 1.2% agarose gel. Uncut PCR product after digestion with both enzymes is mostly due to heteroduplex DNA molecules.

Electrophysiology

Measurements were carried out under halothane anesthesia (without recovery). Sciatic/tibial MNCV was measured with recordings obtained via a fine concentric needle electrode inserted into the muscles of the first interosseous space in the foot and stimulation at the sciatic notch and at the ankle. The mice used were all 12 months old.

Histology

Animals were killed by cervical dislocation. The sciatic nerve was exposed and fixed in situ for 15 min [1% paraformaldehyde, 1% glutaraldehyde in 0.1 M piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPERES) buffer] and then removed and fixed for a further 24 h. Tissues were washed in PIPES buffer + 2% sucrose and then postfixed in 1% osmium tetroxide in PIPES buffer + 2% sucrose, 3% sodium iodate and 1.5% potassium ferricyanide overnight. The specimens were dehydrated in increasing concentrations of ethanol and transferred to epoxy resin via 1,2-epoxypropane as an intermediary. Semi-thin

The pmp22 cDNA and lacZ responder plasmids

A fragment of the mouse pmp22 cDNA was amplified with primers to introduce an EcoRI site at the 5′ end, an XbaI site at the 3′ end and to modify the BssHII site in the 3′–UTR to an Ssfl site by changing a G to a T at position 707 (relative to GenBank accession no. NM_008885). The distal primers used were 5′-GTAGAATTCGAGATACGTTCCCTTTG-3′ and 5′-GTATCTAGTCCATCTCTAGCTGCTCCCTTTG-3′. This cDNA fragment was cloned into pUHD10.3, obtained from H. Bujard (19), and cut with EcoRI and XbaI such that the pmp22 cDNA is positioned between the conditional promoter PhCMV*-1 and the SV40 poly(A) site. The cDNA region of this construct was sequenced to ensure that no mutations had been introduced which would possibly cause a dominant negative effect. The pmp22 coding region was found to be as published though which would possibly cause a dominant negative effect. The pmp22 coding region was found to be as published though
sections were cut and stained with thionin and counterstained with acridine orange.

Measurements of fibre diameter were confined to those with a one-to-one axon/Schwann cell relationship. Many fibres had myelin that was too thin to be identified (less than five myelin lamellae) at the light microscope level. For these, the axon was measured and we have termed them ‘fibres with very thin or absent myelin’. The number of Schwann cell nuclei and tomacula in each section were also counted. All measurements were taken from whole fascicles viewed with a ×100 objective and a ×1.25 optivar on a Zeiss Axioplan microscope (Zeiss) connected on-line through a television camera to a Kontron IBAS AT image analyser (Imaging Associates).

Ultra-thin sections were contrasted with lead citrate and methanolic uranyl acetate for electron microscopy.

Staining for LacZ

Tissues from mice were fixed by immersion in 0.4% paraformaldehyde fixative solution (0.4% paraformaldehyde, 0.1 M PIPES pH 7.3, 2 mM MgCl₂, 5 mM EGTA) for 5–6 h at room temperature and then incubated in PBS with 2 mM MgCl₂ and 30% sucrose at 4°C overnight. The tissues were embedded in tragacanth gum, frozen in a cryostat at –29°C overnight in the dark at 37°C, and then transferred to liquid nitrogen for storage. The tissues were sectioned onto poly-L-lysine (0.1% w/v in water, Sigma P8920) coated glass slides, and postfixed in 0.4% paraformaldehyde fixative solution on ice for 10 min. The slides were rinsed with PBS + 2 mM MgCl₂ and washed for 10 min in the same solution at 4°C. They were then placed in detergent rinse (0.1 M sodium phosphate buffer pH 7.3, 2 mM MgCl₂, 0.01% sodium deoxycholate, 0.02% NP-40) for 10 min at 4°C and stained overnight in the dark at 37°C in staining solution (detergent rinse with 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mg/mL X-gal). Finally, the slides were rinsed gently in PBS and mounted in glycerol/gelatin.

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REFERENCES


