Brain (2000), **123,** 222–233

Charcot–Marie–Tooth disease type 1
Molecular pathogenesis to gene therapy

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Summary
Charcot–Marie–Tooth disease type 1 (CMT1) is caused by mutations in the *peripheral myelin protein*, 22 kDa (*PMP22*) gene, *protein zero* (*P0*) gene, *early growth response gene 2* (*EGR-2*) and *connexin-32* gene, which are expressed in Schwann cells, the myelinating cells of the peripheral nervous system. Although the clinical and pathological phenotypes of the various forms of CMT1 are similar, including distal muscle weakness and sensory loss, their molecular pathogenesis is likely to be quite distinct. In addition, while demyelination is the hallmark of CMT1, the clinical signs and symptoms of the disease are probably produced by axonal degeneration, not demyelination itself. In this review we discuss the molecular pathogenesis of CMT1, as well as approaches to an effective gene therapy for this disease.

Keywords: CMT; myelination; Schwann cell axonal interactions; gene therapy

Abbreviations: AVR = adenoviral vectors; BiP/GRP78 = growth related protein, 78 kDa; CMT = Charcot–Marie–Tooth disease; *EGR-2* = early growth response gene 2; MAG = myelin associated glycoprotein; MBP = myelin basic protein; PMP22 = peripheral myelin protein (22 kDa); P0 = protein zero

Introduction
Charcot–Marie–Tooth disease (CMT), a heterogeneous group of inherited peripheral neuropathies, is one of the most common degenerative neurological disorders with a prevalence of 1 in 2500 (Skre, 1974). The most common form of CMT, CMT1, is associated with PNS demyelination as demonstrated by slowed nerve conduction velocities and segmental demyelination on nerve biopsy (Dyck *et al*., 1993). CMT1 is caused by mutations in one of several genes expressed in Schwann cells, the myelin producing cells of the PNS (Aguayo *et al*., 1978). The majority of patients with CMT1, designated CMT1A, have a duplication in the p11-p12 region of human chromosome 17 (Hoogendijk *et al*., 1991; Lupski *et al*., 1991; Raeymaekers *et al*., 1991) which contains the *peripheral myelin protein 22* (*PMP22*) gene, encoding one of the major PNS myelin proteins. A less common form of CMT1, CMT1B, is caused by mutations in the *protein zero* (*P0*) gene, which encodes the major PNS myelin structural protein (Hayasaka *et al*., 1993). Recently, an additional less common form of CMT1 has been found with point mutations in the *early growth response gene 2* (*EGR-2*), or *Krox 20*, encoding a zinc finger transcription factor expressed in myelinating Schwann cells (Warner *et al*., 1998). Finally, an X-linked form of demyelinating CMT, CMTX, is caused by mutations in the *connexin-32* gene, expressed in myelinating Schwann cells but not incorporated into the myelin sheath (Bergoffen *et al*., 1993). Mutations in the *PMP22*, *P0*, *EGR-2* and *connexin-32* genes thus account for most of the cases of demyelinating CMT.

Although considerable progress has been made during the last 10 years in understanding the molecular basis for CMT1, less is known of how these genetic defects cause disease in patients. As will be discussed below, these mutations disrupt myelination and Schwann cell function in a number of complex ways and consequently produce a wide variety of clinical phenotypes. In addition, they also produce secondary axonal damage, which is the major cause of weakness in CMT1. In this review we will discuss our current understanding of the regulation of myelin-specific gene expression in Schwann cells and the relevance of these findings for the molecular pathogenesis and future treatment of CMT1.

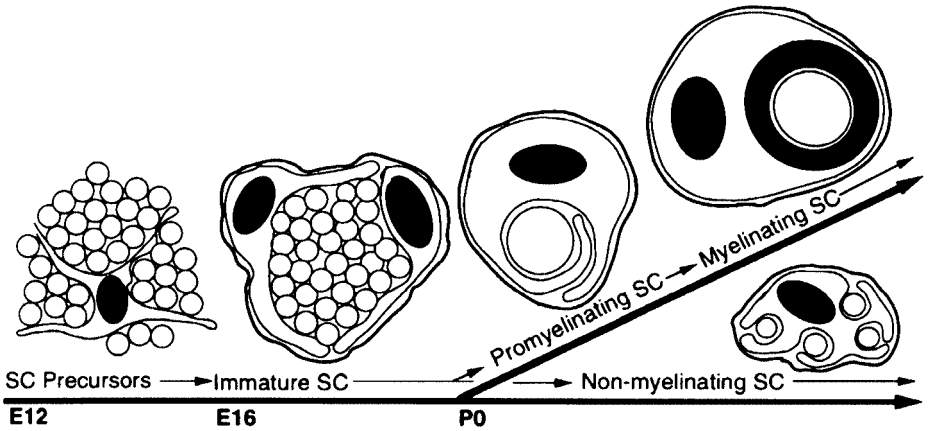

Fig. 1 Schematic representation of the stages of Schwann cell differentiation in rat peripheral nerve; E12 = embryonic day 12; E16 = embryonic day 16; P0 = day of birth. (From Scherer, 1997.)

Biological background

During the development of the PNS, Schwann cell precursors arise from the neural crest, migrate out and contact developing peripheral axons (Harrison, 1924; Le Douarin and Dupin, 1993). These 'immature' Schwann cells then invade and ensheath bundles of developing axons, a process called 'radial sorting' in which they further differentiate into myelinating or non-myelinating Schwann cells (Webster, 1993). During this stage of development, some Schwann cells establish a one-to-one association with an axon, the so-called 'promyelinating stage', a step necessary for myelination to proceed (Webster, 1993). These cells then initiate a programme of myelin-specific gene expression, turning on a set of genes encoding the major myelin proteins and turning off a subset of genes previously expressed (Scherer, 1997). In contrast, immature Schwann cells that do not establish a one-to-one relationship with an axon do not activate the programme of myelin gene expression and become non-myelinating Schwann cells (Webster *et al*., 1983; Mirsky and Jessen, 1990). The differentiation of non-myelinating Schwann cells, however, also depends on continuing interactions with axons, which provide, at least in part, the signal (or signals) necessary to initiate this process (Jessen *et al*., 1987). The stages of Schwann cell differentiation are represented schematically in Fig. 1.

Once myelination has been completed its maintenance also depends on continued Schwann cell–axonal interactions. If a peripheral nerve is cut, severing the axon and its Schwann cells from the neuronal cell body, axons degenerate and demyelination occurs, initiating the process of Wallerian degeneration. During Wallerian degeneration myelinating Schwann cells change their pattern of gene expression, turning off the set of genes encoding the major myelin proteins and turning on the same set of genes expressed prior to myelination as an immature Schwann cell (Scherer and Salzer, 1996). If the nerve is crushed, however, allowing regeneration to occur after Wallerian degeneration, Schwann cell differentiation and myelination can be reinitiated as the axons regenerate through the crushed segment, recontacting 'denervated' Schwann cells (Aguayo *et al*., 1976*a*, *b*; Weinberg and Spencer, 1976). In addition, regenerating unmyelinated axons are ensheathed but not remyelinated, even if the Schwann cells previously synthesized a myelin sheath (Aguayo *et al*., 1976*b*). Both the initiation and maintenance of the myelinating Schwann cell phenotype thus depend on continuing Schwann cell–axonal interactions.

The molecular pathophysiology of CMT1: the role of *Krox 20* and *Oct 6*

The establishment of a one-to-one relationship of a Schwann cell with its axon, the so-called 'promyelinating' stage of development, is a necessary prerequisite for myelination to begin and is regulated through Schwann cell–axonal interactions. For myelination to proceed, however, Schwann cells must then make the transition from the promyelinating to the myelinating stage, a process which depends on Schwann cell–axonal interactions and a sequence of genetically programmed events to upregulate myelin-specific gene expression. In mice lacking the expression of the zinc finger transcription factor, *EGR-2* (also called *Krox 20*), for example, Schwann cells establish a one-to-one relationship with axons but myelination does not occur (Topilko *et al*., 1994), demonstrating that *Krox 20* expression is necessary for this transition. In contrast, in mice lacking the expression of the POU-domain transcription factor, *Oct 6* [also called suppressed cyclic AMP-inducible POU (SCIP) and Tst 1], a similar phenomenon occurs, but myelination is only delayed, not blocked completely (Bermingham *et al*., 1996; Jaegle *et al*., 1996). *Oct 6* is thus not necessary for the transition to myelination, but rather for its timing. Both *Krox 20* and *Oct 6* are important components of the genetically programmed transition from the promyelinating to a myelinating stage of Schwann cell development.

Since *Krox 20* and *Oct 6* are both transcription factors, their role in the transition from the promyelinating to the myelinating stage of Schwann cell development might be to activate the transcription of myelin-specific genes. Consistent

with this notion, new transcription can account for the increase of P0 mRNA and protein both in Schwann cells in culture and during sciatic nerve development (Menichella *et al*., 1999). *In situ* hybridization analysis of Schwann cell gene expression in *Oct 6* and *Krox 20* knockout mice (Murphy *et al*., 1996), however, demonstrates that accumulation of myelin-specific transcripts occurs normally in both animals, even though myelination does not begin. The role of these transcription factors in myelination must therefore be downstream of the initial activation of myelin gene transcription.

Warner and colleagues have recently identified two families with clinical signs and symptoms of CMT1 caused by a point mutation in the *Krox 20* gene. Both of these *Krox 20* mutations segregated as an autosomal dominant trait and both were found in the zinc finger region of the protein which interacts with DNA. A third family with a congenital hypomyelinating neuropathy caused by a *Krox 20* mutation was also identified (Warner *et al*., 1998). This mutation, however, is known to segregate as an autosomal recessive trait and has its location in a region of the protein outside of the DNA binding domain which interacts with a co-repressor, NAB (Svaren *et al*., 1996; Warner *et al*., 1998). The identification of these families thus confirms that *Krox 20* plays an important role in the regulation of peripheral nerve myelination.

Although we do not yet know the molecular mechanisms by which *Krox 20* and *Oct 6* regulate myelination or stimulate remyelination, both proteins clearly are necessary for the timely transition from the promyelinating to the myelinating stage of Schwann cell development. Identification of the downstream genes regulated by *Oct 6* and *Krox 20* will thus be important in the future for understanding the regulation of myelination and to identify molecular targets for the design of treatment strategies for demyelinating neuropathy.

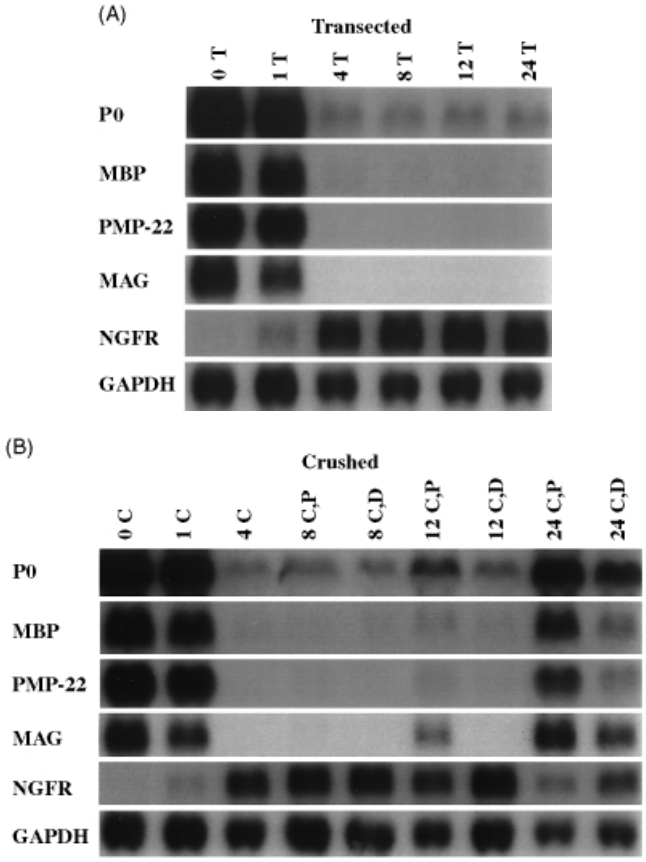

Fig. 2 Northern blot analysis of rat sciatic nerve mRNA. (**A**) RNA isolated distal to a permanent transection; (**B**) RNA isolated distal to a crush injury. The number of days after transection (T) or crush (C) is indicated; the zero time-point is from unlesioned nerves. In crushed nerves, the distal nerve stumps were divided into proximal (P) and distal (D) segments of equal lengths. The blots were successively hybridized with radiolabelled cDNA probes encoding P0, MBP, PMP22 and MAG, expressed by myelinating Schwann cells, and the low affinity nerve growth factor receptor (NGFR), expressed by denervated Schwann cells. Twelve days following crush injury, regenerating axons contract denervated Schwann cells and re-induce myelin gene expression. By 24 days post-crush, regenerating axons have re-induced myelin gene expression in Schwann cells in the distal portion of the distal nerve stump. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were used as a loading control.

The molecular pathophysiology of CMT1: the roles of PMP22 and P0

Once Schwann cells have established a one-to-one relationship with their axon and initiated myelination, the components necessary to assemble a myelin sheath accumulate in a coordinated manner. The mRNAs encoding the myelin proteins, P0, PMP22, myelin basic protein (MBP) and myelin associated glycoprotein (MAG), for example, as well as the mRNAs encoding the cholesterol biosynthetic enzymes, HMG (3-hydroxy-3-methylglutaryl) Co-A reductase and oleoyl-CoA synthase, accumulate with similar kinetics, so that sufficient amounts of both myelin structural proteins and membrane constituents are available during myelin synthesis (Lemke and Axel, 1985; Garbay *et al*., 1998). The pattern of expression of a number of Schwann cell mRNAs during Wallerian degeneration and regeneration is shown in Fig. 2. Since this process is regulated mainly at the transcriptional level, it must be accompanied by the new expression or modification of a unique set of transcription factors. Two of these, *Krox 20* and *Oct 6*, were discussed in the previous section. Other transcription factors involved in this process, however, are not yet known.

The role of PMP22*: the endoplasmic reticulum and the protein misfolding hypothesis*

Regulation of myelination not only requires timely synthesis of sufficient quantities of myelin constituents, but also their coordinated trafficking and assembly. The insertion of PMP22 into the membrane from a pool of protein in the endoplasmic reticulum, for example, is activated by axonal contact through

a sequence on the carboxy terminus of the protein (Pareek *et al*., 1997). Mutations in PMP22 can inhibit transport of the mutant protein through the endoplasmic reticulum to the cell surface (Naef *et al*., 1997; Tobler *et al*., 1999) probably due to protein misfolding (Gow *et al*., 1994; Gow and Lazzarini, 1996). This causes a reduction in the amount of PMP22 available for myelination, producing, at least in part, a 'loss of PMP22 function', similar to that found in mice heterozygous for PMP22 knockout, an animal model for hereditary neuropathy with predisposition to pressure palsy (Adlkofer *et al*., 1997*a*).

Insight into both the regulation of myelin assembly and the molecular pathogenesis of CMT1 has been gained by comparison of the molecular phenotypes among individuals with different *PMP22* mutations. Trembler mice, for example, in which one of the two *PMP22* genes is mutated, are much more severely affected than mice carrying a single copy of a *PMP22* knockout allele (*PMP22+/–*) (Adlkofer *et al*., 1997*b*). Trembler mice have less myelin than *PMP22+/–* animals and the steady-state levels of their myelin-specific mRNAs are dramatically reduced (Garbay *et al*., 1989), giving rise to the 'loss of function' phenotype. Since both the trembler mutation and the *PMP22* knockout allele effectively reduce the amounts of PMP22 available for myelination, there must be an additional, adverse effect of the mutated *PMP22* in trembler other than simple loss of function. This additional effect, called a toxic 'gain of function', is probably caused by interactions of the mutated protein with other cellular constituents, and may account for these differences in phenotype.

One cellular component with which the mutant PMP22 has been shown to interact is normal PMP22 (Tobler *et al*., 1999). Interaction between normal and mutant PMP22 reduces the amount of protein available at the membrane for myelin synthesis, further exacerbating the loss of function phenotype in trembler mice. Other mutant protein interactions are also likely to occur in the endoplasmic reticulum (Naef *et al*., 1997) and may account for other aspects of the trembler phenotype (Perkins *et al*., 1981*a*, *b*), including the dramatic decrease in myelin gene expression. A second cellular component with which PMP22 can interact is P0, the major myelin protein (D'Urso *et al*., 1999). If mutant PMP22 is complexed with P0 during its synthesis or transport, then both proteins could be sequestered in the endoplasmic reticulum. This would also effectively reduce the amount of P0 available for myelin assembly, further exacerbating the loss of function phenotype.

Although accumulation of PMP22 and/or P0 in the endoplasmic reticulum can account for the loss of function phenotype in trembler mice or patients with *PMP22* point mutations, it cannot account for the dramatic decrease in myelin-specific gene expression. A signal transduction cascade between the endoplasmic reticulum and nucleus, however, activated by misfolded proteins, could mediate these changes in gene expression. In yeast, for example, an endoplasmic reticulum-mediated signal transduction pathway has been identified in which a serine-threonine kinase, Ire1, participates (Cox *et al*., 1993). This pathway, called the unfolded protein response pathway, is activated by misfolded proteins in the endoplasmic reticulum and subsequently activates transcription of the gene encoding growth related protein, 78 kDa (*BiP/GRP78*) and other endoplasmic reticulum-resident proteins through activation of the transcription factor, HAC1 (Cox *et al*., 1993; Chapman *et al*., 1998). Treatment of cells with tunicamycin, a drug which inhibits glycosylation of proteins in the endoplasmic reticulum or the overexpression of membrane proteins also activates this pathway (Cox *et al*., 1993). The accumulation of misfolded proteins in the endoplasmic reticulum thus activates a signal transduction cascade which can lead to increased gene transcription of genes encoding resident endoplasmic reticulum proteins.

Treatment of Schwann cells in culture with tunicamycin, as shown in Fig. 3, also dramatically upregulates *BiP* mRNA levels, demonstrating that the unfolded protein response pathway can be activated in these cells. In addition, both PMP22 and P0 levels are reduced in the presence of tunicamycin, suggesting that activation of the unfolded protein response pathway can also affect myelin gene expression. Since Schwann cells *in vitro* are not actively myelinating, however, we also analysed *BiP* and myelin-specific mRNA levels in the sciatic nerves from trembler mice, both during development and in adults after myelination had been completed. Although all the myelin-specific mRNA levels were significantly reduced in trembler sciatic nerve, as predicted (Garbay *et al*., 1989), *BiP* mRNA was not increased (data not shown). Interestingly, recent studies from Naef and colleagues demonstrate that mutant PMP22 is sequestered in the endoplasmic reticulum after transfection into Cos cells and that the protein is localized in the endoplasmic reticulum in trembler sciatic nerve (Naef *et al*., 1997). Other proteins, however, such as the nuclear protein CHOP/GADD153, can be upregulated as a part of the unfolded protein response without the concomitant upregulation of *BiP* (Wang *et al*., 1998), and *BiP* is not always upregulated in response to misfolded proteins (Graham *et al*., 1990). These results thus do not rule out the activation of the unfolded protein response pathway in trembler Schwann cells, and it is likely that misfolded or mutant PMP22 molecules exert their toxic gain of function effect, at least in part, by way of an endoplasmic reticulum-mediated signalling cascade which does not include the upregulation of *BiP*.

Not only does the endoplasmic reticulum participate in the regulation of membrane protein biosynthesis, it is also a central location for the regulation of cholesterol and lipid biosynthesis. As shown by the work of Brown and Goldstein, SREBP, an endoplasmic reticulum-resident membrane protein, is proteolytically cleaved in the absence of cholesterol to release a carboxy-terminal fragment containing a bHLH (basic helix-loop-helix) transcription factor. This factor is then transported to the nucleus where it upregulates the expression of the genes involved in cholesterol biosynthesis,

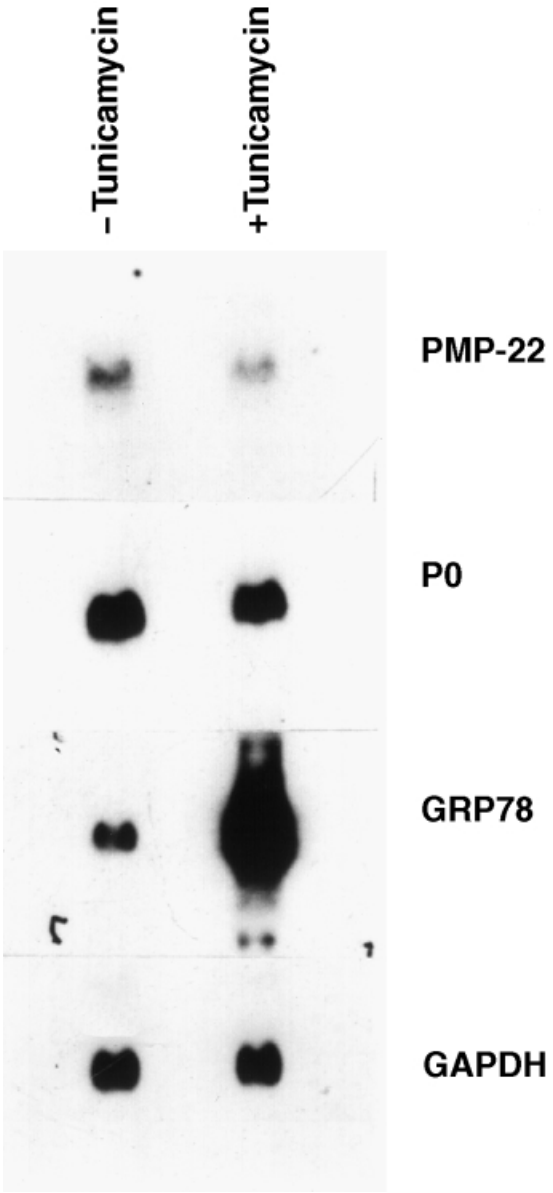

Fig. 3 Northern blot analysis of RNA prepared from secondary rat Schwann cells grown in the presence (+ tunicamycin) and absence (– tunicamycin) of tunicamycin for 8 h. The blots were successively hybridized with radiolabelled cDNA probes encoding P0, PMP22, GRP78 and glyceraldehyde phosphate dehydrogenase (GAPDH).

ensuring the proper concentration of cholesterol for membrane synthesis (Brown and Goldstein, 1997). Since endoplasmic reticulum-mediated pathways can regulate membrane protein folding, and cholesterol and lipid biogenesis, both of which are central to the myelination process, these pathways must also be involved in the regulation of myelination. In addition, they must be able to communicate with each other so that membrane protein and cholesterol synthesis can be coordinated. Consistent with this hypothesis, Toews and colleagues have demonstrated that tellurium, a compound which blocks one of the steps of cholesterol biosynthesis, also produces a demyelinating neuropathy in developing rats, with concomitant changes in myelin gene expression (Toews *et al*., 1991, 1992). Since the overall process of myelination is controlled predominantly at the transcriptional level, it is likely that an endoplasmic reticulum-mediated signal transduction cascade participates in the regulation of myelin gene transcription.

The role of P0*: an adhesion-mediated regulatory pathway?*

P0, the major PNS myelin protein, is a member of the immunoglobulin supergene family of transmembrane proteins and contains a single immunoglobulin domain (Lemke and Axel, 1985). P0 has been demonstrated to mediate homophilic adhesion in cultured cells (Filbin *et al*., 1990), and X-ray crystallographic analysis of the extracellular domain of P0 suggests that it performs a similar function in myelin (Shapiro *et al*., 1996). Interestingly, P0 expression in chickens occurs in the neural crest prior to the appearance of immature Schwann cells, suggesting that P0 may subserve a function other than as a structural protein (Bhattacharyya *et al*., 1991).

In order to evaluate further the role of P0 in myelination, we have analysed the pattern of myelin gene expression in mice lacking P0 due to inactivation of the *P0* gene by homologous recombination (Giese *et al*., 1992). These studies, shown in Fig. 4, demonstrate that the absence of P0 produces a unique Schwann cell phenotype. In contrast to *PMP22* knockout animals in which the myelin-specific gene products are coordinately reduced (Adlkofer *et al*., 1995), *P0* knockout animals do not express PMP22, have a 7-fold increase in both MAG and proteolipid protein expression, but have no change in MBP expression. These data thus confirm that P0 plays a regulatory as well as a structural role in myelination.

Since disruption of other adhesion molecules in the *P0* knockout animals might be involved in producing the unique Schwann cell phentoype, we also analysed the localization of MAG and E-cadherin in these animals. Both of these adhesion molecules are differentially expressed in myelinating Schwann cells and localized within the non-compacted region of myelin, within the paranodal loops and Schmidt–Lanterman incisures (Scherer, 1996). Because β-catenin is also required for cadherin-mediated adhesion (Lilien *et al*., 1997), we also analysed its localization. These data, shown in detail by Menichella and colleagues (Menichella *et al*., 1999), demonstrate that MAG is localized normally in the *P0* knockout nerve in the paranodal loops and Schmidt–Lanterman incisures. In contrast, both E-cadherin and β-catenin are abnormally localized. E-cadherin is not found within the paranodal loops or Schmidt–Lanterman incisures, but within many small punctate areas along the Schwann cell process; β-catenin is not co-localized with E-cadherin, but is found concentrated around the nucleus. These data demonstrate that both E-cadherin-mediated adhesion and signalling have also been disrupted in the *P0* knockout animals.

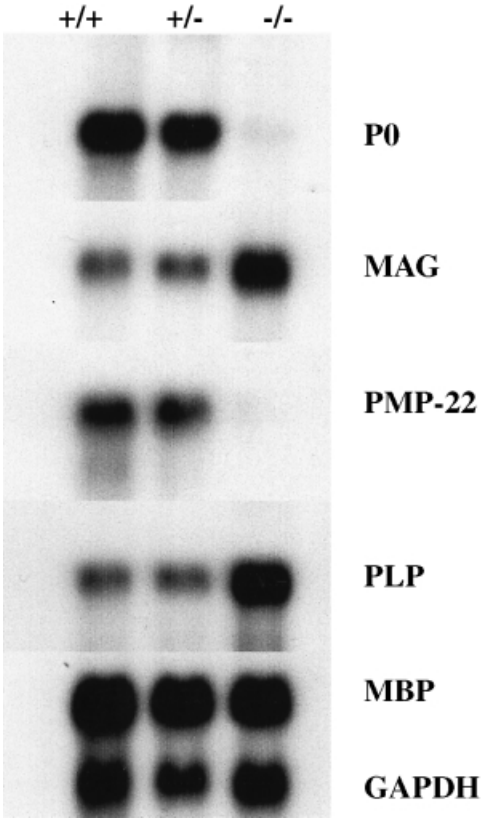

Fig. 4 Northern blot analysis of sciatic nerve mRNA prepared from normal (+/+), heterozygous (+/–) and homozygous (–/–) *P0* knockout mice. In heterozygous knockout mice P0 mRNA levels are reduced by 50% but the levels of other myelin specific mRNAs remain unchanged compared with wild type. In homozygous knockout mice the programme of myelin gene expression is dysregulated: PMP22 mRNA levels are dramatically downregulated, MAG and proteolipid protein (PLP) mRNA levels are upregulated, while MBP levels are unchanged. Glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA levels are used as a loading control. (From Menichella *et al*., 1999.)

How does P0 participate in the regulation of myelination? There are two main possibilities, one direct, the other indirect. P0 could regulate myelination directly through a P0-mediated signal transduction cascade similar to that associated with the cadherins or integrins (Gumbiner, 1996). In this model, P0-mediated adhesion would depend upon the interactions between the P0 cytoplasmic domain and the cellular cytoskeleton. Modulation of these interactions could thus alter both P0-mediated adhesion and myelin gene expression due to a downstream cascade of events triggered by these changes. Consistent with this hypothesis, mutations in the intracytoplasmic domain of P0 have been shown to reduce P0-mediated adhesion *in vitro* (Wong and Filbin, 1994) and to produce particularly severe forms of CMT1B (Warner *et al*., 1996; Mandich *et al*., 1999). P0 could also regulate myelination indirectly by way of cadherin or integrin-mediated adhesion and signal transduction processes. In this model, P0-mediated adhesion would be necessary for both myelin compaction and the correct localization of other adhesion molecules expressed in myelinating Schwann cells, such as E-cadherin (Fannon *et al*., 1995) and/or β4 integrin (Feltri *et al*., 1994). Consistent with this hypothesis, we have shown that E-cadherin signalling is disrupted in the *P0* knockout nerve. In contrast, E-cadherin signalling is not disrupted in the sciatic nerves of trembler and quaking mice (data not shown), both of which have genetically inherited demyelinating peripheral neuropathies but express P0. Regardless of which model is correct, however, a P0-mediated signal transduction cascade is clearly involved in the regulation of myelination, including myelin gene expression and possibly myelin gene transcription.

Summary of the regulation of myelination: relevance to CMT1

Although the overall regulation of myelination is quite complex, a focal point of this process, both during development and remyelination, is the regulation of myelin gene transcription. Myelin gene transcription can be modulated through the activity of at least three signal transduction pathways. One pathway, activated by axon–Schwann cell interactions, is required for the establishment of the promyelinating stage of Schwann cell development, the transition to the myelinating phenotype and the maintenance of this phenotype. Changes in myelin gene expression produced by this pathway, as we have seen with *Oct 6* expression, can also alter the ability of Schwann cells to respond to axonal signals, thus driving forward the process of development or remyelination. A second pathway, localized within the endoplasmic reticulum and sensitive to cholesterol and misfolded membrane proteins, is required for the co-ordination of lipid and protein biosynthesis. A third pathway, mediated in part by P0 and possibly localized within the assembling myelin sheath or at the paranodal region, also participates in the coordinate regulation of myelin gene expression by measuring successful myelin assembly and compaction. A schematic representation of the pathways involved in myelin biogenesis is shown in Fig. 5.

Disruption of each of the three signal transduction pathways can cause CMT1. Loss of *Krox 20* expression, for example, causes CMT1 by blocking the transition from the promyelinating to the myelinating stage of Schwann cell development, a process regulated by an axonally mediated signal transduction cascade. Point mutations in *PMP22* cause CMT1 by disrupting protein folding in the endoplasmic reticulum, where the mutant proteins eventually accumulate. This produces both a decreased amount of PMP22 available for myelin assembly and probably a change in the endoplasmic reticulum-nuclear signal transduction cascade, further downregulating myelin transcription. Loss of P0 expression causes CMT1 by interfering with the myelin compaction process, as well as by causing a change in an adhesion-mediated signal transduction cascade, thereby further dysregulating myelin gene expression. CMT1 is thus

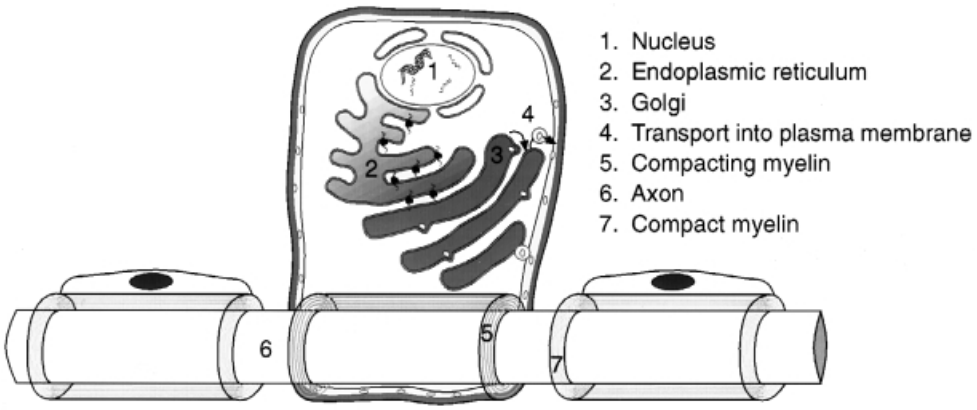

Fig. 5 Schematic representation of Schwann cell myelination. The processes of transcription, translation, protein trafficking, vesicular transport and myelin compaction are indicated. Abnormalities at the level of transcription for *EGR-2* point mutations (1); protein trafficking for *PMP22* point mutations (2–4); and compaction for P0 loss of function mutations (5) are hypothesized to cause CMT1.

the result of the disruption of one of several separate but interacting cellular pathways, each required for normal myelination to occur. Selective molecular therapy to repair the Schwann cell defect in CMT1, an important goal for the future of CMT research, will thus clearly require further understanding of the molecular mechanisms involved in these complex regulatory pathways, in both normal and demyelinated nerves.

The molecular pathogenesis of CMT1: the role of axonal degeneration

Although demyelination is the pathological and physiological hallmark of CMT1, the clinical signs and symptoms of this disease, weakness and sensory loss, are probably produced by axonal degeneration, not demyelination. Children with CMT1, for example, have slow nerve conduction velocities prior to the onset of symptoms and these velocities do not change appreciably as the disease progresses, suggesting that demyelination per se is not sufficient to cause their neurological signs and symptoms (Gutman *et al*., 1983; Garcia *et al*., 1998). In addition, Krajewski and colleagues have demonstrated that compound motor action potential amplitudes, not nerve conduction velocities, correlate best with weakness in patients with CMT1A, again suggesting that axonal loss is the cause of weakness (Krajewski *et al*., 1999). Finally, there is anatomical evidence of progressive length-dependent axonal loss in CMT1 (Dyck *et al*., 1974; Gabreels-Festen *et al*., 1992) as well as in mice overexpressing *PMP22* (Sancho *et al*., 1999), an animal model of CMT1A. Taken together, these data strongly suggest that distal axonal degeneration, not demyelination, is the major cause of clinical disability in CMT1. What is the molecular pathogenesis of this phenomenon?

A number of studies have demonstrated that Schwann cell–axonal interactions are disrupted in demyelinating peripheral neuropathies, including CMT1, causing significant changes in axonal physiology. de Waegh and Brady, for example, have demonstrated that trembler mice, which have a demyelinating peripheral neuropathy due to a point mutation in *PMP22* which is similar to CMT1A, have significant changes in both axonal structure and function, including alterations in neurofilament phosphorylation, increased neurofilament density and decreased axonal transport (de Waegh and Brady, 1990), and similar changes have been found in patients with CMT1 (Watson *et al*., 1994). Transplantation of a segment of trembler (Aguayo *et al*., 1977) or CMT1A (Sahenk *et al*., 1999) nerve into normal nerve also produces similar changes in axons that have regenerated through the nerve graft, but not in the surrounding nerve, demonstrating that this effect is induced by contact with the abnormal Schwann cells. Finally, neurofilament packing density is increased and axonal calibre is decreased at the node of Ranvier of normal nerve, where there is no axonal contact with Schwann cells, compared with the adjacent myelinated internode (Hsieh *et al*., 1994). These data thus convincingly demonstrate that axonal contact with myelinating Schwann cells has a significant effect on underlying axonal physiology in both normal and abnormal nerve. Changes in the nature of this interaction, such as those that occur in CMT1 or other demyelinating peripheral neuropathies, leads to changes in neurofilament packing density, phosphorylation and axonal transport, and subsequently axonal degeneration. Whether the changes in neurofilament packing density, phosphorylation and axonal transport directly cause axonal damage, however, or are merely a marker of some other more fundamental axonal abnormality, is not currently known.

What is the nature of the Schwann cell-derived signal that interacts with axons to modulate neurofilament packing density, neurofilament phosphorylation and axonal transport? Unfortunately, neither the identity of this molecule (or molecules) nor its axonal receptor are currently known. The signal must be altered or modified in demyelinating Schwann cells, since we know these cells can induce changes in axonal

physiology causing axonal degeneration. Since the signal is also produced by normal Schwann cells, however, it is also likely that the molecule (or molecules) necessary for this signal is expressed as a part of the coordinated programme of myelin-specific gene expression.

The fact that demyelinating Schwann cells can produce secondary axonal damage is important for the treatment of CMT1, since this damage is the major cause of weakness and disability in the disease. As we have seen in the previous sections, the regulation of myelination is complex, and different mutations have distinct physiological effects on Schwann cells. For this reason, correction of the Schwann cell defect in CMT1, including CMT1A, will also be complex and might require different interventions for different classes of mutations. Prevention of axonal degeneration in CMT1, however, might be accomplished simply by supplying sufficient quantities of an exogenous growth factor, such as ciliary neurotrophic factor or glial derived growth neurotrophic factor (Oppenheim *et al*., 1995; Yan *et al*., 1995), that is known to rescue degenerating motor neurons. This approach would be applicable to all types of CMT1, regardless of the mutation, and would not depend on understanding the complex molecular pathophysiology of demyelination. In addition, it might prevent the onset of neurological signs and symptoms if given early in the course of the disease, or improve them due to axonal regeneration if given later. Efforts both to understand the effects of myelinating Schwann cells on their axons and to promote axonal regeneration or prevent axonal degeneration are thus central for the future development of a rational molecular therapy for CMT1.

Towards a gene therapy for CMT1

One of the major goals in studying the molecular pathogenesis of CMT1, as stated above, is to develop rational molecular or gene therapies for this disease. The development of a gene therapy approach, however, will also require the development of an efficient genetic delivery system for the PNS. This system should be capable of efficiently delivering gene products to both dividing and non-dividing Schwann cells without significant cellular toxicity.

Recombinant adenoviral vectors (AVR) can be used to deliver exogenous genes to Schwann cells in the PNS of experimental animals and might thus be useful in the future in patients with CMT1. AVR have a number of advantages over other vectors currently used for gene delivery, such as retroviruses. They can be grown to very high titre and express high levels of transgene product. In addition, unlike retroviral vectors, AVR can infect both dividing and non-dividing cells. This is important in the PNS where Schwann cells and neurons are both post-mitotic. Finally, AVR do not integrate into host cell DNA, eliminating the possibility of insertional mutagenesis.

We have demonstrated previously that first generation (E1- and E3-deleted) AVR can transduce expression of the

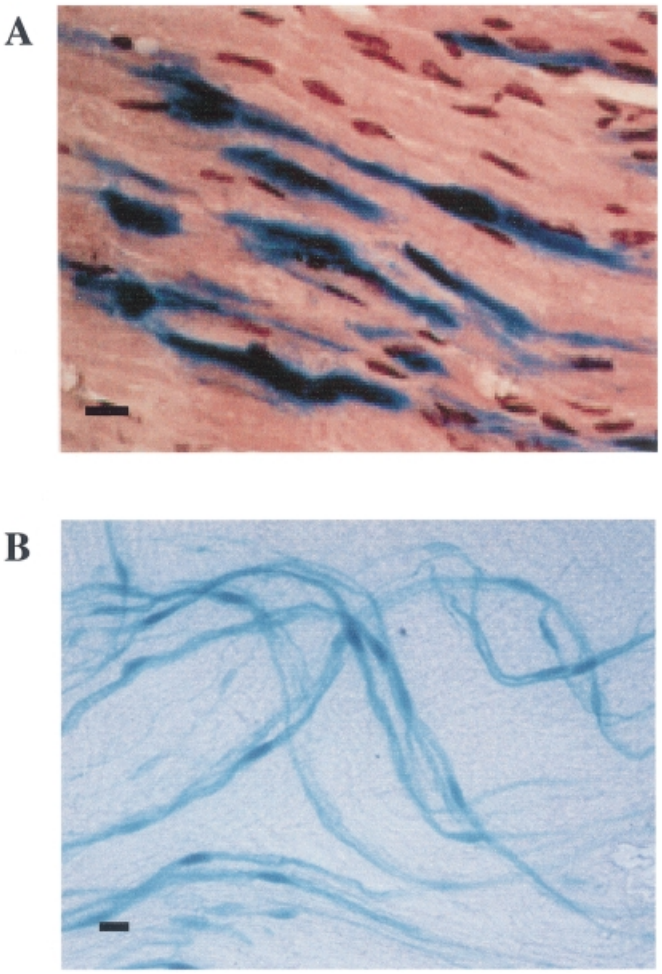

Fig. 6 Adenoviral-mediated gene expression in sciatic nerve of adult mice treated with the immunosuppressing agent FK506 after intraneural injection of recombinant adenovirus, AVRCMV*lacZ*. Transgene expression was determined in sciatic nerve sections (**A**) and teased fibres (**B**) 30 days following injection of 10^9 plaque forming units of AVRCMV*lacZ* by X-gal staining. Scale bar = 10 μm.

Escherichia coli lacZ gene efficiently into Schwann cells, both *in vitro* and *in vivo* (Shy *et al*., 1995). In addition, adenoviral-mediated gene expression persists for >1 month in nerves of adult animals if cellular immunity is suppressed (Jani *et al*., 1999), suggesting that the major barrier to prolonged expression is mainly immunological. AVR have also been used to introduce transgenes into denervated Schwann cells following nerve lesion (Sorensen *et al*., 1998) as well as into Schwann cells of *P0* knockout mice (Guenard *et al*., 1999). An example of adenoviral-mediated gene expression after intraneural injection into rodent sciatic nerve is shown in Fig. 6.

Not only can AVR transduce Schwann cells in the PNS, they can also alter motor neuron gene expression following intramuscular injection of virus (Acsadi *et al*., 1995; Ghadge *et al*., 1995; Gravel *et al*., 1997), perhaps by way of retrograde axonal transport. Consistent with this result, an AVR expressing a secreted form of ciliary neurotrophic factor has

been shown to increase the mean life span and reduce phrenic nerve degeneration after intramuscular injection into *pmn* (progressive motor neuronopathy) mice (Haase *et al*., 1999). Taken together, these data demonstrate that AVR-mediated gene transfer can be used to modify gene expression in the major cellular constituents of the PNS, including motor neurons, Schwann cells and muscle.

The development of a newer generation of AVR with reduced numbers of adenoviral genes will probably reduce the immunological barriers to long-term AVR gene expression (Mitani *et al*., 1995; Clemens *et al*., 1996; Kumar-Singh and Chamberlain, 1996; Lieber *et al*., 1996; Hardy *et al*., 1997) For example, a high capacity ('gutted') vector, in which all viral coding sequences have been deleted, can mediate expression of human a1-antitrypsin in the liver of C57Bl6/J mice for up to 10 months (Schiedner *et al*., 1998). In addition, improved adeno-associated viral vectors, which have no viral-specific gene products but can carry less foreign DNA than AVR, have also been used to mediate long-term gene expression without a significant host immune response (Kessler *et al*., 1996; Clark *et al*., 1997) In fact, Greelish and colleagues have stably restored the sarcoglycan complex in dystrophic muscle of the *Mdx* mouse using intravenous administration of a recombinant adeno-associated viral vector along with histamine (Greelish *et al*., 1999).

Future prospects

Studies of the pathophysiology of CMT1 have demonstrated the existence of several signal transduction pathways, both intracellular and extracellular, involved in the regulation of myelination and which are disrupted in CMT1. Further understanding of the molecular pathophysiology of these pathways will provide targets for the design of molecular therapies. In addition, adenoviral vectors have been shown to be able to mediate gene transfer into Schwann cells, motor neurons and muscle. Although viral-mediated gene expression is mainly limited by the host immunological response, newer vectors with reduced numbers of viral genes may eliminate this problem entirely. Future prospects for the development of an effective and safe therapy for CMT1 are thus promising.

Acknowledgements

M.E.S., J.K. and D.M. were supported by funds from the Muscular Dystrophy Association. A.J. was the recipient of the Armington and Buuck fellowships of the Charcot–Marie–Tooth Association. S.S.S. was supported by funds from the National Institutes of Health.

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Received June 10, 1999. Revised August 27, 1999.
Accepted August 30, 1999