NOTE TO USERS

Regulation of The Peripheral Myelin Protein-22 Gene

Wayel Orfali

Department of Neurology and Neurosurgery
McGill University, Montreal

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to everyone suffering from a myelin disorder

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Abstract

Peripheral Myelin Protein-22 (PMP22) is a relatively major component of peripheral nerve myelin. Either missense mutations or duplication of the *PMP22* gene can be the cause of Charcot-Marie-Tooth disease type 1 A (CMT1A). The control of *PMP22* expression could be a basis for treatment. The aim of my research is to understand the regulation of the *PMP22* gene in a transgenic mouse model. Initially, –8.5 kb 5` flanking sequence of the rat *Pmp22* gene was used to drive reporter gene expressed in transgenic mice. This construct confers tissue specificity, weak developmental regulation, weakly detectable levels of expression in the dorsal root ganglia (DRG), but not the expected up-regulation during nerve regeneration. Secondly, I generated transgenic mice using –21 kb 5` *Pmp22* flanking sequence. This construct confers robust reporter gene expression in the peripheral nervous system (PNS), and little to the DRGs in one line. The other lines show weak expression in the PNS, and stronger expression in the DRGs. Thirdly, I targeted a 21 kb *Pmp22* promoter/reporter transgene to the hypoxanthine phosphorybosyl transferase (*Hprt*) locus in male embryonic stem cells. Using this methodology, I obtained a line carrying –21 kb of *Pmp22* promoter driving β-galactosidase gene in the *hprt* locus. This line confers expression similar to the four lines derived by pronuclear injection carrying the same construct. Fourthly, I introduced the *LacZ* reporter gene at the start codon of *Pmp22* in a 100 kb bacterial artificial chromosome (BAC). Six transgenic lines were produced and analyzed, showing embryonic and postnatal developmental regulation, and high level of expression in the PNS similar to endogenous *Pmp22* expression. From my results and the literature, I suggest that (1) the stretch of –10.5 to –21 kb could carry repressor(s), (2) there is (a) positive element(s) that lie(s) between –8.5 and –10.5 kb, and (3) 3` element(s) are important for *Pmp22* expression.

Résumé

La proteine de myeline peripherique-22 (PMP22) est relativement une majeure composante de la myeline du systeme nerveux peripherique. Les mutations ou la duplication de la *PMP22* semble etre la principale cause de la maladie Charcot-Marie Tooth de type 1A (CMT1A). Le contrôle de l'expression genetique de la PMP22 pourrait etre une modalite de traitement a cette maladie. L'objectif de ma recherche est de comprendre la regulation du gene de la PMP22. Premierement, une sequence de -8.5 Kb 5' du gene de la *Pmp22* chez le rat a ete utilise pour amener l expression le gene indicateur chez des souris transgenique. Cette construction produit la specificite du tissu, une faible regulation developpementale, un niveau decelable de l'expression dans la racine dorsale ganglionnaire (RDG) mais non la haute regulation pendant la regeneration du nerf qui etait attendue. Deuxiemement, j'ai utilise une sequence de –21 kb 5` chez les souris pour obtenir des souris transgeniques. Cette construction produit une forte expression genetique dans le systeme nerveux peripherique et une faible expression dans la racine dorsale ganglionnaire dans une simple lignee de souris. Les autres lignees montrent une faible expression dans le systeme neveux peripherique and une plus forte expression dans la racine dorsale ganglionnaire. Troisiemement, j'ai cible a l'aide d'un gene indicateur/promoteur de 21 kb de la *Pmp22* le locus d' hypoxanthine phosphorybosyl transferase (*hprt*) dans les cellules stem enbryonnaires males. En utilisant cette methodologie, j ai obtenu une lignee, contenant un gene promoteur de –21 kb de la *Pmp22* produisant au gene β-Galactosidase dans le locus de *hprt*. Cette lignee a une expression similaire aux quatre lignees derivees de l'injection pronucleaire contenant la meme construction. Quatriemement, j'ai introduit le gene indicateur *LacZ* au premier codon de la *Pmp22* dans un « Bacterial Artificial Chromosome » (BAC) de 100 kb. Six lignees transgeniques sont produites. Leur analyse rapporte une regulation developpementale embryonnaire et postnatale ainsi qu'une niveau eleve de l'expression dans le systeme nerveux peripherique similaire a l'expression de PMP22 endogene. Base sur la litterature anterieure et mes resultats, je suggere que la region entre –10.5 kb et 21 kb pourrait porter le ou les represseurs. De

plus, il y a un ou des elements positifs qui sont situes dans le region entre –8.5 kb et -10.5 kb ainsi que des elements 3` tres important pour l'expression de la *PMP22*.

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Abbreviations

BAC	Bacterial Artificial Chromosome
cAMP	Cyclic Adenosine Monophosphate
CAT	Chloramphenicol Acyl Transferase
CHN	Congenital Hypomyelination Neuropathy
CMT	Charcot-Marie-Tooth disease
CMTX	Charcot-Marie-Tooth X-linked disease
CNS	Central Nervous System
CRE	cAMP Response Element
CREB	cAMP Response Element Binding protein
Cx32	Connexin 32
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
DSS	Dejerine-Sottas syndrome
EGR2	Early Growth Response Protein 2
EM	Electron Microscopy
Gas-3	Growth Arrest Specific Gene-3
HMSN	Hereditary Motor-Sensory Neuropathy
HNPP	Hereditary Neuropathy with liability to Pressure Palsies
hprt	Hypoxanthine Phosphorybosyl Transferase
MAG	Myelin Associated Glycoprotein
MBP	Myelin Basic Protein
MPZ	Myelin Protein Zero
mRNA	Messenger ribonucleic acid
MTMR2	Myotubularin-related protein-2
NDRG1	N-myc Downstream-Regulated Gene 1
NFL	Neurofilament protein of Light molecular weight
P0	Myelin Protein Zero
PCR	Polymerase Chain Reaction

PLP	Proteolipid Protein
PMP22	Peripheral Myelin Protein 22 KD
PNS	Peripheral Nervous System
PRX	Periaxin gene
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SCIP	Suppressed cAMP-Inducible POU Protein
UTR	Untranslated Region
YAC	Yeast Artificial Chromosome

Chapter I: General Introduction

Charcot-Marie-Tooth (CMT) disease is the most common hereditary peripheral neuropathy in humans with a prevalence of approximately 1/3000 (Skre, 1974). In 1886, Jean Martin Charcot, Pierre Marie and separately Howard Henry Tooth described a unique progressive hereditary syndrome causing weakness and atrophy most strikingly in the peroneal muscles of the lower legs, hence the original name of peroneal muscular atrophy (Charcot and Marie, 1886.), later to become known as Charcot-Marie-Tooth disease (CMT). Typically, patients with CMT present in the second decade of life with progressive weakness and atrophy of their distal extremities (for review, see Lupski et al., 1993). Clinical signs of a hereditary neuropathy such as hammertoes and pes cavus deformity are often present. Sensory deficits referable to peripheral nerve dysfunction also coexist. There is significant clinical variability within kindreds and even between identical twins with CMT (Garcia et al., 1995).

1-1 PMP22 is a CMT Disease Gene

Despite the marked clinical variability, it is clear, both from a clinical and a genetic standpoint, that CMT is a heterogeneous group of diseases. CMT can be classified into subtypes on either a clinical or genetic basis. The existence of two parallel classification schemes, however, creates some confusion. Clinically, CMT has been subdivided into CMT1 and CMT2, predominantly on the basis of the conduction velocities of affected nerves, with CMT1 having markedly reduced conduction velocities while patients with CMT2 display near normal conduction velocities. Hence, CMT1 is often referred to as a "demyelinating" neuropathy. CMT1 also shows clear genetic heterogeneity, CMT1 has been linked to over 12 loci where to date 6 genes have been identified, peripheral myelin protein of 22 kD (*PMP22*), connexin 32 (*Cx32*), myelin protein zero (*MPZ*), early growth response gene 2 (*EGR2*), myotubularin-related protein-2 gene *MTMR2* (Bolino et al., 2000), and N-myc-downstream-regulated gene 1 *NDRG1* (Kalaydjieva et al., 2000). CMT2 has been linked to four loci. Recently the low molecular weight neurofilament gene (NF-L) has been identified as a CMT2 gene

(Mersiyanova et al., 2000). In addition, rare mutations affecting *MPZ* and several mutations affecting CMTX also cause a CMT2 phenotype. CMT2A is linked to 1p35-36, CMT2B to 3q13-22, CMT2D to 7p14, and HMSNP to 3q13.1. CMT2C could not be linked to any of these loci suggesting the existence of at least one additional locus (Nagamatsu et al., 2000). CMT3 appears to be a more severe form of CMT1 and, indeed, mutations in *PMP22* and *MPZ* genes have been identified in this severe early-onset peripheral neuropathy (also known as "Dejerine-Sottas syndrome"). CMT4 is an autosomal recessive demyelinating type of CMT. CMT4 is genetically heterogeneous: CMT4A is linked to 8q13-21.1 (Ben Othmane et al., 1993); CMT4B which includes CMT4B1 linked to 11q22 (Bolino et al., 1996) is caused by mutations in the *MTMR2* gene encoding myotubularin-related protein-2 (Bolino et al., 2000), and CMT4B2 linked to 11p15 (Ben Othmane et al., 1999); CMT4C linked to 5q23-33 (LeGuern et al., 1996), CMT4D (HMSNL) linked to 8q24 (Kalaydjieva et al., 1996) as N-myc Downstream-Regulated Gene 1 (8q24.3) mutation in *NDRG1* has now been identified as the cause for HMSNL (Kalaydjieva et al., 2000); and CMT4E is linked to 10q21-22; and CMT4F is linked to a locus on 19q13.1-13.3 (Delague et al., 2000) now known to contain the periaxin gene (*PRX*) locus. In 3 unrelated patients with Dejerine-Sottas neuropathy, Boerkoel et al. (2001) identified recessive mutations in the *PRX* gene that can cause Dejerine-Sottas neuropathy (Boerkoel et al., 2001). Clearly, further investigation of the Charcot-Marie-Tooth related neuropathies would identify a large number of genes that specifically affect the function of the peripheral nervous system.

The majority of patients with Charcot-Marie-Tooth disease have autosomal dominant CMT1A (Lupski et al., 1993). The discovery that *PMP22* is the major gene responsible for this neuropathy arose from two parallel lines of inquiry. First, Shooter and colleagues (Welcher et al, 1991 or 1992 PNAS) cloned differentially expressed genes that are down-regulated by nerve injury. One of these genes, *PMP22* (also known as SR13, *gas-3*, PAS-II, and CD25) was characterized as a protein component of compact myelin (Snipes et al., 1992). Shortly thereafter, Shooter's group identified mutations in the murine *Pmp22* gene that are responsible for the hereditary neuropathies in the

Trembler and *Trembler-J* mice (Suter et al., 1992; Suter et al., 1992). Concurrently, Lupski showed that human CMT1A was caused by a 1.5 Mb intrachromosomal duplication at Chr 17p11.2-12 (Lupski et al., 1991; Lupski et al., 1993). These lines of inquiry converged when these (Patel et al., 1992) and several other laboratories (Matsunami et al., 1992; Timmerman et al., 1992) demonstrated that *PMP22* mapped within the CMT1A duplicated region. That *PMP22* is the CMT1A disease gene was confirmed by the demonstration that mutations in *PMP22* are associated with a CMT1A phenotype (Roa et al., 1993) and by the demonstration of a peripheral neuropathy in transgenic mice expressing multiple copies of the *PMP22* gene (Magyar et al., 1996; Sereda et al., 1996). In humans, approximately 50-70% of CMT1 patients harbor the 1.5 Mb Chr 17p11.2-12 duplication (Nelis et al., 1996). It appears likely that overexpression of wild type PMP22 mRNA and protein, secondary to the changes in gene dosage, is the major disease-causing mechanism in CMT1A . A similar situation occurs with Pelizaeus-Merzbacher disease (Suter and Patel, 1994), a dysmyelinating disease of the central nervous system, in which either deletion of the entire locus encoding proteolipid protein (PLP; Raskind et al., 1991), or duplication of the PLP locus (Cremers, 1987) can cause Pelizaeus-Merzbacher disease.

Two additional human diseases have been associated with *PMP22* gene mutations. First, though rare, some mutations S72L, M69K (Roa et al., 1993) in *PMP22* cause a more severe neuropathy presenting in early childhood, which had been termed Dejerine-Sottas syndrome (DSS). Point mutations in *PMP22* may also cause CMT1 S79C (Roa et al., 1993), codon 94fs, (Ionasescu et al., 1997); L147R, (Navon et al., 1996). Secondly, another autosomal dominant disease, hereditary neuropathy with liability to pressure palsies (HNPP), is shown to be due to an intrachromosomal deletion of the same region on Chr 17p11.2-12 that is duplicated in CMT1A (Chance and Pleasure, 1993). The 1.5 Mb region is flanked by repeating elements that apparently misalign during meiosis and following cross-over generates either duplication or a deletion (Pentao et al., 1992). Like CMT1A, HNPP is a later onset peripheral neuropathy predisposing to prolonged peripheral nerve dysfunction after often minimal traumatic nerve injury. Pathologically,

HNPP is characterized by the presence of numerous focal regions of sausage-shaped myelin thickening, termed tomacula (Latin, tomaculum=sausage) which affect a small portion of the internodal segment of myelin produced by a single Schwann cell. Also, as in CMT1A, HNPP appears to be specifically due to a *PMP22* gene dosage effect since presumed loss of function frame-shift mutations in *PMP22,* codon 94fs, (Young et al., 1997); exon 1, (Nicholson et al., 1994) causes the HNPP phenotype. Furthermore, HNPP appears to be adequately modeled by homozygous and heterozygous *Pmp22* null mice.

Decreased PMP22 gene dosage/gene deletion

Heterozygous *Pmp22* deficient mice (*Pmp22*$^{+/0}$) generated by targeted gene disruption show a phenotype very similar to that seen in patients with HNPP. Like HNPP, these mice had an adult onset neuropathy, appearing at 12 months of age with subtle electrophysiological abnormalities. In *Pmp22*$^{+/0}$ mice, again like in HNPP, the number of tomaculae in peripheral nerves increased with age (Adlkofer et al., 1997). By EM, the morphology of the hypermyelinated "tomaculous" segments was consistent with either repetitive ensheathing of the axons, or by internal or external wrapping of redundant myelin loops. Examination of single teased myelinated fibers stained with phalloidin revealed marked disarray of the Schmidt-Lanterman clefts in the peripheral nerves of *Pmp22*$^{+/0}$ mice. It appears that the myelin sheaths in these animals are unstable since many fiber profiles showed morphological changes of myelin degeneration with splitting and vacuolization of the major dense lines, and excessive onion bulb formation. Homozygous *Pmp22*-deficient mice (*Pmp22*$^{0/0}$) showed early onset severe dysmyelination with focal hypermyelination and abundant onion bulbs accompanied by axonal loss (Adlkofer et al., 1995). Maycox et al. (1997) have described a novel model for HNPP in transgenic mice using an antisense PMP22 cDNA driven by the P0 promoter. These mice seem to have clinical and pathological features intermediate between the *Pmp22*$^{+/0}$ and *Pmp22*$^{0/0}$ mice. In summary, all of these mice appear to provide excellent animal models for HNPP and confirm the central role of reduced *PMP22* dosage in this neuropathy. Hopefully, further study of these animals will yield a

better understanding on how the decreased dosage of *PMP22* leads to myelin abnormalities.

Increased PMP22 gene dosage/gene duplication.

Transgenic rat and mouse models of CMT1A have been produced by pronuclear injection of either cosmids containing the mouse *Pmp22* gene (Magyar et al., 1996; Sereda et al., 1996) or YACs carrying the human *PMP22* gene (Huxley et al., 1996). To date, all of these animal models carry multiple copies of the PMP22 gene (from 6-60) though Huxley et al. (1996) have reported the existence of mice with low copy number PMP22 insertions, one copy (Huxley et al., 1996; Huxley et al., 1998). In the transgenic mice created by Magyar et al. (1996) there were 16-30 copies of a cosmid containing 43 kb of the mouse *Pmp22* gene, which had approximately 7 kb of 5` flanking sequence. At 21 days of age, these mice had a twofold increased expression of PMP22 mRNA, compared to P0 mRNA expression, but only minimal myelin formation even though the Schwann cells had formed basal laminae and had established a normal 1:1 relation to axons. By 72 days, the rudimentary compact myelin was no longer present. Histological analysis of the quadriceps muscles showed fiber type grouping, which is interpreted as evidence of denervation and reinnervation, indicating concurrent axonal damage.

Animal models of CMT have been created that reproduce more of the features of CMT, particularly with regards to myelination. Sereda et al. (1996) developed a transgenic rat model for CMT1A that contains three copies of a 43 kb mouse *Pmp22* genomic cosmid in addition to endogenous *Pmp22*. These rats had 1-4 fold increase in PMP22 mRNA when normalized to P0 mRNA. At 2 months of age, these rats had unsteady gait, loss of muscle strength, and failed in motor performance tests. Electrophysiologically, their nerves had reduced conduction velocities and reduced amplitudes, indicating myelin loss and possible axonal loss. Histologically, the sciatic nerves initially only showed myelin loss, but later demonstrated onion bulb formation, a pathologic hallmark of CMT1. Neuronal cell bodies in dorsal root ganglia and ventral roots, which project into

the peripheral nerve, were not affected, and axonal degeneration was a rare event. Homozygous *Pmp22* overexpressing rats were more severely affected and died within one month with completely amyelinated peripheral nerves. All of these transgenic animals showed evidence of a very severe peripheral neuropathy that appears to correlate with the copy number of the PMP22 transgene, which, in general, exceeds that found in humans. In humans, patients homozygous for the *PMP22* duplication (4 copies of the *PMP22* gene) show a more severe phenotype than heterozygotes, but with significant clinical overlap (LeGuern et al., 1997).

1-2 PMP22 is a component of compact myelin in peripheral nerve.

PMP22 is a putative four transmembrane protein of 160 amino acids with the C- and N-termini oriented intracellularly. The first transmembrane domain consists of an uncleaved signal sequence. *In vitro* translation studies have established that PMP22 (*gas-3*) is N-glycosylated (N41) in the first extracellular loop (Figure 1-1). PMP22 has been localized by immunohistochemistry at the light and ultrastructural level to the compact portion of the myelin sheath in the peripheral nervous system (Figure 1-2). The function of PMP22 is unknown, but cell fractionation studies suggest that it is a relatively major component (approximately 2-5% of total myelin protein) of compact PNS myelin. Recently, based on *in vitro* transcription/translation assays and immunohistochemical analysis of transfected cells of chimeric proteins consisting of PMP22 domains fused to reporter genes or tagged internally (Taylor et al., 2000) proposed that PMP22 could have only two transmembrane domains.

1-3 Regulation of PMP22

The temporal and spatial patterns of regulation of PMP22 are very complex. Although PMP22 is clearly a major protein component of compact myelin (Haney et al., 1996), low levels of PMP22 protein and mRNA have been detected in other tissues such as lung, intestine, brain, spinal cord and heart (Spreyer et al., 1991; Welcher et al., 1992; Suter and Snipes, 1995). Within the brain and spinal cord, PMP22 mRNA and protein has been identified in motorneurons (Parmantier et al., 1995), which may have

important implications for explaining the axon loss that has been noted in various mouse models of PMP22-associated neuropathies.

PMP22 is Expressed Prenatally

Significant PMP22 mRNA expression is detectable in mouse embryos as early as embryonic day 9.5 (E9.5) in the epithelial ectodermal layer (Parmantier et al., 1997). At E11.5, PMP22 mRNA expression is observed in the capsule surrounding the liver and the forming gut, while low levels are detected in precartligeous condensations forming the vertebrae and the subventricular layer of the myelencephalon (Baechner et al., 1995). By E14.5 to 16.5, PMP22 expression is observed in several mesoderm-derived tissues such as connective tissue of the face region, bones, and the lung mesenchyme and in diaphragmatic and intercostal muscles. High levels of expression were also found in ectoderm-derived tissues including epithelia of the lens, and the skin. There is low expression in the gut, and no PMP22 mRNA is detected in the liver, thymus, kidney, or heart. In late embryogenesis, PMP22 mRNA expression is reduced to low levels in all tissues. For example, PMP22 expression in skin and vertebrae stops at E18.5. At the same time, PMP22 expression can be detected in PNS ganglia (vestibulocochlear ganglion and spinal ganglia for example). The prenatal and non-neural postnatal expression of PMP22 suggests that PMP22 has a function outside of myelin. Furthermore, the characterization of normal PMP22 expression is an important prerequisite for studies aimed at dissecting the PMP22 gene control region into positive and negative elements that regulate PMP22 expression

PMP22 is Expressed in Neural and Non-Neural Tissue in Adult Rodents

The highest level of PMP22 expression, however, is in peripheral nerves at postnatal day 14 (P14). Interestingly, significant PMP22 expression is detected in the vibrissae, and in the villi of the gut in adult animals. PMP22 is expressed in other parts of the nervous system in post-natal animals. In 21 day-old animals, there is significant PMP22 expression in some motor nuclei such as the trigeminal, facial, ambiguous, vagus, hypoglossal, and accessory nerves and in the ventral horn of the spinal cord, whereas

there is low expression of PMP22 in the oculomotor and trochlear nuclei, and PMP22 expression is absent in the abducent nucleus. Parmantier et al. (1995) were not able to detect PMP22 expression in the sensory cranial nuclei and dorsal root ganglia.

It is perhaps not surprising that PMP22 is found outside of peripheral nerves. Indeed, PMP22 is the homologue of the *gas-3* (*g*rowth *a*rrest *s*pecific gene-3) cDNA that was first identified in NIH 3T3 fibroblasts by virtue of the fact that it is upregulated during cellular growth arrest induced by either contact inhibition or serum deprivation. Thus, PMP22 appears to have an important role outside of myelin. Several transfection and mRNA microinjection studies correlating PMP22 expression with cellular growth (Zoidl et al., 1995; Zoidl et al., 1997) or an apoptotic-like phenotype (Fabbretti et al., 1995) support the possibility that PMP22 could serve to regulate these events.

The dual role hypothesized for PMP22 in myelinating Schwann cells and other PMP22-expressing cell types is reflected in the organization of the PMP22 gene which has two promoters (promoter 1 and promoter 2) upstream of two alternatively utilized 5' noncoding exons, exons 1A and 1B, respectively; see Figure 1-4 (Suter et al., 1994). RNase protection studies show that PMP22 mRNA transcripts containing exon 1A are the predominant PMP22 transcripts found in myelinating Schwann cells while exon 1B-containing transcripts are favored in non-neural tissues, which show much lower levels of PMP22. By RT-PCR, exon 1B-containing mRNA is found in the CNS whereas exon 1A-containing mRNA is reported to be found in the brain stem and the spinal cord (Parmantier et al., 1995).

There have been reports of a 1C mRNA transcript for PMP22 (first published by Patel et al., 1992; and more recently, Huhne et al., 1999; and Huehne et al., 2001) in a glioma cell line and in human fetal lung cDNA library. Surprisingly, the start point of the 1C transcript corresponds to the previously described 5` UTR of exon 2. Furthermore, the PMP22 1C transcript has not been shown to be a major component of PMP22 expression in the peripheral nervous system.

1-4 Developmental regulation of PMP22.

The expression levels of myelin genes, including PMP22, correlate with myelination. Quantitative analysis of Northern blots and RNase protection studies in the rat sciatic nerve show that PMP22 expression starts after birth with the initiation of myelination and rises dramatically as myelination progresses. The expression of PMP22 reaches its peak between the 14^{th} and 21^{st} day of age. The most prominent PMP22 mRNA transcript encountered in the PNS is that of 1A type. After myelin has been established, the level of PMP22 transcripts falls to maintenance level (Suter et al., 1994). Thus, PMP22 expression levels during postnatal development highly correlates with the onset and duration of myelination.

1-5 Regulation of PMP22 During Regeneration.

The response to crush injury in nerves allows us to study regeneration and the effect of axonal contact on Schwann cells. After focal crush injury, the axon degenerates distal to the crush site leaving the Schwann cells without axons. PMP22, like other myelin genes, is found to be sharply down-regulated distal to the site of crush injury, immediately after injury. Then, as axons start to re-grow and reinnervate they recontact Schwann cells. These "reinnervated" Schwann cells seem to be triggered to reproduce myelin and wrap the axon and myelinate. As expected, PMP22 is strongly upregulated while remyelination occurs, and myelin is being restored. Thus, PMP22 expression during regeneration strongly correlates with axonal contact and remyelination. In addition, the correlation of myelin gene upregulation with axon contact suggests that axon-Schwann cell interactions are important for PMP22 expression.

1-6 Axon-glial interaction

Expression of the PMP22 gene is probably regulated by axonal contact with the myelinating Schwann cell (Suter et al., 1994). There is evidence that axons play an important part in regulating the expression of *PMP22* gene during development and regeneration (Suter et al., 1994), as well as regulating other myelin genes, such as the

Myelin Basic Protein (*MBP*) and Myelin Protein Zero (*MPZ*) gene (Stahl et al., 1990). In addition, there is evidence that axons might also be involved in regulating the translocation of PMP22 from the golgi apparatus to the plasma membrane (Pareek et al., 1997).

The presence or absence of myelin also affects axonal properties. In the *Trembler* (PMP22 G150D) mouse, axons display a smaller caliber, slower rates of axonal transport, less neurofilament phosphorylation, and higher neurofilament density. The microtubule (MT) composition and its phosphorylation are altered and the MT stability is reduced compared to wild-type nerves (de Waegh et al., 1992). This result is unlikely to be due solely to the PMP22 mutation since this finding is not unique to the *Trembler* mouse. For example, in the *Shiverer* mouse, which is defective in the production of myelin basic protein (*Mbp*), optic nerve axons display altered slow axonal transport and increased microtubule number and density (Brady et al., 1999). Also, even normal nerves have reduced diameter and increased number of neurofilaments in the nodes of Ranvier and in the unmyelinated axon stems in dorsal root ganglia where axons have no myelin. These results suggest that Schwann cells/oligodendrocytes (and likely intact compact myelin) modulate the local axonal architecture. Interestingly (see below), progesterone was found to be severely reduced in *Trembler* sciatic nerves, compared to wild type. Even though the function of progesterone in myelin gene regulation is poorly understood (possibly it provides trophic support to myelin), one of the hypothesized mechanisms for this reduction in progesterone levels is the loss of axonal contact (Koenig et al., 1995).

On the other hand, clearly axons are required for Schwann cells to upregulate myelin genes and form myelin. Axons also seem to provide direct trophic support to Schwann cells. Normally, many Schwann cells undergo apoptosis during early postnatal development (Nakao et al., 1997). If developing nerves are axotomized, Schwann cell apoptosis increases. Together theses result suggest that axons control Schwann cell

function at several levels, by providing trophic support and by supporting or directing differentiation.

1-7 Sensory versus motor regulation aspects.

Evidence is accumulating that there are differences between motor and sensory axons in their capacity to regulate myelination. Even though CMT1A patients exhibit both altered sensory and motor nerve conductive velocities, there might be differences in the extent of the pathology between sensory and motor fibers. This concept is supported by studies of the *Trembler-J* mice that show more severe pathological findings in the ventral versus dorsal spinal nerve roots (Robertson et al., 1997; Robertson et al., 1999). Similar observations on the differences between motor and sensory spinal roots were made in homozygous *Pmp22* knockout (Adlkofer et al., 1997) and *Pmp22* over-expressing mice (Sancho et al., 1999). These studies raise the intriguing question of how and why a myelinating Schwann cell recognizes differences between motor and sensory axons.

1-8 The regulation of PMP22 is tight regulation.

The fact that the addition (150%) or the deletion (50%) of one copy of the *PMP22* gene can cause a disease phenotype suggests that PMP22 expression is tightly regulated. This tight regulation must also accommodate the approximately 200-fold induction in the expression of PMP22 during postnatal development. Thus, the regulation of the PMP22 gene should provide an interesting model for how gene dosage is regulated in mammalian cells.

1-9 General lessons from gene regulation

The majority of promoter analyses focus on the proximal 1-3 kb of DNA upstream (5') of the coding region, most likely due to the limited capabilities of conventional cloning vectors to carry larger pieces of DNA. Thus, most of the *cis*-regulatory elements that have been described are in the proximal 5` region of the genes studied. Many genes, however, contain elements that can be in the 5` region as far as 50 kb upstream

(McCormick and Nielsen 1998), in the introns, or the 3` region of the gene (Mandemakers et al., 2000), highlighting that the elements controlling the expression of a gene cannot be predicted to be in a certain location. For example, the apolipoprotein B (Apo B) gene contains an enhancer-like sequence within its third intron (Levy-Wilson et al., 1992) and the second intron contains a tissue-specific enhancer (Brooks et al., 1991). Moreover, intestinal expression of Apo B is controlled by DNA sequences over 50 kb 5` from the gene (McCormick and Nielsen, 1998; Nielsen et al., 1999; Nielsen et al., 1999). On the other hand, the human *Apo E* gene is known to contain an enhancer within the +44 to +262 region of the first intron (Paik et al., 1988). Also, sequences from the 3`UTR of the human tissue plasminogen activator (tPA) gene inhibited the expression of a CAT reporter gene in CV1 cells, mammalian cells (Sarafanov et al., 1999). As presented above, elements that control the expression of a gene can be anywhere in the gene without following a specific rule on where it is to be located. An element can be located in the 5` or 3`portion of the gene, close to or far away from the coding region, or anywhere in between.

1-10 Other Myelin Genes

It has been hypothesized that myelin genes should share similar mechanisms for regulation, since their expression patterns and functions are so closely related (Suter et al., 1994; Notterpek et al., 1999). It is germane to discuss the salient features of the regulation of the two other major peripheral myelin genes; myelin protein zero (P0, gene designation *MPZ*), and myelin basic protein (*MBP*). Despite the fact that regulatory elements can be far from the proximal promoter, most of the work on the *MPZ* has been done using approximately 1 kb of 5` sequence *in vitro* although some recent work has been done using larger DNA fragments *in vivo*. A number of studies of the *MBP* gene have used a transgenic model approach. Elucidation of the MBP-Golli transcription unit as well as a number of studies in transgenic mice have underscored the importance of far-upstream elements in the regulation of the MBP gene.

Myelin Basic Proteins (MBP)

The regulation of the *MBP* gene has been studied more extensively than *PMP22*. Though our understanding of the regulation of the *MBP* gene is incomplete, significant progress has been made in defining some of the features responsible for its temporally and spatially restricted expression to myelinating cells. The Golli/Myelin basic protein gene encodes for several developmentally regulated soluble proteins that are present in compact myelin of the central and peripheral nervous systems and elsewhere. It is expressed by oligodendrocytes in the CNS and Schwann cells in the PNS. The tissue specificity of expression of MBP is coded in separable *cis*-elements that direct the expression to the CNS or PNS (Gow et al., 1992). For example, a fragment of DNA that contains the 5` 10 kb of the MBP gene confers expression in the myelinating oligodendrocytes and Schwann cells, while -3.2 kb sequence yields expression in the oligodendrocytes only (Foran and Peterson, 1992), but the addition of a ~500 bp fragment that is at ~ -10 kb results in strong expression in Schwann cells and oligodendrocytes (Farhadi et al., 1999). It is clear that there is a *cis* oligodendrocyte-targeting element in the -3.2 kb proximal promoter. The region of MBP promoter from +36 to –1907 includes the core promoter and the oligodendrocyte-targeting element (Gow et al., 1992), while the Schwann cell targeting element is present at around –10 kb. Furthermore, Forghani et al. (2001) showed that a 600 bp fragment located at –10 kb of MBP promoter can robustly target expression of a reporter gene independent of other 5` flanking sequence of MBP. A cAMP responsive element has been identified between –85 and –77 in a transient transfection paradigm in rat Schwannoma cell line D6P2T (Li et al., 1994). cAMP and its effect on myelination will be discussed below.

Myelin Protein Zero (MPZ, P0)

Progress has also been made in understanding some the *cis*-elements in the *MPZ* gene. The regulation of myelin protein zero is important not only because it is the major protein component in peripheral myelin, but, like PMP22, P0 is a compact myelin protein whose temporal and spatial expression pattern parallels that of PMP22 in the PNS (Snipes et al., 1992; Doyle and Colman, 1993; Notterpek et al., 1997). *MPZ*

expression in the PNS correlates with myelination. The *MPZ* gene is upregulated in primary Schwann cells by forskolin, (see Lemke and Chao 1988; also Mirsky and Jessen, 1996; Mirsky et al., 1996) and in myelinating Schwann cell/neuronal cocultures with progesterone. DNA sequence of 1.1 kb proximal to the transcription start of MPZ is sufficient to drive Schwann cell specific expression in transgenic mice with appropriate developmental regulation. The strength of the promoter activity in this system is unclear. Within the proximal promoter (-550 to +45), in cultured Schwann cells, a basal promoter region made of two regulatory elements, a G/C element in –48 to –59 is found to be essential for binding nuclear factor Sp1 (a ubiquitous general transcription factor that binds to a G/C box and is involved in cellular processes as cell cycle regulation and chromatin remodeling). And the region of the *MPZ* promoter between –66 to –79 contains a CAAT element that binds to NF-Y (a ubiquitous transcription factor, binds to CCAAT box). No silencer element has been identified in the *MPZ* proximal promoter that shuts off expression in non-neuronal tissue. But the *MPZ* gene may contain an element where cAMP may act indirectly (Brown and Lemke, 1997). Multiple SOX10 binding sites have been identified in the *MPZ* promoter and shown to be functionally important in transient transfection paradigms (Peirano et al., 2000). In conclusion, the expression profile of the *MPZ* gene in the PNS is similar to that of *PMP22* gene. It seems likely that both *PMP22* and *MPZ* will be regulated by many of the same mechanisms involving axon-glial interactions and transcription factor(s). Another reason for studying the regulation of other myelin genes is that these genes can be influenced by similar or identical transcription factors. Similar to *MPZ*, the promoter upstream of exon 1A of *PMP22* contains an inverted CCAAT box and potential NF1 binding sites, whereas the promoter upstream of exon 1B contains a GC-rich sequence. Further studies will be required to determine if these similar regions subserve similar functions in these two myelin genes. Feltri et al. (1999) generated several transgenic lines using the full length *Mpz* gene and found out that the 3` region of the gene is important to have a faithful expression of the *Mpz* promoter.

1-11 Transcription factors that influence myelin gene regulation

As discussed earlier, PMP22 is tightly regulated, and the PNS is apparently very sensitive to small changes in PMP22 production, and cannot accommodate even two-fold changes, without causing a disease phenotype. Within this system, control must be very precise. It might be expected that any mutation that interferes with the regulation of the *PMP22* gene would also be associated with a disease phenotype. Thus, it is anticipated that mutations in critical regions of the *PMP22* promoter would cause HNPP if they interfere with *PMP22* expression or CMT if they augment *PMP22* expression. So far, no mutations have been identified in the *PMP22* promoter region (Nelis et al., 1998; Nelis et al., 1999a; Nelis et al., 1999b). However, the important functional elements in the *PMP22* promoter have not yet been identified. And, as mentioned earlier, the majority of the CMT1 patients have altered dosage of the *PMP22* gene, so the possibility for treating these patients might evolve from understanding how to control the expression of the *PMP22* gene.

In summary, the identification of these *cis* or *trans* acting factors that regulate the *PMP22* gene are important for several reasons: 1) The tight regulation of the *PMP22* gene is intrinsically interesting. How is *PMP22* gene dosage maintained even though it is upregulated over 200 fold during development? 2) Understanding the regulation of the *PMP22* gene should provide important insights into axon-glial interactions and the relevant signaling pathways. 3) Identifying important regulatory elements in the *PMP22* gene should direct searches in regulatory regions for mutations that cause CMT or HNPP. Thus, understanding the regulation of the *PMP22* gene may be of diagnostic value for rare cases of HNPP or CMT. Alternatively, if disease-causing mutations are identified first, they may direct attention to important regulatory regions. 4) Finally, treatment strategies for HNPP and/or CMT to modulate under or over-expression of the *PMP22* gene will require knowledge of its regulation.

In the past decade, several transcription factors were identified as regulators of myelination or myelin gene expression. Thus, it is pertinent to consider if these factors might be involved in the regulation of the *PMP22* gene.

1-11-1 EGR2/Krox-20

The human *E*arly *G*rowth *R*esponse gene (*EGR2*), and its mouse homologue Krox-20, is a transcription factor that influences myelination in the PNS as revealed by studies of transgenic mice and of human disease. The human *EGR2* is a cys2his2 zinc finger transcription factor (Topilko et al., 1994). Expression of Krox-20 is prominent in the developing hindbrain, whereas its expression in the PNS correlates with the appearance of embryonic Schwann cells. Gene ablation studies demonstrate that Krox-20 is involved in the development of the hindbrain. Most homozygous *Krox-20* knock-out mice die shortly after birth (Swiatek and Gridley, 1993); the survivors showed hypomyelination and arrested development of Schwann cells in the premylinating stage. Heterozygous Krox-20 knockout mice have no functional abnormality or pathological finding (Topilko et al., 1994). *Krox-20* expression is confined to motor and sensory roots of cranial and spinal nerves, and is restricted to myelinating Schwann cells and Schwann cells precursors (Topilko et al., 1994). Neither homozygous nor heterozygous *Krox-20* knockouts show any abnormality in the CNS myelin, and Krox-20 has never been detected in oligodendrocytes. Histology of the PNS of *Krox-20* null mice shows Schwann cells at 1:1 ratio with axons, intact Schwann cell basal laminae, and arrest at the promyelination state as the Schwann cell plasma membrane makes about a round and a half around the axon. MAG expression is detected but not late myelin markers such as P0 and MBP. These results suggest that Krox-20 is involved in the completion of the myelination program rather than initiating it. *SCIP* (see below) is found to have prolonged expression in the Schwann cells of the *Krox-20* knockout mice, which correlate with the lack of terminal differentiation of these cells. Co-transfection studies of Krox20 and P0 promoter constructs showed modest enhancement of P0 promoter activity. Whether *Krox-20* has a significant direct effect on the regulation of myelin genes has to be further investigated.

Recently, several dominant and recessive mutations in *EGR2* have been identified as causing CMT, CH and DSS. The dominant mutations were in any of the DNA-binding

zinc finger domains, while one recessive mutation was identified in the inhibitory (R1) domain that interacts with *NAB 1* (Svaren et al., 1998; Russo et al., 1995), a transcriptional co-repressor (Warner et al., 1999). A luciferase reporter gene fused downstream of a promoter containing two EGR binding sites is not activated when cotransfected in the CV-1 cell line with either R409W or R405X (in the zinc finger) mutated *EGR2* constructs, while low activity is noticed using S382R, D383Y, and R359W (in the zinc finger domain) EGR2 mutants. On the other hand, the I268N mutation of *EGR 2* (in the R1 domain) caused increased activity in the reporter gene expression.

Two *cis* acting elements have been identified (Ghislain et al., 2002) in the promoter of *Krox-20*. An immature Schwann cell element (ISE) is found to be active in immature but not myelinating Schwann cells, and a myelinating Schwann cell element (MSE) is active from the onset of myelination to adulthood in myelinating Schwann cells. Interestingly, the *Krox-20* MSE may require the *Oct 6/tst-1/SCIP* transcription factor (see below) during myelination (Ghislain et al., 2002). Several candidate sites for binding with *Oct-6* are found in the sequence of the 1.3 kb of the MSE suggesting the possibility that *Oct6* directly controls the expression of *Krox-20*.

1-11-2 SCIP (suppressed cAMP-inducible POU protein, homologue to Tst-1, and Oct-6).

SCIP/Tst-1/Oct-6 is a POU transcription factor that has also been implicated in the regulation of myelination, and to have an effect on myelin gene expression. SCIP was first described as a possible regulator for myelin genes, because it is found that in cultured Schwann cells, the addition of adenyl cyclase activator (forskolin) induced SCIP in a dose-dependent manner. SCIP expression starts after one hour of exposing Schwann cell cultures to forskolin. SCIP expression increases dramatically thereafter, peaking after 36 hours, and remains elevated as long as there is cAMP stimulation. P0, MBP, and PLP expression increases 18-24 hours after cAMP elevation. These results

establish that SCIP expression precedes myelin gene expression, and suggests that SCIP might be a *trans*-activator for myelin genes (Monuki et al., 1989; Monuki et al., 1990). SCIP belongs to the POU family of transcription factors. POU transcription factors are known to participate in determining cell fate (regulator of cell specific genes; Jaegle and Meijer 1998). They are also important for DNA replication and cell proliferation (Monuki et al., 1993). The POU domain, the major feature of the POU proteins, is composed of 2 parts, the POU homeodomain and the POU-specific domain. Later, several reports showed that SCIP has a repressor effect on myelin genes in co-transfections with myelin P0 promoter in Schwann cells, and it represses other genes like the low affinity nerve growth factor receptor (Monuki et al., 1993; He et al., 1991). So, SCIP is a POU family transcription factor, it is expressed in Schwann cells *in vivo* and it can modulate the promoter activity of myelin genes

Further investigations reveal that the role of SCIP in myelination is complex. The trans-activation domain of *SCIP/Tst-1/Oct-6* is likely in the glycine- and alanine-rich amino-terminal third, though deleting parts of it did not completely abolish its activity, possibly due to functional redundancy (Meijer et al., 1992). Lemke's group found that both an intact amino-terminus and a POU domain (regardless of the origin of this domain, e.g. from another POU protein) are needed for maximal function. Furthermore, the amino-terminus of SCIP fused to GAL4 was able to confer repression at lesser potency, suggesting that the POU domain doesn't function as a DNA binding domain. Several potential SCIP DNA binding sites have been identified by DNA footprinting. Many of them do not confer SCIP repression ability in transfected cells, whereas a low affinity site was able to mediate repression. These data may also be compatible with an indirect *trans*-repression by SCIP on myelin genes. In Schwann cells, SCIP expression is transient and high with a maximum right after birth, then it is down-regulated as the expression of myelin genes starts to peak. It was suggested that the initial high level of SCIP is dependent on axonal contact (Monuki et al., 1989). In support of this is the fact that SCIP is downregulated in the distal nerve stump following nerve transection after birth. On the other hand, another study (Scherer et al., 1994) showed that, in adult

animals, regenerating nerves up-regulate SCIP expression for a protracted period (for at least 58 days post injury). Furthermore, the effect of nerve transection (without regeneration) was an initial moderate up-regulation that peaked the first day post transection and became down-regulated afterward (Scherer et al., 1994). In summary, SCIP is expressed in Schwann cells, and it seems to have an influence on myelin gene expression, though it is uncertain whether this is a direct effect. Axons seem to influence the expression of SCIP in Schwann cells, but the nature of axonal interaction with Schwann cells needs to be further investigated. Thus, 1) the initial up-regulation of SCIP may be mediated through axonal contact. 2) the effect of SCIP on myelin gene regulation is most likely an indirect effect (next paragraph gives more detail about the interaction with SOX10), and 3) *SCIP* is likely to be part of a complex that controls myelin genes.

1-11-3 Sox10.

Human genetic studies have recently implicated the transcription factor SOX10 in the pathogenesis of CMT1, and therefore in myelination. Sox10 (characterized by the possession of a DNA-binding domain and the similarity to the high mobility group domain of the sex determining factor, SRY) is a transcription factor thought to have an important role in myelinating glia (oligodendrocytes and Schwann cells; Wegner, 2000). Sox10 expression starts early in embryogenesis in the neural crest, then becomes confined to glia in the central and peripheral nervous system throughout development and after terminal differentiation. Sox10 has no inherent transactivating activity, but acts synergistically with SCIP to significantly activate a model promoter that contains an FXO element (an element identified in FGF-4 enhancer where SOX2 and Oct3/4 are capable of synergistic action). A recent study (Kuhlbrodt et al., 1998) concluded that Sox10 and SCIP must bind DNA in separate binding sites to work synergistically. Not only it is possible that SOX10 interacts with SCIP, but Sox-10 is also able to repress the effect of EGR2 on a promoter that contained contiguous elements for both of SOX10 and EGR2. It seems that SOX10 lacks transcriptional activities by itself, but it may modulate the activity of other transcription factors. In summary, SOX10 does not seem to have the ability to

modulate myelin gene expression alone, on the other hand, SOX10 can work in synergistic fashion with other transcription factors.

Interestingly, SOX10 has been identified as a gene causing CMT1. Normally, most mutations in SOX10 cause Waardenburg-Hirschsprung disease. A patient was reported with a SOX10 mutation who presented with leukodystrophy compatible with Pelizaeus-Merzbacher disease and peripheral neuropathy compatible with CMT1 in addition to Waardenburg-Hirschsprung syndrome (Inoue et al., 1999).

1-12 Progesterone

Progesterone may be an important co-factor in stimulating myelin formation. This is exciting because it suggests a relatively simple procedure for regulating myelination-one of the goals in treating CMT and related neuropathies. Unfortunately, the role of progesterone in myelination is complicated. Consider the following evidence: 1) exogenous progesterone or its precursors enhanced the myelin formation, while trilostane (an inhibitor of the conversion of progenenolone to progesterone) and RU-486 (a competitive progesterone antagonist at the level of the receptor) decreased the thickness of myelin sheath (Koenig et al., 1995). 2) Following a cryolesion in the sciatic nerve, the amount of progesterone and its precursors increased prominently at 15 days post lesion suggesting that nerves were capable of progesterone autoregulation. 3) It has been reported that progesterone enhanced myelination by six-fold when it was added to neuron-Schwann cell co-cultures (Baulieu et al., 1996). The following represents a brief review of the role of progesterone in Schwann cells and myelin formation. The progesterone receptor has been identified in Schwann cells (see Melcangi et al., 1999). In addition, the important enzymes needed for progesterone biosynthesis (such as 3α-hydroxysteroid dehydrogenase 3αHSD, 5α-reductase) are also found to be present and active in myelinating Schwann cells. Thus, Schwann cells may be responsive to endocrine or autocrine progesterone.

The effect of progesterone, its precursors and derivatives on the expression of myelin genes has been evaluated *in vitro* and *in vivo*. In general, these steroids were capable of up-regulating myelin genes mRNA, *in vivo* and *in vitro*, but one should be cautious when interpreting the results of those experiments. Progesterone stimulates MPZ promoter (1 kb 5`flanking sequence) activity two-fold in cultured rat Schwann cells, but not in T47D cells which is progesterone receptor positive human mammary carcinoma cells (Desarnaud et al., 1998). Similarly, progesterone (1 μM) produced a two-fold increase in *Pmp22* promoter activity when cultured rat Schwann cells were transfected with *Pmp22* promoter 1 (2.5 kb 5` flanking exon 1A) driving a luciferase reporter gene, whereas the isolated *Pmp22* P2 promoter (1B with 3.4 kb) failed to respond to progesterone. Another study showed that the endogenous *Pmp22* mRNA level was not significantly increased by the application of either progesterone or dihydroprogesterone (DHP; both at a concentration of 10 nM), in cultured Schwann cells *in vitro*. However, tetrahydroprogesterone (THP; 10 nM; does not act on the progesterone receptor but rather on $GABA_A$ receptor) gave significant increase in the PMP22 mRNA level, leading to the suggestion that the effect of THP might be driven through the $GABA_A$ receptor rather than progesterone receptor (Melcangi et al., 1999). How can we explain that the *Pmp22* 1A promoter was enhanced 2-fold by progesterone while the endogenous gene was not? Either, 1) progesterone enhances PMP22 expression through a complex of positive and negative enhancer elements, directly or indirectly, and not all these elements are present in the cloned DNA used to promote the P1 luciferase construct. And/or, 2) the result was influenced by technical differences between the studies (for example, the amount of progesterone, 10 nM vs. 1 μM). On the other hand, *in vivo* systemic administration of progesterone (P), DHP, and THP to rats at the age of 3-4 months caused a moderate increase in PMP22 mRNA; however, only THP showed a statistically significant effect, in contrast to older rats (22-24 months old) that did not show any change in the level of PMP22 mRNA level in response to any of these steroids. Whereas RU-486 reduces remyelination during regeneration post cryoinjury *in vivo* and reduces the rate of myelination and myelin formation in Schwann cell and neuron co-cultures (Koenig et al., 1995), another study claims that RU-486 has an agonist effect on the 1A promoter in

transfected Schwann cell cultures (Desarnaud et al., 1998). The latter study suggested that perhaps the antagonist-agonist effect of RU-486 might be related to cAMP elevation. It is difficult to accommodate all of the data outlined above and to conclude that progesterone directly regulates myelin gene expression. To reiterate: 1) in Schwann cells, *in vitro* and *in vivo*, endogenous PMP22 mRNA is up-regulated in response to THP, but not to progesterone, while the "P1 promoter" of PMP22 responded to progesterone (2 fold increase); 2) *in vivo*, progesterone is upregulated in Schwann cells during regeneration post injury; and 3) application of PR blockers inhibits remyelination post injury, whereas progesterone enhances myelination. Progesterone could be influencing myelination through a direct or indirect mechanism. Whether $GABA_A$ receptors play an important role in myelination, will require further investigations. Further complicating the issue is whether the 2- fold upregulation observed *in vitro* represents a significant change in the expression of PMP22 when we know that the regulation of PMP22 shows a 200-fold increase during myelination *in vivo*.

Axons may influence endogenous progesterone production and metabolism in Schwann cells. Progesterone levels in Schwann cells increase to a peak at 15 days post sciatic nerve lesion; around the time axons have re-established contact with Schwann cells. Further evidence that axons influence progesterone production by Schwann cells comes from studies of the *Trembler* mice. *Trembler* mice have a very low progesterone concentration in Schwann cells and produce a very small amount of myelin. The correlation between axon-glial contact and progesterone levels might be secondary to myelination that might indirectly control progesterone metabolism, especially considering that ageing rats (over 22 months old) exhibit a low production of these enzymes in the PNS at the same time when the levels of myelin genes mRNA level is low (Melcangi et al., 1998).

For progesterone to work on a promoter, it needs a response element to exist on the target promoter, or it can influence the promoter indirectly by enhancing or activating transcription factors. There is no evidence yet for a hormonal response element in the promoter of P0 or PMP22 but it has been shown that MBP mRNA, too, can be elevated

in response to progesterone. It is notable that a steroid-responsive element that modulates the translation of MBP was identified in the 5` untranslated region of MBP mRNA (Campagnoni et al., 1990; Verdi and Campagnoni, 1990). The modulation of mRNA translation rates by steroids is a more general phenomenon that may serve as another mechanism by which steroids can regulate gene expression (Verdi and Campagnoni, 1990). There is no evidence to suggest progesterone initiates myelination, but the data presented above suggests that progesterone and possibly its derivatives stimulate ongoing myelination by one or more of the following mechanisms, 1) an autocrine trophic effect, 2) a systemic effect, 3) both, an autocrine and a systemic effect, 4) or alternative pathways (e.g. $GABA_A$)

Glucocorticosteroids are shown to have a specific effect on *Pmp22* and *Mpz* promoters in primary rat Schwann cells. One report showed that 2.5 kb of *Pmp22* promoter upstream of 1A and 3.4 kb of of *Pmp22* promoter upstream of 1B, and –1 kb of *Mpz* respond to corticosterone or dexamethasone by upregulation in a dose dependent fashion, and the effect was Schwann cell specific (Desarnaud et al., 2000). Glucocorticosteroid caused a 2-fold increase in the activity of 2.5 kb 1A *Pmp22* promoter, and 1 kb *Mpz* promoter. Since no glucocorticoid response elements have been found in these promoters, it was suggested that glucorticosteroids may upregulate myelin genes indirectly through inducing Schwann cell-specific transcription factors.

1-13 The effect of cAMP on myelination

Based on the fact that myelin genes are up-regulated *in vitro* as a result of stimulating Schwann cells or oligodendrocytes with cAMP activators such as forskolin, cAMP has been posited to mimic axonal contact in its effect on glia in tissue culture. cAMP levels, however, do not always correlate with myelination and myelin gene expression *in vivo*. For example, in rat sciatic nerve, a crush injury (or transection) causes a dramatic decrease in the content of cAMP distal to the site of injury; the levels are reported to be 6-10% of the control intact nerve value. For comparison, at 14 days post crush injury, P0 mRNA level returns to 60% of the control level, and 100% of the P0

control level is reached in 21 days, but the level of cAMP does not rise until 35 days post crush and at 27% of control value. cAMP is triggered 1 week after P0 levels have reached its peak in regenerating nerves (Poduslo et al., 1995). Thus, cAMP is unlikely to mediate myelin gene expression triggered by axonal contact *in vivo*. Indeed, cAMP stimulates myelin gene expression *in vitro*, but under certain conditions, such as serum starvation, and density-dependent inhibition to prevent proliferation. cAMP was not able to induce myelin gene expression in the distal part of a transected sciatic nerve *in vivo* (LeBlanc et al., 1992a; LeBlanc et al., 1992b).

Recently, a report suggested the presence of two CREB sites at –1699 and –1725 of human *PMP22* (Saberan-Djoneidi et al., 2000), and suggested that these sites act as negative regulators in the absence of cAMP. On the other hand, ΔCREB constructs showed up-regulation compared to the undeleted counterpart in cultured Schwann cells with and without cAMP, which raises the question about the real function of the deleted sequences. No other cAMP response element has been identified in any other myelin gene. The above mentioned results are consistent with the hypothesis that cAMP, *in-vitro*, may partially mimic axonal contact, but it does not explain *in vivo* events, suggesting caution in dealing with data obtained using cAMP *in vitro*.

In summary,

PMP22 gene is a disease gene for several classes of peripheral neuropathies. Mutations in the coding region as well as alteration in the dosage of the gene cause a disease phenotype. The majority of CMT1A patients have a duplication of the gene coding for *PMP22* as the mechanism for increased gene dosage. Understanding the regulation of the *PMP22* gene is important for several reasons: 1) the tight regulation of the *PMP22* gene is intrinsically interesting. How is *PMP22* gene dosage maintained even though it is upregulated over 200 fold during development? 2) Understanding the regulation of the *PMP22* and other myelin genes should provide important insights into axon-glial interactions and the relevant signaling pathways. 3) Identifying important regulatory elements in the *PMP22* gene should direct searches in regulatory regions for mutations

that cause CMT or HNPP. Thus, understanding the regulation of the *PMP22* gene may be of diagnostic value for rare cases of HNPP or CMT. Alternatively, if disease-causing mutations are identified first, they may direct attention to important regulatory regions. 4) Finally, treatment strategies for HNPP and/or CMT to regulate under or over-expression of the *PMP22* gene will require knowledge of its regulation. The temporal and spatial patterns of regulation of PMP22 are very complex. PMP22 is expressed prenatally in several neural and non-neural tissues. Postnatally, PMP22 is preferentially expressed in myelinating Schwann cells, under the influence of the contacting axons. The sum of the previous discussion indicates that the PMP22 gene most likely contains elements that confer: 1) developmental regulation, 2) regulation in response to progesterone, 3) regulation in response to injury (regeneration), 4) tissue-specific regulation, 5) motor versus sensory regulation, 6) axon responsiveness. For each of these modalities there might be one or more element(s)/module(s) controlling or modifying its actions. To date, several transcription factors that affect myelin genes or modulate myelination have been identified, and most likely more remain to be uncovered. These transcription factors may govern myelin gene expression, either alone or by a cooperative effect combined with other transcription factors.

1-14 Objectives and rationale

I believe that it would be difficult to study the mechanisms underlying the axon-glial interactions governing myelination, the developmental regulation of myelin, the functional recovery following injury, or embryonic expression patterns of the PMP22 gene *in vitro*. Because of the strict requirement of myelination for axonal contact, and the inherent difficulties related to the study of myelination *in vitro*, I have undertaken a study to define the major *PMP22* gene control regions *in vivo*. This methodology would allow me to reveal the complex interactions at the promoter level of *PMP22* and would establish model system for further analysis of transcription factors that influence the regulation of *PMP22*. Furthermore, adopting the insertion of one copy of a transgene at a defined neutral locus, the *hprt* locus, in the genome would allow for qualitative and quantitative assessment of transgene activities among different transgenic animals.

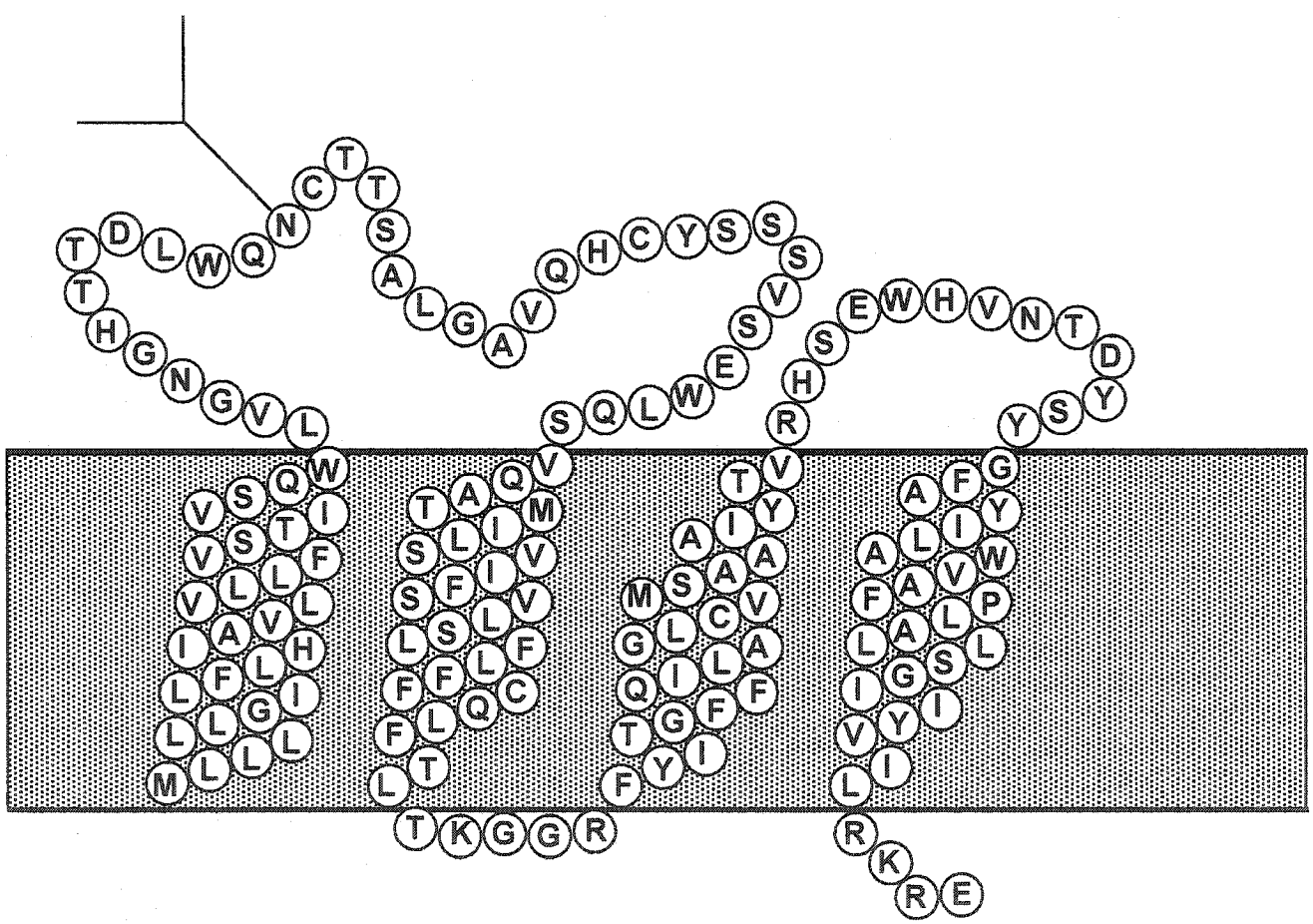
From there it should be possible to further narrow down the important transcriptional regulation sites for PMP22, regulators of myelin gene expression and myelination in general.

Figure 1-1.

The proposed structure of PMP22 includes four putative hydrophobic domains with the N and the C termini intracellular. PMP22 is glycosylated at N41.

It is controversial whether the 2nd and the 3rd hydrophobic domains are truly transmembrane or whether they are extracellular (Taylor et al., 2000).

Figure 1-1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T D L W Q N
T H G N G V L
S Q L W E S H R
V S Q W I
V S T I
V S T F
V L L L
V L A V L
I A V H
L F L I
L L G L
M L L L
T A Q V
S L I M
S L I V
S F I V
S S L V
L S L F
F F L Q C
F L
L T
T K G G R
T V
A I Y
A I A
M S A V
G L C V
Q I L A
T G F F
F Y I
A F G
A L I Y
A L V W
F A V P
L A L L
I G S
V Y I
L I
R K R E

Figure 1-2

Schematic drawing of a cross section of myelinating Schwann cell ensheathing an axon fiber showing the relationship between the wraps of myelin, the major dense lines (MDL) which are contiguous with the cytoplasmic space, and the intra period lines (IPL) which are contiguous with the extra-cellular space.

Figure 1-2

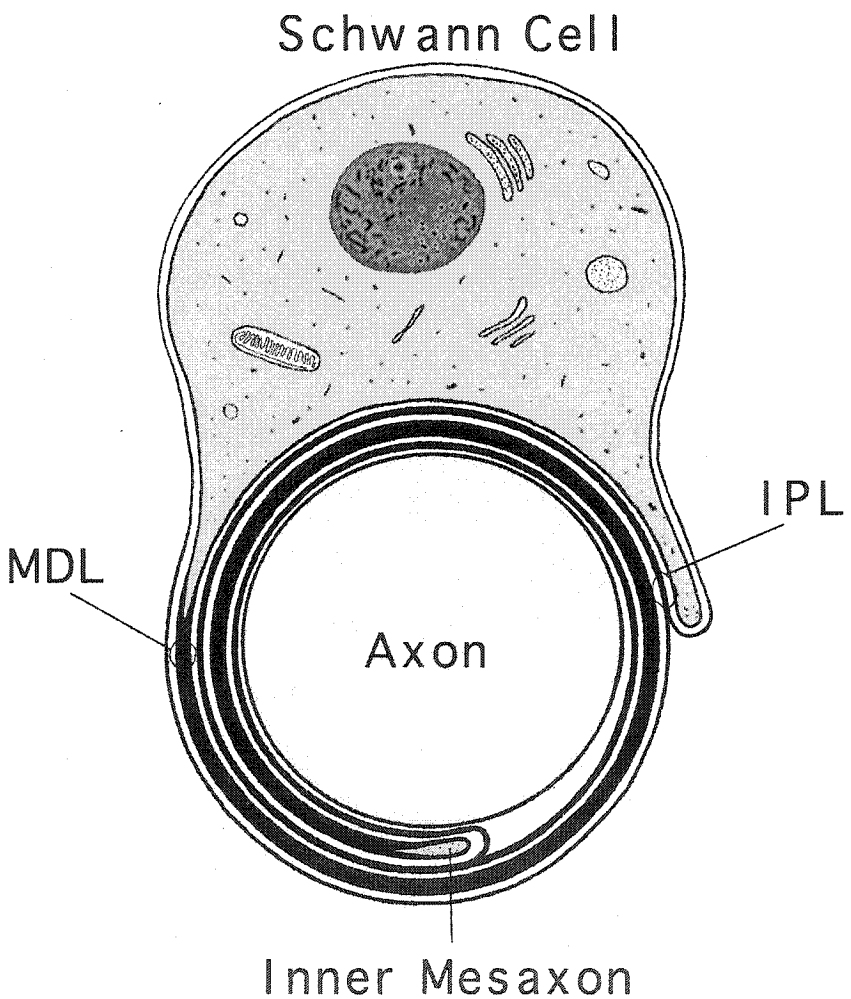


Figure 1-3

Schematic drawing showing the relationship between the compact and non-compact (Schmidt-Lanterman clefts and paranodal loops) portion on the myelin sheath in relation to the axon, the Schwann cell body and the nodes of Ranvier.

Figure 1-3

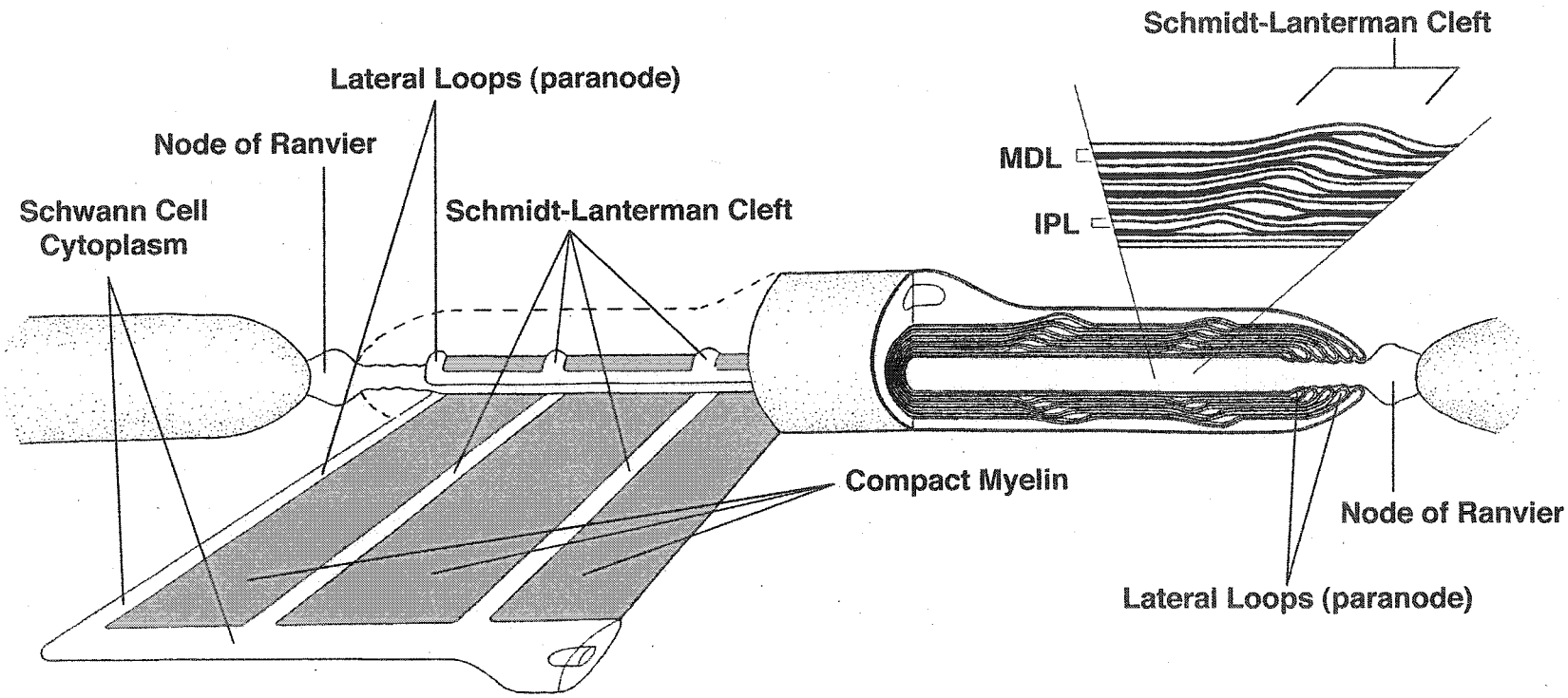
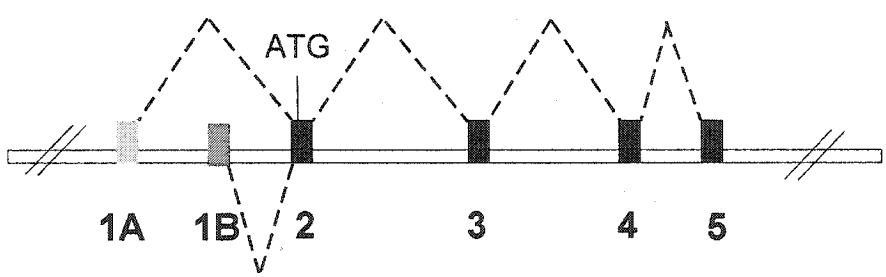

Schmidt-Lanterman Cleft
Lateral Loops (paranode)
Node of Ranvier
MDL
IPL
Schwann Cell Cytoplasm
Schmidt-Lanterman Cleft
Compact Myelin
Node of Ranvier
Lateral Loops (paranode)

Figure 1-4.

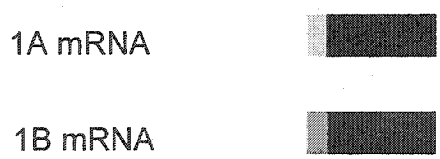
The structure of PMP22 gene (a) includes 4 translated exons named 2, 3, 4, and 5; and two alternatively transcribed 5` untranslated exons named 1A and 1B, leading to two mRNA transcripts (b) that differ in their 5` region.

Figure 1-4

a



b



Chapter 2: General Materials and Methods

2-1 Construction of the –8.5 kb rat Pmp22 promoter/reporter transgene.

The PMP22 promoter transgene used for these studies consists of -8.5 kb of the rat *Pmp22* gene (5' to the translation start site (+1) in exon 2) driving a bicistronic reporter cassette (Figure 2-1) containing chloramphenicol acetyl transferase (CAT) and *LacZ* genes separated by a 612 bp internal ribosomal entry site (IRES) from polio virus (Meerovitch et al., 1991). The 8.5 kb contig of the rat *Pmp22* promoter (pBSP1P2) was assembled in pBluescript KS+ (Stratagene, La Jolla, CA) from clones isolated from a lambda Dash II phage rat genomic library (Stratagene, La Jolla, CA) screened with the SR 29 probe (from the rat PMP22 cDNA; A.Welcher, Amgen, Thousand Oaks, CA).

The CAT open reading frame was isolated from pSV2-CAT (Pellitier et al., 1988) by *Ban* I digestion. Following treatment with the Klenow large fragment of DNA polymerase I (NEB, Beverly, MA), and the addition of *Hin*d III linkers with T4 DNA ligase (NEB), the CAT fragment was ligated into the *Hin*d III site in pBluescript to create pBS-KSCATH3+. Then, the CAT coding region was modified by two silent mutations at codons 6 and 7 to create an *Age* I site to coincide with a similarly modified PMP22 start site in exon 2, so that CAT could be ligated in frame with the ATG of PMP22. This *Age* I site was introduced into pBSCATH3+ by PCR using the following primers (*Age* I site is underlined) 5`-GGG AAT TCG GTA CCG GTT ATA CCA CCG TTG-3`, 5`-CGG AAT TCA AGC TTG GCG CCT CAC GCC CCG CCC TGC C-3`. pBSP1P2 was similarly modified to introduce the initial 7 codons of CAT and the *Age* I site immediately after the PMP22 start codon using the following primers (*Age* I site is underlined, and the start codon is in bold) 5`-GG GAA TTC GGT ACC GGT GAT TTT TTT CTC **CAT** GGT GGC TGG-3` in combination with a forward primer 5`-GGA TGG CGG CGG CCG CCC CC-3` the product of this PCR was cloned in pBluescript KS+ to create pBS PMPAge. Then, a *Not* I/*Age* I fragment of pBS PMPAge was ligated upstream of CAT into the modified *Age* I site in pBS CATAge to create pBS PMPAgeCAT. A *Sac* II (blunted) /*Hin*d III fragment from pBS PMPAgeCAT was

subcloned into the *Stu* I/ *Hin*d III site in pSV-β-Gal (Promega, Madison, WI). Next, the polio IRES (a 612 bp *Hin*d III fragment of FYK612 plasmid, kindly provided by Dr. N. Sonenberg, McGill University) was ligated into the *Hin*d III site between CAT and *LacZ* to create pSV CPG. Then the *Not* I/*Sal* I fragment from pSVCPG containing proximal PMP22, CAT, IRES, *LacZ* and poly adenylation signal was introduced at the *Not* I/*Sal* I sites pBSP1P2 to give the final construct P1P2CPG that was used for the generation of the transgenic animals.

P1P2CPG was linearized by *Sal* I restriction digestion and injected into paternal pronuclei at a concentration of 2.5 ng /μl of injection medium (10 mM Tris, pH 8, 0.25 mM EDTA, pH 7.4). The injected ova were implanted in pseudo-pregnant female mice. Lines were established from four transgenic founder mice.

2-2 Cloning mouse genomic Pmp22 gene

A bacterial artificial chromosome (BAC) library (Genome Systems, St. Louis, Missouri) is used to clone BACs carrying the mouse *Pmp22* gene. The advantage of this vector is that it can carry inserts up to 300 kb and it has high fidelity replication in bacteria. The disadvantage is that the bacterium that carries a BAC would carry only one copy of this vector, leading to low yield when amplifying by growing these bacteria. The library used is a three hit ES-129/SvJ mouse library (i.e. the possibility of three clones). The library was screened for *Pmp22* by PCR (94° C denaturing for 40 sec, 66° C annealing for 1 min, 72° C for 1 min, 30 cycles) using the following sets of primers: 1) Forward NOT I 5`: 5' GGC CTC GGC TCA TCG TTC AGG; Reverse NOT I 3`: AGT TGG GCT CGG GAT CAG AGG (recognizes the 5` of the *Pmp22* gene as shown on Figure 2-2 and 2) Forward primer M22E5F: 5' CTG AAC ACT TGA CCC TGT AGA CGG; Reverse M22E5B: AAG TCA TCG CCA GAC AGT CCT TGG (recognizes exon 5 at the 3` of the gene Fig. 2-2).

Three clones were identified as positive by PCR using the first primer pair and one of them was positive using the second primer pair. That is, we identified three BAC clones

(99M12, 132C18, and 196E18) that carry murine *Pmp22* gene: one of them (132C18) contains the entire murine *Pmp22* gene while the other two are truncated at their 3` ends. Further analysis of the clones made it possible to estimate the size and orientation of the clones as shown in figure (2-2), clone 132C18 contains all *Pmp22* exons (1A-5) including approximately 21 kb 5' of the translation start site in exon 2. Clone 99M12 carries approximately 30 kb 5` of the start codon (ATG) and 12 kb downstream. Clone 196E18 carries approximately 60 kb upstream and 12 kb downstream of the start codon. The 21 kb of *Pmp22* promoter is released from clone (132C18) by a *Not* I restriction digest, gel purified and then ligated in *Not* I site in pBluescript (pBS21) to be used in further strategies in my study for PMP22 regulation.

2-3 -21 kb promoter construct

The next step was to identify if sequences further upstream of the *Pmp22* promoter contribute to the regulation of PMP22. To test this, we created a transgene that carries 21 kb 5`of *Pmp22* driving the *LacZ* gene and introduced it into mice by standard transgenic technique of pronuclear injection. The transgene was constructed as a BAC due to the size of the insert, and for easier subcloning. To do this, pBeloBACII was partially digested with *Not* I and filled in with Klenow fragment to get rid of one *Not* I site at nucleotide 631 to produce pBACΔN. The *LacZ* gene for these studies was obtained from the vector pGNA (Fig 2-3), a gift from Dr A Peterson, Molecular Oncology Group, Royal Victoria Hospital, Montreal, Canada.

The ATG of PMP22 was mutated into an *Sph* I site by PCR overlap mutagenesis using the following primer pair: forward primer NOT I 5`: GGC CTC GGC TCA TCG TTC AGG and reverse primer ATGSphIB: AAG AGT AGA AGCATGCTG GCT GGG (*Sph* I site is underlined, ATG is in bold) using the plasmid pKS3.5 as a template. (pKS3.5 was constructed from a 3.5 kb *Xba* I fragment from clone 132C18 and subcloned into the *Xba* I site of pBluescript. This fragment spans 1.5 kb 5` and 2 kb 3` of the translation start site. The resulting 300 bp PCR product was digested with *Sph* I and filled in with Klenow large fragment to eliminate the recessed ATG. The gel-

purified product was ligated into the *Xmn* I site of pGNA, giving the plasmid pNGNA which was verified by cycle sequencing. pNGNA was digested with *Xho* I and filled in with Klenow large fragment and then digested with *Not* I releasing the *Not* I piece of the proximal promoter with *LacZ* gene and a polyadenylation signal. This was ligated into pBACΔN that had previously opened at *Hind* III, blunted with Klenow, then opened at *Not* I, giving rise to pBACNLAC. Finally, a 21 kb *Not* I fragment from BAC clone 132C18 containing contiguous sequence upstream of the *Pmp22* proximal promoter region was ligated into the *Not* I site of pBACNLAC to create the final transgene which was checked for correct orientation by test digestion and sequencing. The resulting construct can be linearized using *Xho* I digestion. As mentioned earlier for the -8.5 kb, transgenic mice were generated using the same methodology and genotyping was performed the same way.

2-4 Homologous recombination on 100kb BAC in bacteria.

In order to test for the importance of downstream sequences in the regulation of the murine *Pmp22* gene, we created a transgene that contained the *LacZ* gene under the control of 21 kb of upstream and 80 kb of downstream *Pmp22* sequence comprising the entire *Pmp22* gene. BAC clone 132C18 was used as the template for this construct as it contained the entire region of interest. Because of the difficulty of subcloning in BACs that requires modifying the translation start of *Pmp22* and inserting the *LacZ* reporter gene, I developed a methodology to introduce the insert of the reporter cassette into BAC clone 132C18 by homologous recombination in bacteria. To do this, I created a targeting vector for homologous recombination that carries homologous sequence flanking the translation start site of *Pmp22* at either end of the *LacZ* gene and a selection gene, the kanamycin resistance gene. Accordingly, I isolated a 3.5 kb *Xba* I fragment containing 1.5 kb 5` and 2 kb 3' of the translation initiation site of *Pmp22* from clone 132C18 and ligated into the *Xba* I site in pBS KS+ to create pKS3.5 (as described above). As seen in Figure 2-4, this piece includes the 3.5 kb fragment of mouse genomic DNA that spans –1.5 kb to +2 kb. Then, pGNA (from Dr. Alan Peterson, Royal Victoria Hospital, McGill University) was opened at *Kpn* I, blunted by Klenow

large fragment then digested with *Xba* I to receive the 3` recombination arm which was prepared by digesting KS3.5 using *Bam*H I blunted with Klenow large fragment and then digested with *Xba* I. The *Bam*H I/*Xba* I fragment from KS3.5 of about 2 kb was gel purified and ligated into the prepared pGNA to create p3`GNA.

Then, KS3.5 was double digested with *Xba* I/*Not* I to release a 1.5 kb fragment containing the 5` recombination arm that was ligated into *Xba* I/*Not* I treated p3`NPGNA to create the targeting vector p3`5`GNA (TV1). p3`5`GNA was linearized by an *Xba* I digestion. Initial attempts at homologous recombination in bacteria failed, so I optimized the Kanamycin concentration for growth and selection of resistant BAC transformed bacteria, at approximately 12.5 ug/ml. Even after optimizing antibiotic concentration, the recombination did not work. It seemed that the reason for unsuccessful recombination lies within the targeting vector. I hypothesized that the presence of an origin of replication of the plasmid is not compatible with the replication machinery of the BAC. So, I made few modifications on TV1 to create TV3 as follows. pBluescript was modified by killing the *Sal* I and *Xho* I sites (restriction digests, Klenow treatment, re-ligation) creating pBSΔSalΔxho. *Xba* I linearized TV1 (see above) was ligated into *Xba* I treated pBSΔsalΔxho. The resulting plasmid was digested with *Xho* I/*Sal* I to release the origin of replication of pGNA and then ligated closed creating TV3, the final targeting vector. The final targeting construct is prepared for electroporation by *Xba* I digestion, which releases the arms for recombination with the insert of *LacZ*, polyadenylation signal and kanamycin resistance gene, but excludes the backbone of pBluescript.

In order to further increase the likelihood of successful homologous recombination, we electroporated RecA$^+$ *E. coli* bacteria strain BJ5183 with BAC clone 132C18. Transformed colonies were characterized and made competent for electroporation. 200 ng of the targeting vector was used to electroporate 40 microliters of electrocompetent recombinant bacteria BJ5183 transformed with BAC clone 132C18 (Gene Pulser II, Biorad; 200 Ohms, 25uF, at 1.8KV) using 0.1 mm electroporation cuvettes (Biorad).

Following electroporation, the transformed bacteria were incubated with shaking at 37° C for 40 minutes and then plated on three LB plates for double selection (kanamycin and chloramphenicol at a concentration of 12.5 ug/ml for each). Colonies were screened by restriction digest on 0.4% agarose gels in 1X TBE at 35 Volts. I found that the majority of colonies demonstrated recombination of the BAC, but only 10-20% had the desired recombination in the targeted area, without any deletions or other unwanted recombination. Recombinant BACs showing the desired recombination event were isolated and then electroporated into DH10b bacteria for further growth and amplification.

The recombinant BAC was then linearized using CosN lambda DNA terminase, gel purified, and then injected to B6/C3H F1 fertilized ova at a concentration of 4 ng/ul. 6 founder animals were generated, four of them expressed very high levels of β-galactosidase in the PNS as assessed by β-galactosidase histochemistry on frozen sections (see below). Transgenic mouse line BMW428 demonstrated the highest level of β-galactosidase histochemical staining followed by lines 419, 413, then 422 in order of decreasing levels of expression. Figure 2-4 summarizes the recombination strategy. Panel A illustrates the targeting vector while panel B illustrates the expected product of a successful recombination event and the site of the probe used for subsequent Southern blot screening. The agarose gel in panel C depicts the result of a homologous recombination. Comparison with the Southern blot using the probe outlined in panel B indicates the clone that has undergone a successful recombination while retaining the essential restriction digestion pattern of the parental BAC clone.

2-5 Targeting to hprt locus in ES-Cells

As discussed in the introduction, the strategy of transgenesis that targets recombination to the *hprt* locus in ES cells was introduced to facilitate analysis of different promoter sequences by holding constant the site of insertion and the number of copies inserted when producing transgenic mice and the only variable is the promoter used for analysis. This allows for quantitative and qualitative comparison between promoter constructs.

The *hprt* site has been tested (Cvetkovic et al., 2000) and found to be neutral for several genes. In addition, in collaboration with Dr. Alan Peterson, we demonstrated that this site of insertion was able to reproduce expression patterns obtained by conventional pronuclear injections using a 3.1 kb *mbp* promoter construct driving *LacZ* reporter gene. This was the first evidence that insertion into the *hprt* locus can be neutral for myelin gene promoter expression. One problem of the *hprt* strategy is that the *hprt* arms used for recombination in ES cells and reconstruction of the *hprt* gene span approximately 16 kb. This is an inconvenient size for subcloning in plasmids such as pBluescript. The difficulty of working with large inserts in conventional plasmids limits the size of promoter constructs that can be investigated.

To overcome this size limitation, I opted to use pBeloBACII as a cloning vector for generating large insert-containing targeting vectors for recombination in ES cells at the *hprt* locus. First a polylinker containing a number of 8 bp restriction enzyme recognition sites was designed and ligated into *Hin*d III and *Bam* HI sites of pBeloBACII that had both *Not* I sites deleted creating the vector B8 as shown in Figure 2-5a. Then the *hprt* recombination arms were isolated from pMP8SKB (Bronson et al., 1996) and ligated into pBluescript and flipped in orientation so that linearization of the vector can occur between the 2 arms (Fig 2-5b) as follows: pBSΔN (*Not* I site deleted) was opened using *Bam*H I and *Eco*R I to receive *Bam*H I/ *Eco*R I piece of pMP8SKB that consist of the 5` arm creating pBS5`. pBS5`in turn, is opened with *Sac* I and made blunt by treatment with Klenow to receive blunted *Not* I/*Sal* I piece from pMP8SKB that contained the 3` arm to create pBS3`5`. pBluescript KS was prepared by adding a *Pac* I linker at the *Sac* II site then opened at *Eco*R I to receive the 3`5`arms from an *Eco*R I digest of pBS3`5` to create pBSP3`5`. pBSP3'5' was digested with *Pac* I to release the 3`5` arms that were then ligated into the *Pac* I site in our cloning BAC, B8, creating B8H. A novel *Sac* II site is used for linearization.

Twenty one kb of *Pmp22* promoter driving *LacZ* reporter gene was introduced into the B8H cloning vector in 2 steps: first, the *Xho* I (blunted)/*Not* I piece from pNGNA

containing the *LacZ* gene and poly adenylation signal and the proximal 300 bp of the *Pmp22* promoter starting at the *Not* I site was introduced into *Hind* III (blunted)/*Not* I site in the polylinker. Then the 21 kb promoter *Not* I piece from p21 containing the 21 kb promoter stretch up to the *Not* I site was introduced into the unique *Not* I site to create the BMH vector, and confirmed by test digestion and sequencing. The resulting BAC was prepared for electroporation in *Hprt*-deficient ES cells by the following treatment: 50 ug of the BMH vector is linearized using *Sac* II restriction digestion in 50 ul volume for 5 hours. Then, the enzyme was heat inactivated by exposure to 65° C for 20 minutes, and dialyzed using Millipore 0.025um filter membranes (Catalogue #VSWP02500, Millipore, Bedford, MA) against double-distilled water. A sample of the reaction was run on agarose gel to confirm the completion of the digestion, and 40 µl (40 µg) was retained for electroporation as described elsewhere in this chapter.

2-6 Transgenesis

Pronuclear injection,

All animal work was performed under the guidelines of the Canadian Council for Animal Care, and all surgical procedures were approved by the McGill University Animal Care Committee. B6/C3F2 fertilized ova were collected for pronuclear injection. The transgene is linearized and suspended in injection medium (10 mM Tris, 0.25 mM EDTA, pH 7.4) at a concentration of 4 ng /ul. Paternal pronuclei are injected using an air driven injection apparatus under a differential interference contrast DIC microscope (Carl Zeiss Inc., Thornwood, NY). The injected ova were implanted in pseudo-pregnant female mice.

ES cell recombination

Murine fibroblasts (Alan Peterson) are prepared as feeders by gamma irradiation (3000-4000 rads) and plating on gelatinized (0.1% gelatin) 10 cm plates at least 4 hours prior to adding ES cells. 40 µg of linear DNA targeting construct resuspended in 40 µl of double-distilled H_2O is mixed with approximately 2 x 10^6 ES cells in 800 µl of either ice cold PBS or ES medium (DMEM, 10 % decomplemented fetal bovine serum, 0.5%

gentamycin, 1% non-essential amino acids, 1% sodium pyruvate, β-mercaptoethanol, 100 units/ml Leukemia Inhibitory Factor (GIBCO BRL) is transferred to 0.4 mm electroporation cuvette (Biorad 165-2088) and electroporated at 240 V and 500uF at 0°C, then left on ice for 20 min and transferred to one plate of feeder cells in regular ES medium. 24-48 hours after electroporation the ES medium is substituted by the selection medium, HAT medium (ES medium supplemented with 0.016 mg hypoxanthine/ml, 0.01 mM amniopterine, 0.0048 mg thymedine/ml; Bronson et al., 1996).

Clones resistant to the HAT selection (presumably the ES clones that had a successful reconstruction of the *hprt* gene and insertion of the transgene) generally become prominent after 7-14 days of selection. Then, individual clones are isolated for expansion and further analysis before preparation for injection in blastocysts.

2-7 Genotyping of transgenic animals

DNA was isolated from tail biopsies as described by Foran and Peterson (1992). Briefly, 0.5 cm of mouse tail was digested with 100 μg/ml proteinase K enzyme (Promega, Madison, WI) in 300 ul lysis buffer (100 mM Tris, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, and Proteinase K 100 ug/ml) at 55°C overnight, then extracted twice in phenol/chloroform followed by ethanol precipitation. The DNA was resuspended in 10 mM Tris HCl, pH 8.0. Approximately 400 ng of DNA was used as a template for PCR for the bacterial *LacZ* gene utilizing the primers: 5` GAA AAC CCT GGC GTT ACC CAA CTT, and 5`- CTG AAC TTC AGC CTC CAG TAC AGC, for 30 cycles (94°C 1 min, 66°C 1 min, 72°C, 1 min, followed by 72°C for 10 min). This primer pair amplifies an approximately 700 bp fragment of the *LacZ* gene.

Homozygous animals were identified using Southern blot (by dosage), and confirmed by the Mendelian inheritance pattern of the homozygous transgene to their progenies.

2-8 Chloramphenicol Acyl transferase (CAT) Assay

Freshly isolated sciatic nerves from mice were pooled, homogenized in a Polytron (type PT10/35, Kinematica GmbH, Switzerland) at half maximal speed for 3 min at room temperature in Passive Lysis Buffer (Promega, Madison, WI). CAT activity was assayed using ^{14}C chloramphenicol (ICN, Irvine, CA) and N-Buteryl CoA (Promega, Madison, WI) at 37°C for 12 hrs using the CAT Enzyme Assay System (Promega, Madison, WI) according to the manufacturer's instructions. CAT activity was normalized to total protein content as determined by BCA protein assay (Pierce, Rockford, IL).

2-9 β-Galactosidase Assay

Pools of sciatic nerves from transgenic animals were collected at postnatal days 3, 7, 14, and 21, and homogenized using a Polytron (type PT10/35, Kinematica GmbH, Switzerland) at half maximal speed for 3 min at room temperature in 1X reporter Lysis Buffer (Promega, Madison, WI). Two separate assay buffers are used for this reaction; they differ in presence of the active ingredient of the reaction, o-nitrophenyl β-D-galactopyranoside (ONPG). -ONPG assay buffer is made by adding 10 mls 1M Na_2HPO_4, pH~7.2, 100 µl 1M $MgCl_2$, 350 µl β-mercaptoethanol, and water to 50 mls. The +ONPG assay buffer is identical, except for the addition of 67 mg ONPG (Sigma N1127)/50 mls of buffer.

The assay is performed in a 96 well microtiter plate (Falcon 35-3072), on ice at 0°C. Fifty µl of ice cold cell lysate, or tissue homogenate (approximately 3 sciatic nerves from adult mice), in 1X reporter lysis buffer is added to 150 µl assay buffer +ONPG (experimental) and another 50 µl is added to 150 µl -ONPG assay buffer (background). The reaction is incubated at 37°C for 30 min. The microtiter plates are read on a Biorad Microplate Reader Model 450 at a wavelength of 405 nm. The results are read from a standard curve generated from serial dilutions of known amounts of β-galactosidase enzyme.

2-10 β-Galactosidase Histochemistry

Wholemount- Adult animals were perfused transcardially with Webster fixative (Foran and Peterson, 1992; 0.5% paraformaldehyde (PFA), 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4) and then dissected and post-fixed for 1 hr at 4° C, washed with ice-cold 0.1 M phosphate buffer, pH 7.4 twice and then stained overnight at 37° C in stain solution (0.4 mg/ml Bluogal; Gibco, Grand Island, NY) 0.01% sodium deoxycholate, 0.02% IGEPAL CA-630 (Sigma, St. Louis, MO), 3.1 mM potassium ferricyanide, 3.1 mM potassium ferrocyanide, 1 mM MgCl2, in 0.1 M phosphate buffer, pH 7.4 (Foran and Peterson, 1992).

Tissue sections-Animals were perfused with Webster's fixative as above, dissected and postfixed by immersion for 1 hr at 4°C. The tissue was equilibrated with aqueous 30 % sucrose overnight at 4°C, then embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan) and frozen in isobutanol/dry ice (-50°C). Serial 10-16 μm sections were cut on a Leitz cryostat (Kryostat 1720 digital, Leica, Bannockburn, NY). The sections were stained in 0.4 mg/ml X-gal (Gibco, Grand Island, NY) in 0.01% sodium deoxycholate, 0.02% IGEPAL CA-630 (Sigma, St. Louis, MO), 3.1 mM potassium ferricyanide, 3.1 mM potassium ferrocyanide, 1 mM $MgCl_2$, in 0.1 M phosphate buffer, pH 7.4, at 37°C overnight. Selected sections were counterstained with nuclear fast red.

2-11 Sciatic nerve crush injury

Mice were anaesthetized by intraperitoneal (I.P.) injection of avertin (0.066 M 2,2,2-tribromoethanol (Sigma, St. Louis, MO) in 2-methyl-2-butanol (Aldrich, St. Louis, MO); 0.1 ml per 20 gm of body weight). The right sciatic nerve was exposed at the mid-thigh level under aseptic conditions. Single focal crush injury was administered by manual pressure applied through #5 jeweler's forceps twice for 15 seconds. The skin was closed using metal clips. Crushed sciatic nerves and contralateral control nerves

were taken at selected time points.

2-12 Southern Blot analysis

Genomic DNA was isolated from mice tails as outlined above. Purified DNA was digested with *Eco*R I enzyme and resolved on a 1% agarose gel in TBE and transferred to a positively charged nylon membrane (Nylon Membranes, positively charged, Roche Diagnostics GmbH, Indianapolis, IN). The blot was hybridized overnight at 42°C in hybridization buffer (0.5% skim milk powder, 4X SSPE, 50% deionized formamide, 1% SDS, and 0.1% dextran sulfate) with a 421 bp *Sma* I probe representing part of intron 2 of the *Pmp22* gene that was labeled with ^{32}P dCTP (3000 Ci/mmol) introduced by DNA polymerase extension of random hexamers (Highprime, Boehringer, Indianapolis, IN).

2-13 RT-PCR

The primers used to perform the reverse transcription PCR are as follow: 1A(for): 5` CTC CGA GTC TGG TCT GCT GTG, 1B(for): 5' ACC CGA GTT TGT GCC TGA GGC; CAT (Rev): 5' TCC CAT ATC ACC AGC TCA CCG; Exon2 (Rev): 5` TGG CTG ACG ATG GTG GAG ACG. For all RT experiments, total RNA was isolated from pooled sciatic nerves of 30-day-old transgenic animals using Trizol (Gibco, Grand Island, NY) according to the manufacturer's instructions.

Detection of the endogenous PMP22 transcripts.

RT-PCR was performed on 1 μg random hexamer (Roche, Indianapolis, IN) primed total RNA reverse transcribed with Omniscript (Qiagen, Valencia, CA) at 37° C for 75 min. The cDNA was used as a template for two PCR reactions alternatively using the primers 1A, and 1B as forward primers in combination with Exon2 (Rev) as a reverse primer using Taq polymerase (Promega, Madison, WI) at 94°C for 30 sec, 54°C for 45 sec, and 72°C for 45 sec, 25 cycles.). The reverse primer (Exon2Rev) is common to both 1A- and 1B-containing transcripts. 1AFor and Exon2Rev reaction gives a product of 220 bp, while using 1BFor and Exon2Rev primers amplify a 170 bp product.

Detection of the transgene transcripts.

CAT: Because of the low abundance of transgene mRNA, reverse transcription was primed with a CAT specific primer (CATRev) instead of random hexamers. Semi-quantitative PCR was performed under identical conditions for the detection of endogenous PMP22 transcripts except that the reverse primer specific for endogenous PMP22 (exon2Rev) is replaced by a primer specific for CAT in the transgene (CAT Rev). The PCR product for the transgene is predicted to be of 340 bp and 290 bp using the 1A and 1B primers, respectively.

LacZ: For animals carrying constructs that have only *LacZ* gene as reporter gene RT-PCR was performed to detect the expression of *LacZ*. The reverse transcription was primed with a specific *LacZ* primer (LacZ Rev) TCAGGCTGCGCAACTGTTGGG. Semi-quantitative PCR was performed under identical conditions for the detection of the endogenous PMP22 transcripts except for the reverse primer specific for the endogenous PMP22 (exon2Rev) is replaced by a primer specific for *LacZ* in the transgene. The PCR product for the transgene is predicted to be of 170 bp and 120 bp using 1A and 1B primers, respectively.

Figure 2-1

The –8.5 kb *Pmp22*/Cat/*LacZ* construct consisted of about –8.5 kb of rat *Pmp22* promoter, from *Hin*d III at –8.5 kb up to the start codon driving a bicistronic reporter cassette of chloramphenicol acyl transferase and β-galactosidase (*LacZ*) genes utilizing the polio virus internal ribosomal entry site (IRES). Note the position of the probe used for the Southern blot in Fig 3-1.

1A is exon 1A, 1B is exon 1B, and E2 is exon 2 of PMP22. The segment from ATG to the *Bam*HI -2067 is from exon2M3 clone. The segment Hind III –8500 bp to the *Bam*HI –2067 bp is subcloned from the clone SR29. IRES is the polio virus internal ribosomal entry site. P/A is polyadenylation signal. CAT is chloramphenicol acyl transferase coding sequence. β-Gal is the bacterial β galactosidase coding sequence.

Figure 2-1

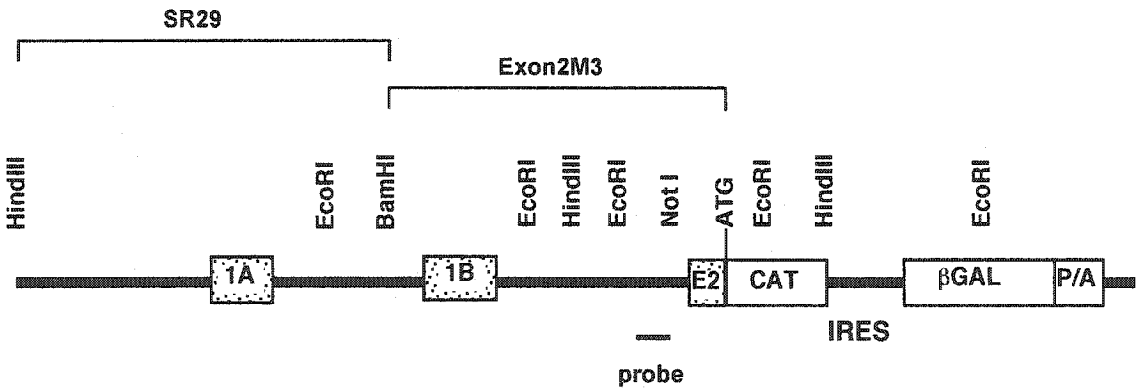

SR29
Exon2M3
HindIII
EcoRI
BamHI
EcoRI
HindIII
EcoRI
Not I
ATG
EcoRI
HindIII
EcoRI
1A
1B
E2
CAT
βGAL
P/A
IRES
probe

Figure 2-2

Bacterial artificial chromosome clones, containing *Pmp22*, isolated from a mouse genomic DNA library. Note that only the first BAC includes the full PMP22 gene.

Figure 2-2

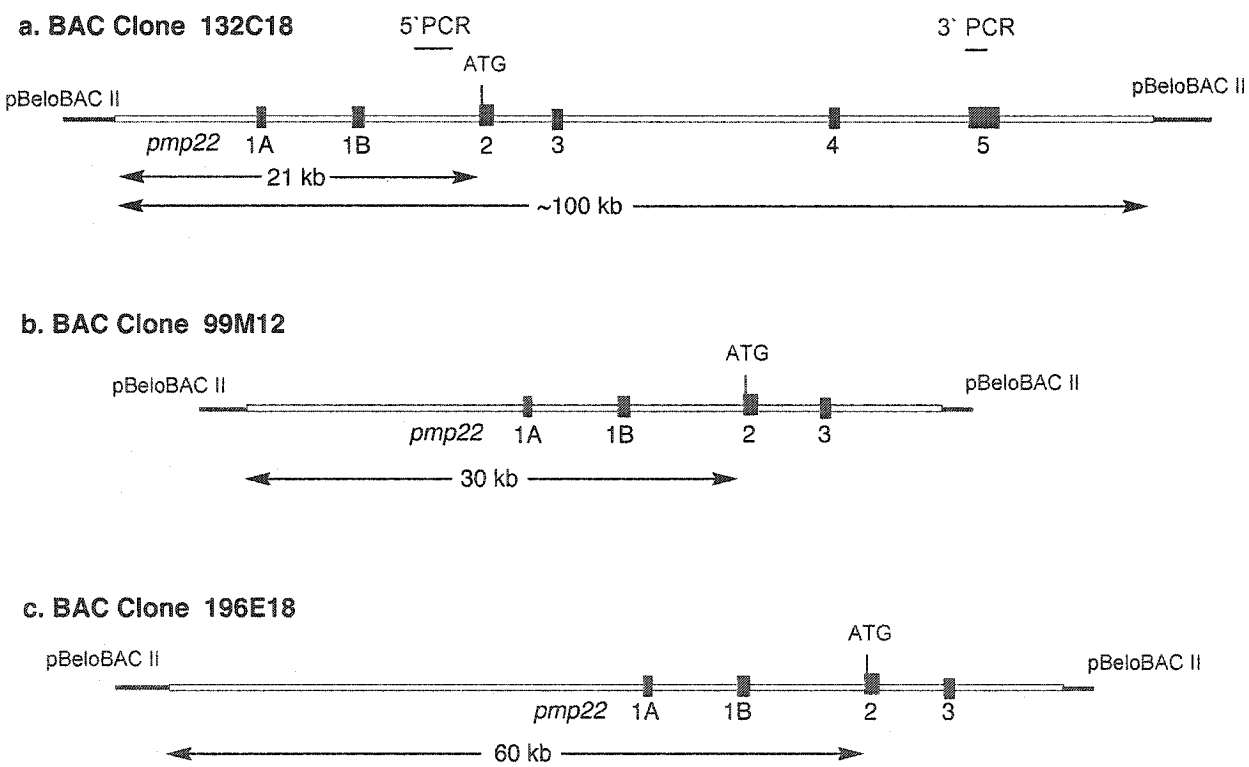

a. BAC Clone 132C18
5`PCR
3` PCR
ATG
pBeloBAC II
pBeloBAC II
pmp22
1A
1B
2
3
4
5
21 kb
~100 kb
b. BAC Clone 99M12
ATG
pBeloBAC II
pBeloBAC II
pmp22
1A
1B
2
3
30 kb
c. BAC Clone 196E18
ATG
pBeloBAC II
pBeloBAC II
pmp22
1A
1B
2
3
60 kb

Figure 2-3

-21 kb mouse *Pmp22* promoter, subcloned from clone 132C18, driving *LacZ* reporter gene.

Figure 2-3

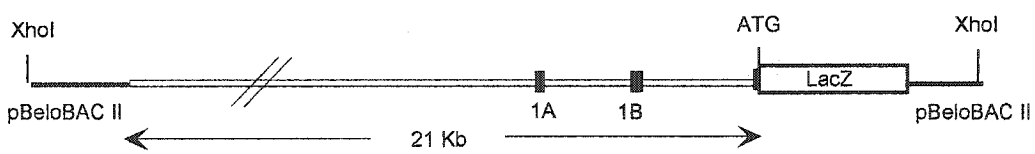
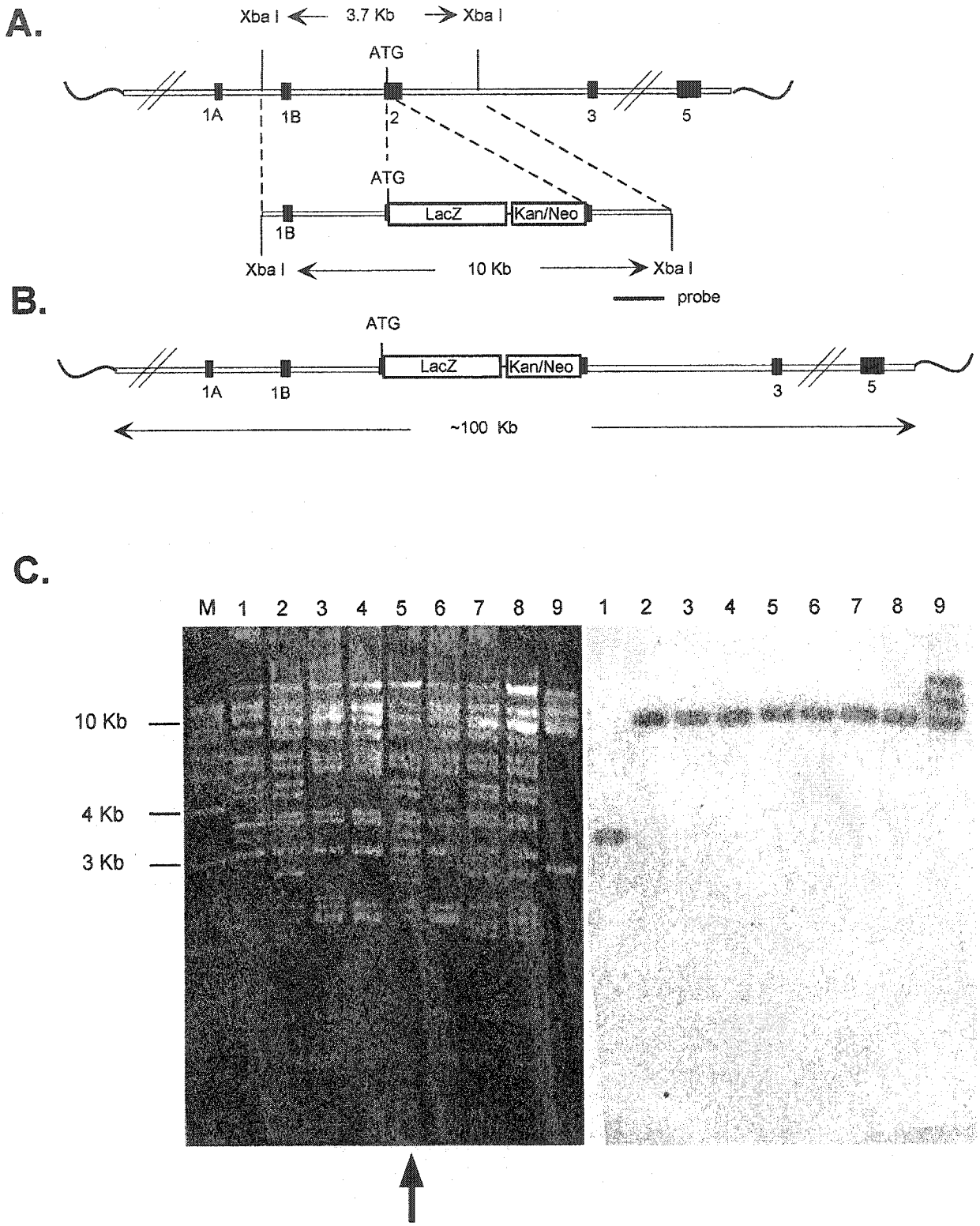


Figure 2-4

Panel A- schematic drawing depicting the targeting vector and the strategy used for recombination in bacteria to introduce the *LacZ* gene at the start codon of pmp22 in clone 132C18. Panel B- shows the result of a successful recombination event. Panel C is *Xho* I restriction digestion of the clones resulting from recombination. On the right is the Southern blot from that gel. On this gel there is one clone with the sought recombination event indicated with an arrow.

Figure 2-4



1 The original genomic PMP22 clone

9 Targeting vector

2-8 Recombinant clones. The bold arrow indicates the correct recombination

Figure 2-5 a

A map of the vector B8H, this vector is based on pBeloBACII, carrying the *Hprt* recombination arms vector for targeting to the *Hprt* locus in ES cells. Notice that *Sac* II site between the two recombination arms is used for linearization of the construct.

Figure 2-5a

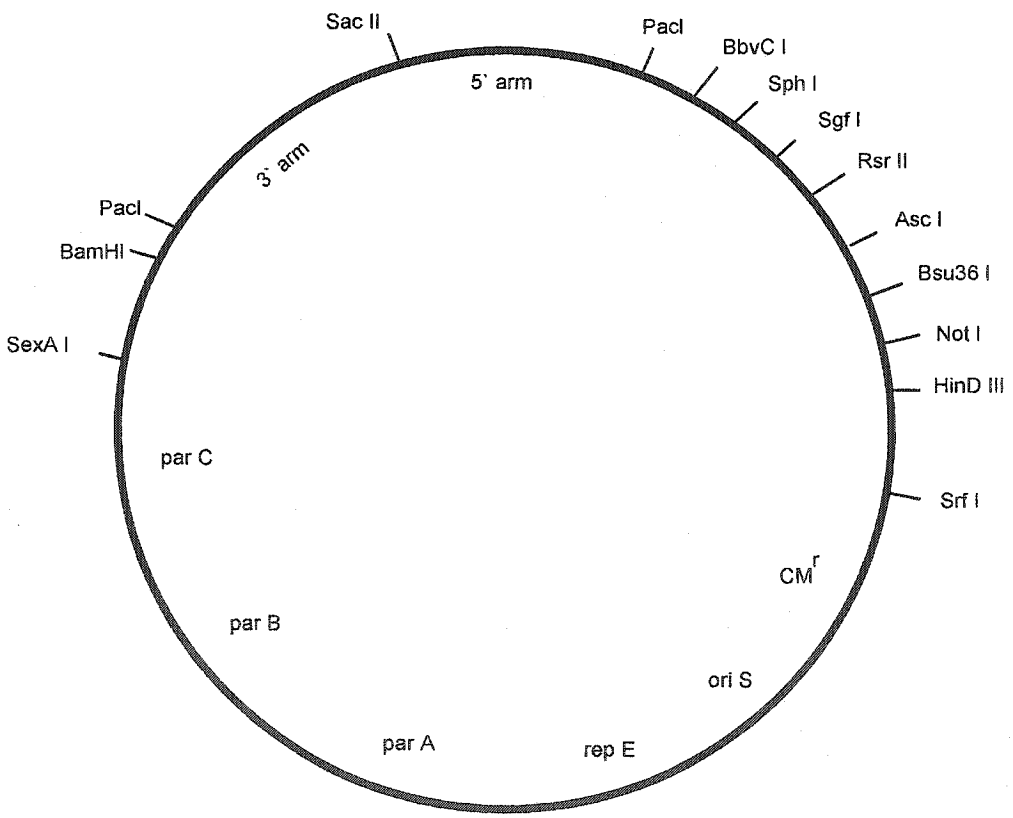
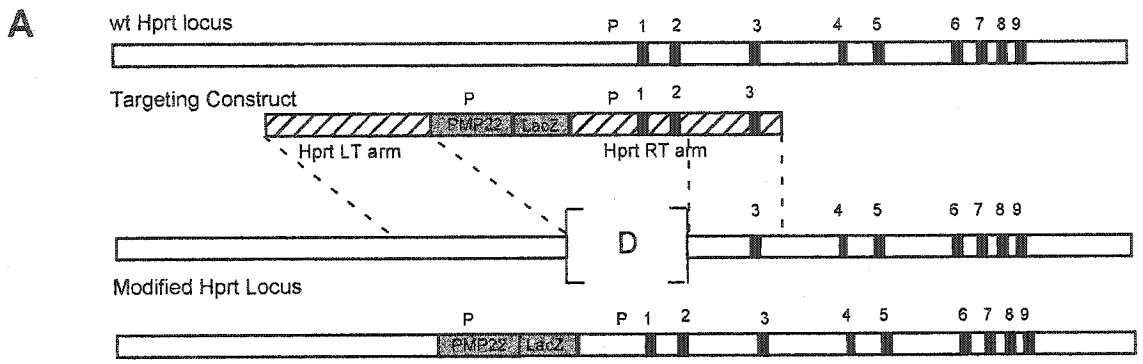
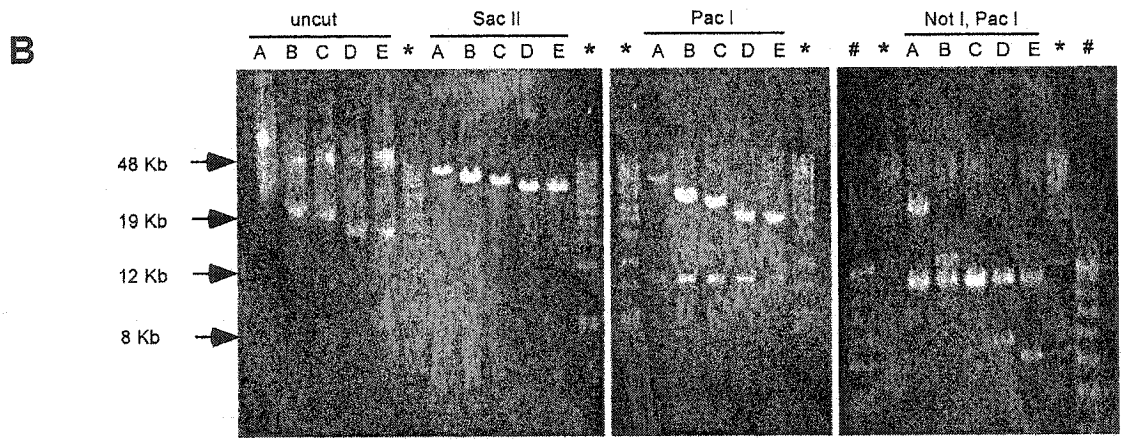

Sac II
PacI
BbvC I
Sph I
Sgf I
Rsr II
Asc I
Bsu36 I
Not I
HinD III
Srf I
CM^r
ori S
rep E
par A
par B
par C
SexA I
BamHI
PacI
3` arm
5` arm

Figure 2-5b

Panel A is a schematic drawing of the targeting strategy to the *Hprt* locus in ES cells. Panel B shows a restriction digest of nested deletions of the –21 kb mouse *Pmp22* promoter/reporter construct in the *Hprt* targeting vector and the resulting clones of –13 kb, -11 kb, -8 kb and –7 kb of mouse *Pmp22* promoter constructs. These constructs are drawn in more detail in panel C.

Figure 2-5b


A
wt Hprt locus
P 1 2 3 4 5 6 7 8 9
Targeting Construct
P
P 1 2 3
PMP22
LacZ
Hprt LT arm
Hprt RT arm
D
3 4 5 6 7 8 9
Modified Hprt Locus
P
P 1 2 3 4 5 6 7 8 9
PMP22
LacZ


B
uncut
Sac II
Pac I
Not I, Pac I
A B C D E * A B C D E *
* A B C D E *
# * A B C D E * #
48 Kb
19 Kb
12 Kb
8 Kb
*, High Molecular Weight Marker (Gibco)
#, 1Kb DNA Marker (Gibco)

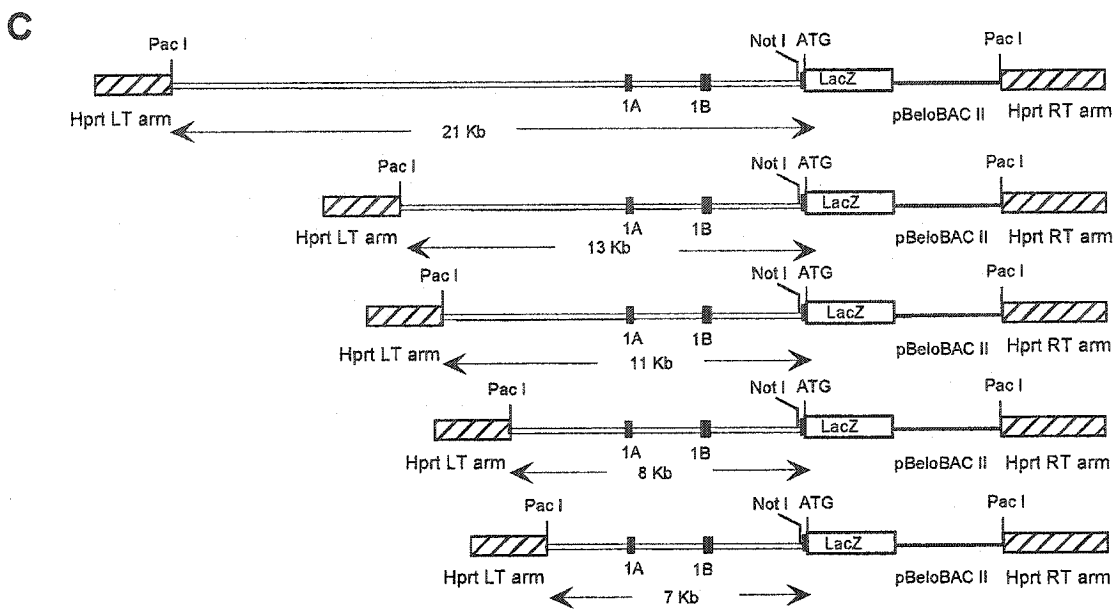
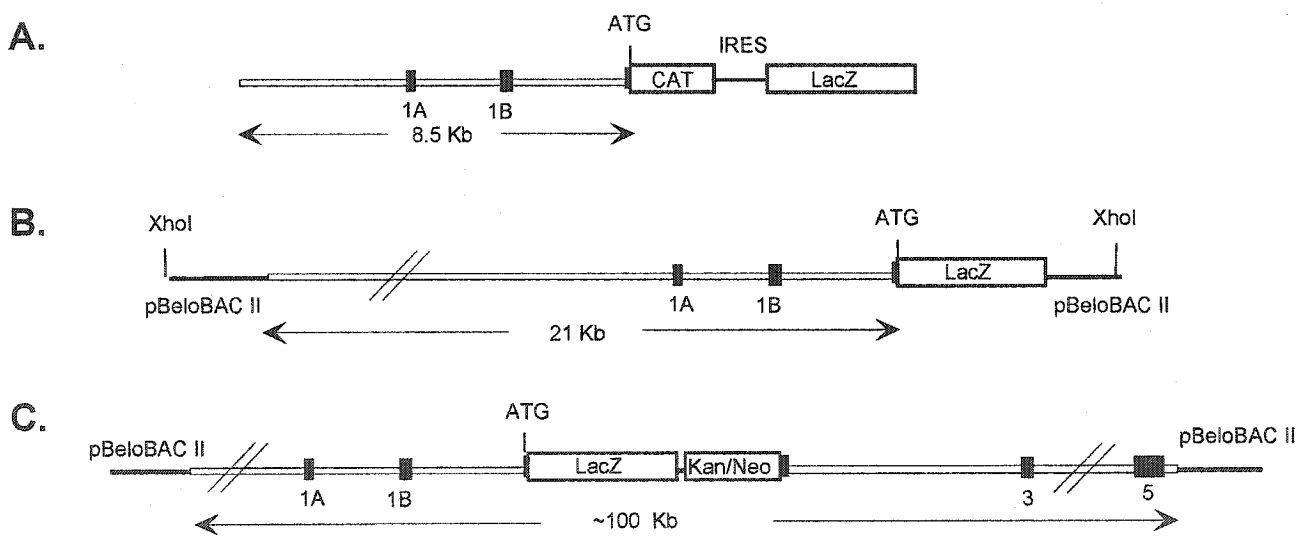

C
Pac I
Not I ATG
Pac I
LacZ
1A
1B
Hprt LT arm
21 Kb
pBeloBAC II
Hprt RT arm
13 Kb
11 Kb
8 Kb
7 Kb

Figure 2-6

The constructs used for the production of transgenic mice are shown in this figure. A) –8.5 kb is the construct discussed in detail in chapter 3. B) –21kb pmp22 promoter/reporter construct discussed in chapter 4. And C) 100 kb BAC containing the full *Pmp22* gene with *LacZ* as reporter gene at the start codon is discussed in chapter 5.

Figure 2-6


A.
ATG
IRES
CAT
LacZ
1A
1B
8.5 Kb
B.
XhoI
ATG
XhoI
LacZ
pBeloBAC II
1A
1B
pBeloBAC II
21 Kb
C.
ATG
pBeloBAC II
pBeloBAC II
LacZ
Kan/Neo
1A
1B
3
5
~100 Kb

Chapter 3: The Expression of –8.5 kb Promoter Construct

In order to begin to address the requirement for axon-Schwann cell interactions in *PMP22* gene expression, we have begun to analyze the *PMP22* control region in transgenic mice. In this study, we have analyzed a transgene that contains all elements upstream of the translation start site to approximately -4 kb upstream of the PMP22 transcription start site for exon 1A driving a compound report construct containing chloramphenicol acyltransferase (CAT) and the β-galactosidase (*LacZ*) genes. This transgene incorporates all of the regions identified as important in the transient transfection studies outlined above in the introduction. Our findings confirmed that this transgene contains *cis*-acting elements that target expression to peripheral nerves, though at relatively modest levels. Expression promoted by this construct is weakly developmentally regulated and is responsive to denervation, but unlike the endogenous *PMP22* gene, it does not get reactivated during nerve regeneration. Further analysis revealed that the inability of this transgene to recapitulate the expression pattern of the endogenous *PMP22* gene correlates with its inability to fully activate transcription from the myelin-associated promoter, P1, strongly suggesting that additional *cis*-regulatory elements extending beyond the 8.5 kb examined here are likely to be involved in *PMP22* gene regulation.

3-1 Production and Characterization of transgenic mice

As shown schematically in figure 3-1a, the design of the initial transgene construct preserves the alternative transcription from the two putative promoters, P1 and P2, upstream of exons 1A and 1B, respectively, and their alternative splicing to the first coding exon, exon 2. This construct also preserves the translation start site of *Pmp22* by fusing chloramphenicol acyl transferase (CAT) gene to the start codon of *Pmp22* (see Chapter 2). This controls for comparable translation initiation between the transgene and the endogenous *Pmp22* gene. This is important since 5' untranslated regions of mRNAs can have marked effects on translatability (Sonenberg, 1994). Also shown in

figure 3-1a, the construct includes a second reporter gene, the β-galactosidase (*LacZ*) gene. The construct was made bicistronic to convey expression of two reporter genes: CAT for more sensitive quantitation and *LacZ* for histochemical analysis. To allow this, we cloned an internal ribosomal entry site (IRES) derived from Poliovirus between the two reporter genes to provide an alternative site for translation initiation. The rationale for preserving the alternative transcription configuration of the proximal *Pmp22* control region in this construct is to initially test whether the 8 kb upstream of the translation start site is capable of faithfully conveying the innate capability of promoters P1 and P2 to target developmentally-regulated and tissue-preferred expression observed for the endogenous *Pmp22* gene as demonstrated by previous RNase protection studies (Suter et al., 1994).

Before constructing the transgenic mice, we validated that the bicistronic reporter gene cassette was functional. To do this, we subcloned the bicistronic reporter gene cassette under control of the SV40 promoter. This test plasmid was transiently transfected by calcium phosphate into COS-7 cells. We observed that the level of expression of chloramphenicol acyl transferase and β-galactosidase were comparable to the level of expression of these genes following parallel transfections of the parental plasmids, pSV2CAT and pSV-β–Galactosidase Control Vector, (both utilizing the SV40 promoter) under the same conditions.

Having validated the reporter cassette, the PMP22-CAT-LacZ construct (P1P2CPG) was injected into fertilized mouse ova. Four independent lines (designated 8, 16A, 16B, and 18) were established from four founder transgenic mice. As part of the initial characterization of these mice, we quantitated the level of expression of CAT in sciatic nerve homogenates from each of these lines and normalized the activity of CAT to the total protein content of the homogenate. As shown in figure 1b, all of the lines had significant levels of CAT expression compared to control (3 pools totaling 27 mice for each line), but the lines differed somewhat in their levels of CAT expression. Line 16A expressed the strongest CAT activity; followed by line 16B, then line 18. Line 8

demonstrated the lowest *Pmp22* promoted expression of CAT. Most of the remainder of the characterization was performed on line 16B because of its relatively increased level of CAT expression and the fact that preliminary histological and biochemical studies indicated that line 16B was qualitatively similar to the other lines. Thus, line 16B was bred to homozygosity and used for further analysis.

We determined the approximate copy number of the -8.5 kb transgene in the transgenic mice in order to confirm that the transgenes were intact and to evaluate whether transgene expression levels correlated with transgene copy number. Thus, Southern blot analysis was performed on *Eco*R I digested genomic DNA prepared from the tails of lines 16A, 16B, 18, and 8 using a ^{32}P labeled 421 bp *Sma* I fragment (from murine *Pmp22* but homologous to rat *Pmp22* located approximately 200 bp 5' to the start codon of rat *Pmp22*, see figure 3-1a) as a hybridization probe. The Southern blots from all lines showed the expected size of the hybridization signal for the wild type and transgene *Pmp22*. The copy number of the transgene was estimated based on quantitation of the Southern blot by phosphoimaging (Storm Phosphoimager, Molecular Dynamics Inc, Sunnyvale, CA) using ImageQuant (Molecular Dynamics Inc, Sunnyvale, CA) software. The -8.5 kb *Pmp22*transgene copy number was estimated as 130 copies for line 8, 6 copies for line 18, 3 copies for line 16B, and 2 copies for line 16A (data not shown). Clearly, the transgene copy number does not correlate with the relative CAT activity measured in the sciatic nerves from each line of transgenic mice (line 16A>16B>8>18; see figure 3-1). If anything, there is a negative correlation between increasing transgene copy number and CAT activity. The different levels of transgene expression (CAT activity) observed between the different lines of transgenic mice likely reflect the different sites of insertion rather than copy number of the transgene. Despite the quantitative differences between the expression levels of the transgenes, as noted above, the different lines of transgenic mice using the -8.5 kb *Pmp22* promoter were qualitatively similar with regards to their expression of the transgene in different tissues and in their responses to nerve injury (see below).

3-2 -8.5 kb of 5` flanking Pmp22 promoter targets reporter expression preferentially to peripheral nerve and Schwann cells.

Previous RNase protection, immunoperoxidase, and *in situ* hybridization studies demonstrate that the PMP22 gene has a complex pattern of temporal and spatial expression. As expected for a peripheral nerve myelin protein gene, these studies demonstrated that the expression from the endogenous *PMP22* gene is very high in sciatic nerves, but can be detected at much lower levels in many non-neural tissues (Suter et al., 1994). To test whether our transgene also exhibited a similar tissue-restricted pattern of expression, we assayed CAT activity in homogenates from a variety of tissues from the *Pmp22* CAT/*LacZ* mice, in triplicate, and compared to similar samples from control mice (same strain). As shown in figure 3-2, maximal levels of CAT activity were readily detected in peripheral nerves, but significant, albeit very low, (<5% of sciatic nerve CAT activity) levels of CAT activity were detectable in intestine, lung, heart, and muscle compared to non-transgenic controls. It is important to emphasize that the peripheral nerve specimen sampled in the transgenic mice consisted only of the distal portion of the sciatic nerve, without dorsal root ganglia or spinal roots. Since these results are qualitatively similar to previous RNase protection studies on the endogenous *Pmp22* gene expression (Suter et al., 1994), we conclude that the -8.5 kb fragment of the *Pmp22* promoter contains at least some of the elements that confer tissue-preferred expression of *PMP22* in peripheral nerves.

We stained transgenic mouse tissue for β-galactosidase expression using Bluo-Gal on whole mounts and grossly observed moderate staining in the dorsal root ganglion and barely detectable levels in peripheral nerve (figures 3-3a and 3-3b, respectively). Frozen sections of dorsal root ganglia and sciatic nerve were stained overnight with Bluo-Gal and X-Gal, respectively. In dorsal root ganglia, β-galactosidase-catalyzed X-Gal reaction product was readily apparent in sensory neurons (figure 3-3c), but was barely detectable in peripheral nerves, observable in only a subset of myelinating Schwann cells (figure 3-3d). The low level of expression of the β-galactosidase driven by the -8.5

kb *Pmp22* promoter in whole mounts and histologic sections of mouse sciatic nerves is in stark contrast to the robust β-galactosidase expression observed in MBP promoted *LacZ* transgenic mouse sciatic nerve sections stained in parallel (data not shown, transgenic mouse nerves kindly provided by Dr. A. Peterson, McGill University). Since our transient transfection control experiments indicated that the bicistronic reporter cassette was fully functional and did not bias towards expression of one or the other reporter genes, the apparent discrepancy between significant levels of CAT activity and the low level of *LacZ* staining probably reflects the increased sensitivity of the radiochemical CAT assay compared to the histochemical β-galactosidase assay (and validates the choice of using the bicistronic reporter). In summary, these results indicate that the -8.5 kb *Pmp22* promoter construct preferentially targets expression of reporter genes to Schwann cells, but it promotes expression at levels that are much below those expected for myelin gene expression.

3-3 Expression of the –8.5 kb Pmp22/reporter construct is weakly developmentally regulated

In rodents, myelination of peripheral nerves occurs over the first four postnatal weeks. After birth, during active myelination, Schwann cells up-regulate myelin genes, including PMP22 at high levels. The approximately 200-fold increase in PMP22 mRNA peaks around 14-21 days postnatal. To test whether the –8.5 kb *Pmp22* transgene was similarly regulated during postnatal development, pools of sciatic nerves from homozygous line 16B animals were collected at 3, 7, 14 and 21 days of age. CAT activity was assayed using ^{14}C-labeled chloramphenicol and normalized to the total protein content of the homogenate. As shown in figure 3-4, the level of CAT detected in the sciatic nerve increases 2-fold between 3 and 14 days. This represents a relatively modest developmental regulation of the expression of the transgene during the peak of myelin gene expression. Clearly, the 2-fold up-regulation does not compare to the 200-fold up-regulation of the endogenous PMP22 mRNA (Snipes et al., 1992). This partial up-regulation of the CAT reporter gene indicates that while some regulatory elements responsive to developmental regulation are present in the transgene, significant

developmental regulatory sequences are not activated in our transgene. This relatively modest degree of developmental regulation might reflect 1) the absence of important enhancer element(s) in the -8.5 kb *Pmp22* construct, or 2) the unopposed effect of (a) repressor(s), and/or 3) it might reflect the modest regulation of the 1B promoter in the absence of 1A promoter activity, or 4) the failure of the transgene to insert into a genomic site permissive for developmental regulation. In order to understand the regulation of the *PMP22* gene, it will be important to distinguish among these possibilities. The latter possibility is partially controlled by the analysis of four independent transgenic mouse lines made from this construct each presumably representing a unique site of insertion in the mouse genome.

3-4 The –8.5 kb Pmp22/reporter construct is not reactivated during nerve regeneration

The expression of myelin genes, including *PMP22*, is highly dependent on axonal contact (Snipes et al., 1992). This is clearly demonstrated in two situations. The first is the marked developmental upregulation of myelin genes in Schwann cells during postnatal development *in vivo* which is not present to anywhere near the same extent in similarly aged Schwann cells grown *in vitro* (Snipes et al., 1992; Suter et al., 1994). The second is the marked down-regulation of myelin genes that occurs during Wallerian degeneration, when Schwann cells are deprived of contact with viable axons (Snipes, et al., 1992). Within 72 hours of lesion, both PMP22 mRNA and protein are sharply down-regulated distal to a focal crush injury in sciatic nerves (Snipes et al., 1992). If axons are allowed to regenerate, the PMP22 levels are up-regulated over the ensuing 14 to 21 days as the regenerating axons are remyelinated (Snipes et al., 1992). We tested whether the –8.5 kb *Pmp22* promoter construct expression in Schwann cells showed a similar responsiveness to axonal denervation and reinnervation in the nerve crush injury model. We found that four days after crush injury the expression of CAT was sharply down-regulated in the sciatic nerves of transgenic mice compared to the contralateral uninjured sciatic nerve (figure 3-5a). However, unlike the endogenous *Pmp22* gene, significant expression from the transgene did not return during regeneration at twenty-

one days post injury. Figure 3-5a shows that the level of CAT activity in the regenerated nerve 21 days following injury was not significantly different than CAT activity in distal sciatic nerves 4 days after injury. The lack of increased CAT activity in injured sciatic nerves after 21 days of regeneration did not reflect the absence of regeneration. As shown in 0.5 μm epon sections depicted in figure 3-5b and 3-5c, there is significant the regeneration taking place in the sciatic nerves of the transgenic mice 21 days after crush injury (figure 3-5c), as compared to the uninjured normal appearing contralateral sciatic nerve (figure 3-5b). Histologic sections of the regenerating nerve (figure 3-5c) shows abundant axonal sprouts with thin myelin sheaths admixed with a number of phagocytic cells (Schwann cells and macrophages) with "foamy" cytoplasm. These findings indicate that the -8.5 kb *Pmp22* transgene is responsive to degeneration secondary to axonal loss, but not to remyelination that occurs during peripheral nerve regeneration. Also, this result suggests that regulation of myelin gene expression during development differs from induction of myelin gene expression during regeneration and that these two methods of PMP22 up-regulation (development and regeneration) are controlled through separate mechanisms.

3-5 The –8.5 kb Pmp22/reporter construct preferentially utilizes transcription from exon 1b

There are at least two PMP22 mRNA species that are generated from the *PMP22* gene by alternative transcription of either one of the 5` untranslated exons, exon 1A or exon 1B. The exon 1A-containing transcript is expressed at high levels almost exclusively in the peripheral nervous system, while the expression of the exon 1B-containing PMP22 transcript is more widespread, though at much lower levels in several different tissues (Suter et al., 1994).

To test whether the expression pattern delivered by the –8.5 kb construct recapitulates the endogenous PMP22 mRNA expression, we performed semi-quantitative RT-PCR on RNA isolated from sciatic nerves of transgenic animals to determine the relative amounts of the two major nerve transcripts, containing either exon 1A and/or 1B. As

shown in figure 3-6, when we used primers specific for endogenous PMP22 transcripts, we obtained a ratio of 1A transcript: 1B transcript of 3:1, which is comparable to previous RNase protection studies. However, when we amplified the products of the transgene using identical PMP22 exon 1A and 1B transcript specific forward primers, but a CAT-specific reverse primer (on exon 2), the 1A transcript: 1B transcript ratios were 2:3. These experiments were performed in triplicate. Although these experiments were not designed to quantitate absolute amounts of any specific transcript, it is clear that the ratio of PMP22 exon 1A-containing transcript compared to the exon 1B-containing transcript shifted in favor of the exon 1B-containing transcript when expressed from the -8.5 kb *Pmp22* transgene.

In this study, I have begun to functionally define the position of elements of the *Pmp22* promoter in transgenic mice. The -8.5 kb fragment of the *Pmp22* control region included in this study extends from –4 kb of P1 upstream of the transcription start site for exon 1A, and includes P2, exon 1B, intron 1B/2 and exon 2 up to the translation start site. I have demonstrated that 8.5 kb upstream of the translation start site targets expression to peripheral nerve in a weakly developmentally regulated fashion. In addition, the –8.5 kb construct contains elements that down-regulate PMP22 expression in response to the loss of axonal contact secondary to focal axonal lesion in the crush injury model. The 8.5 fragment of *Pmp22*, however, appears to lack elements that are responsible for significant upregulation during development, and response to remyelination during nerve regeneration. Most significantly, this -8.5 kb construct appears to lack elements necessary for high level expression in peripheral nerve, which correlates with its ability to transcribe mRNA from the non-myelin-associated promoter, P2.

Figure 3-1.

Construction and expression of the PMP22 reporter transgene. Panel a depicts the *Pmp22/CAT/LacZ* construct used for this study. The transgene consists of 8.5 kb of the rat *Pmp22* control region relative to the translation start site. The compound reporter encoding for a bicistronic mRNA containing chloramphenicol acetyl transferase (CAT), an internal ribosome entry site (IRES) derived from Polio virus and the bacterial β-galactosidase *(LacZ)* gene is fused so that the open reading frame for the CAT is in frame with the start codon for *Pmp22*. The approximate position of the probe derived from the *Pmp22* gene that was used for Southern blot analysis is depicted at the bottom of the panel a. Panel b shows the relative expression CAT activity (average counts x 1000/ μg of total nerve protein) of the transgene in peripheral nerves of four independent lines of transgenic mice derived from pronuclear injection of the construct depicted in panel a. The intensity of the bands identified by Southern blot analysis of *Eco*RI digested genomic DNA derived from each of the lines of transgenic mice probed with the fragment indicated in panel a (panel c), gives the relative copy number of the transgene in each line of transgenic mice.

Figure 3-1

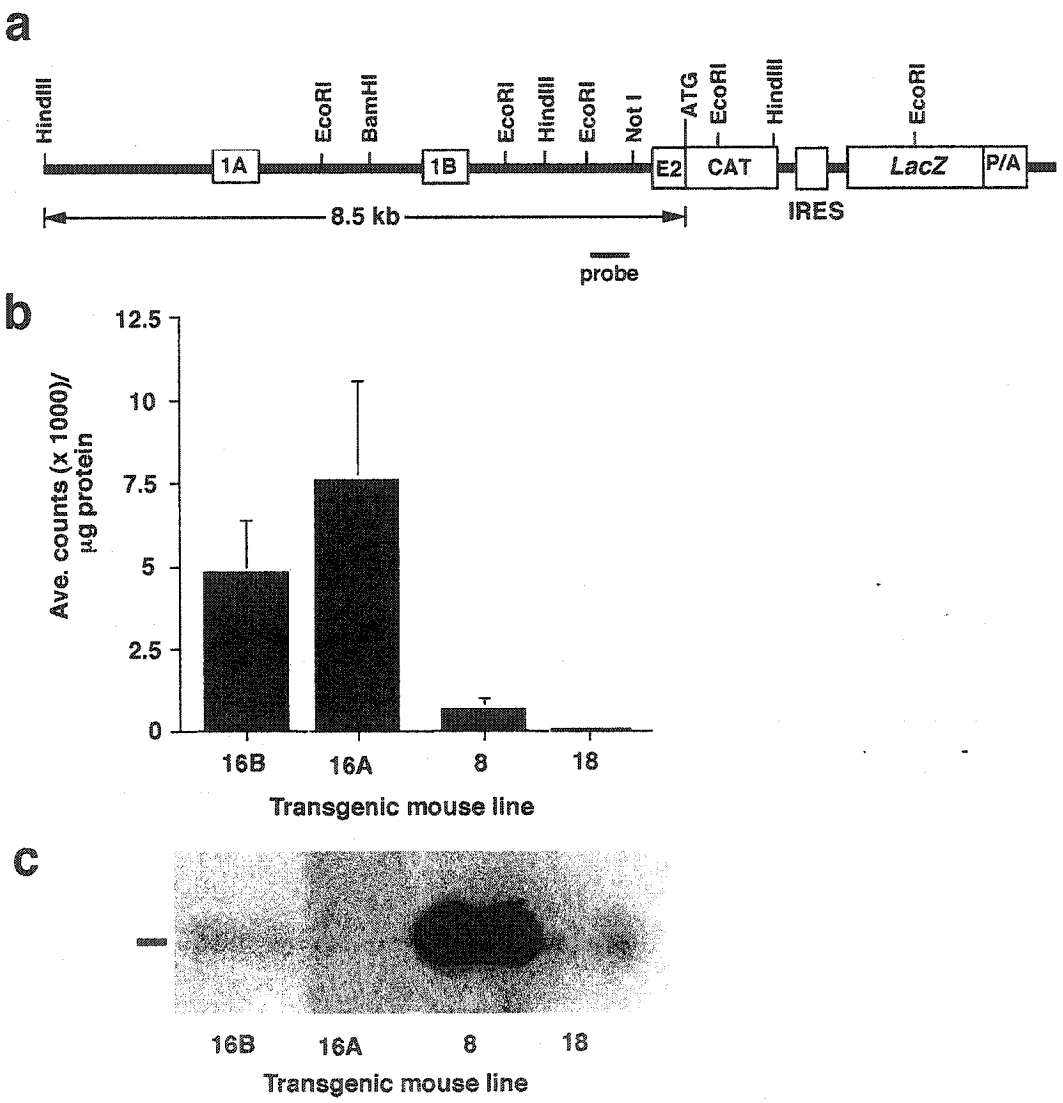

a
HindIII
EcoRI
BamHI
EcoRI
HindIII
EcoRI
Not I
ATG
EcoRI
HindIII
EcoRI
1A
1B
E2
CAT
LacZ
P/A
8.5 kb
IRES
probe
b
12.5
10
7.5
5
2.5
0
Ave. counts (x 1000)/ µg protein
16B
16A
8
18
Transgenic mouse line
c
16B
16A
8
18
Transgenic mouse line

Figure 3-2.

-8.5 kb of the PMP22 control region targets expression to peripheral nerve. Lysates from representative tissues of transgenic mice (Tg) and control (wt) mice were assayed for CAT activity. The CAT activity/μg of protein in the different tissues is expressed in arbitrary units relative to sciatic nerve (100%).

Figure 3-2

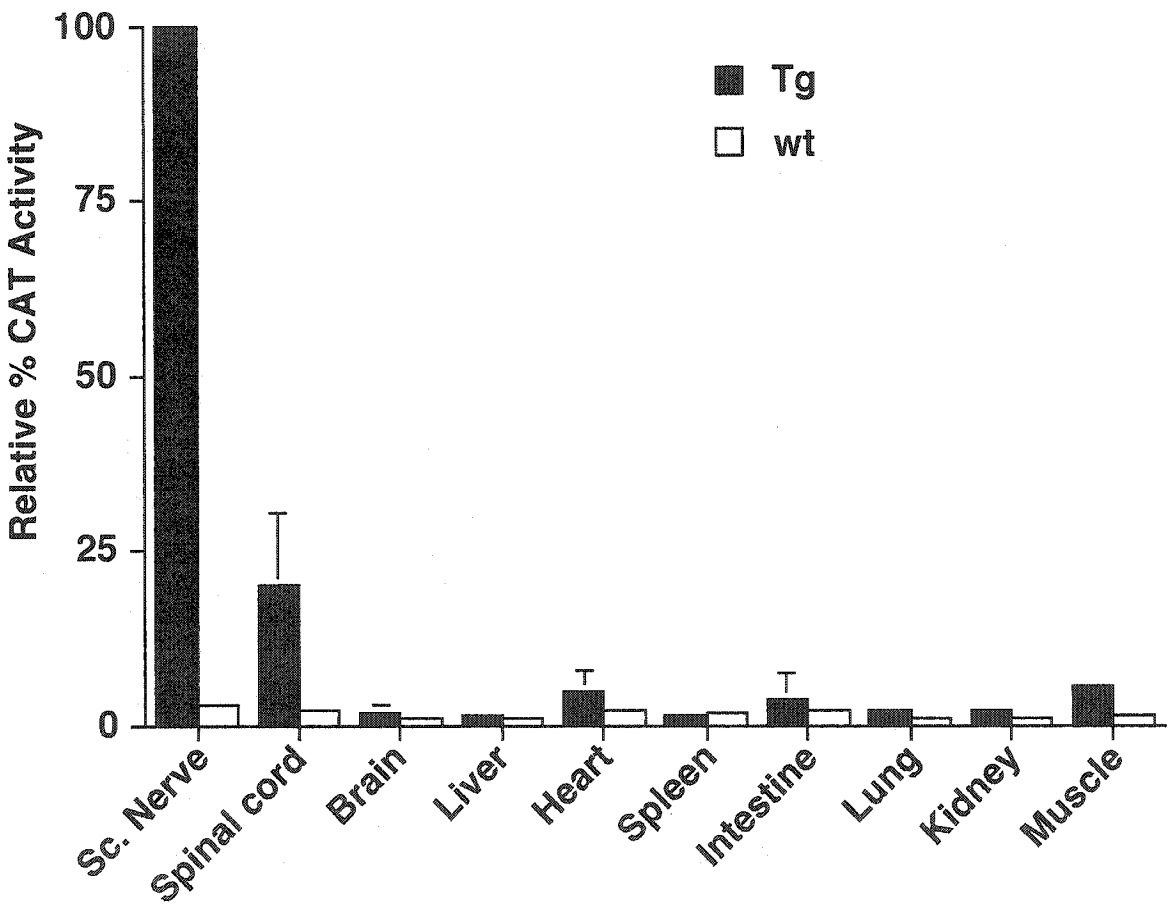

100
75
50
25
0
Relative % CAT Activity
Tg
wt
Sc. Nerve
Spinal cord
Brain
Liver
Heart
Spleen
Intestine
Lung
Kidney
Muscle

Figure 3-3.

Whole mount (a, b) and histological (c,d) preparations of nervous tissue from –8.5 kb *Pmp22*/CAT/*LacZ* transgenic mice for β-galactosidase activity stained with Bluo-gal (a,b,c) and X-gal (d). Panel a shows punctate Bluo-gal staining in dorsal root ganglia (DRG) of transgenic mice. Histologic sections of dorsal root ganglia (panel c) reveal Bluo-gal reaction product in the cytoplasm of sensory neurons of DRGs counterstained with nuclear fast red. Whole mounts of sciatic nerves stained with Bluo-gal (panel b) show barely detectable β-galactosidase activity even after staining for 24 hrs. Cross sections of the sciatic nerve reveal scattered small deposits of X-gal reaction product within the cytoplasm of Schwann cells (arrowheads) closely apposed to myelinated axons.

Figure 3-3

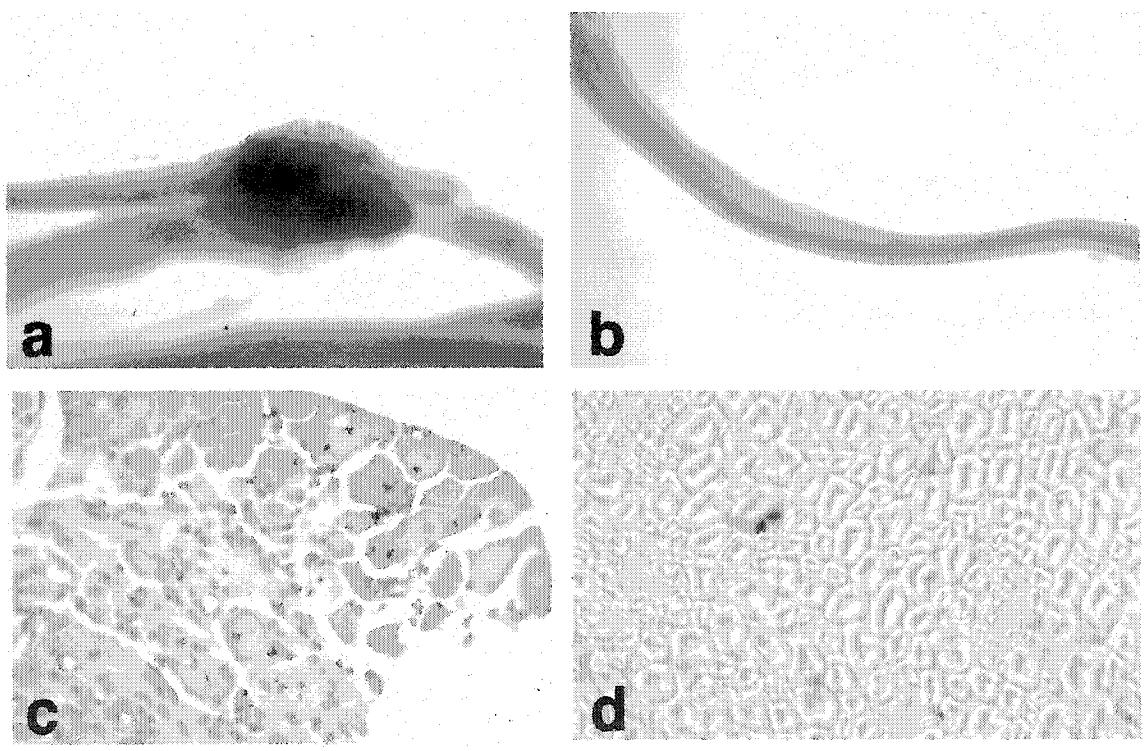


Figure 3-4.

The 8.5 kb PMP22 promoter construct is weakly regulated during sciatic nerve development. Lysates from sciatic nerves harvested from transgenic mice at the indicated ages were assayed in triplicate for CAT activity expressed as counts/μg of total protein.

Figure 3-4

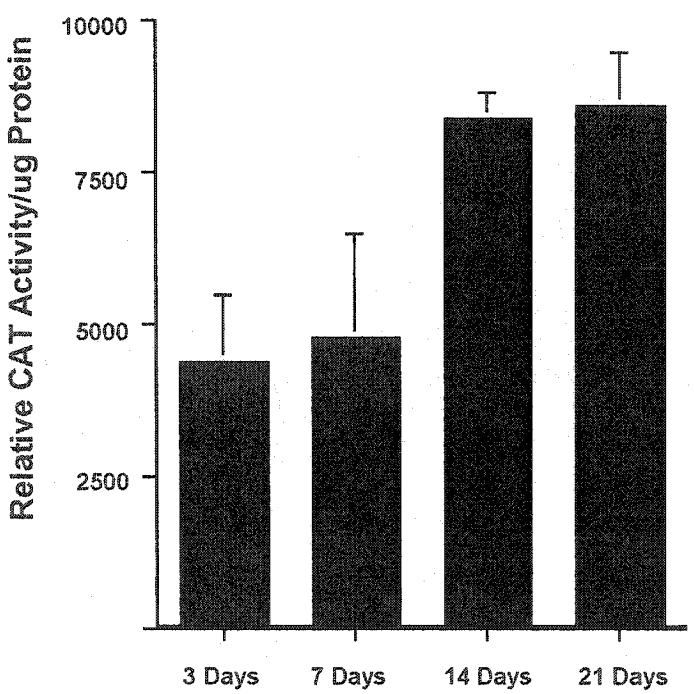
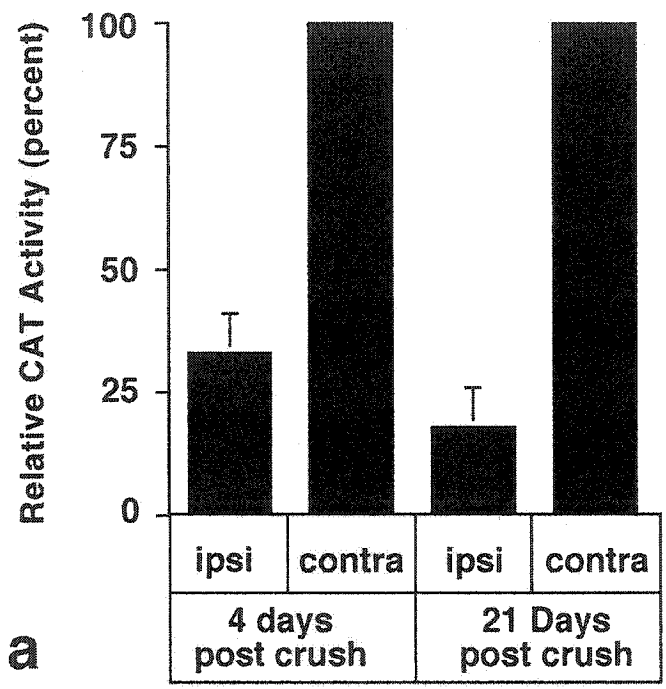

Relative CAT Activity/ug Protein
10000
7500
5000
2500
3 Days
7 Days
14 Days
21 Days

Figure 3-5.

Expression from the 8.5 kb PMP22 promoter construct is down-regulated at four days after focal crush injury and is not up-regulated in distal nerve segment by 21 days post-crush during nerve regeneration. Sciatic nerves from adult 8.5 kb *Pmp22*/CAT/*LacZ* mice were focally crushed at the mid-thigh level. At the indicated times (4 days or 21 days) after lesion, the portion of the sciatic nerve distal to the crush was harvested (ipsi) and CAT activity (in triplicate) was compared to the contralateral (contra) uninjured nerves (panel a). Representative 0.5 μm thick epon sections of the regenerating sciatic nerve 21 days following crush is shown in panel c compared to a control contralateral nerve in panel b. These sections are stained with paraphenylenediamine and visualized under phase contrast optics. Note the presence of numerous phagocytic cells with foamy cytoplasm (thick arrowhead) and the significant degree of regeneration evident by the number of thinly myelinated small caliber axons (thin arrows).

Figure 3-5


Relative CAT Activity (percent)
100
75
50
25
0
ipsi
contra
ipsi
contra
4 days post crush
21 Days post crush
a

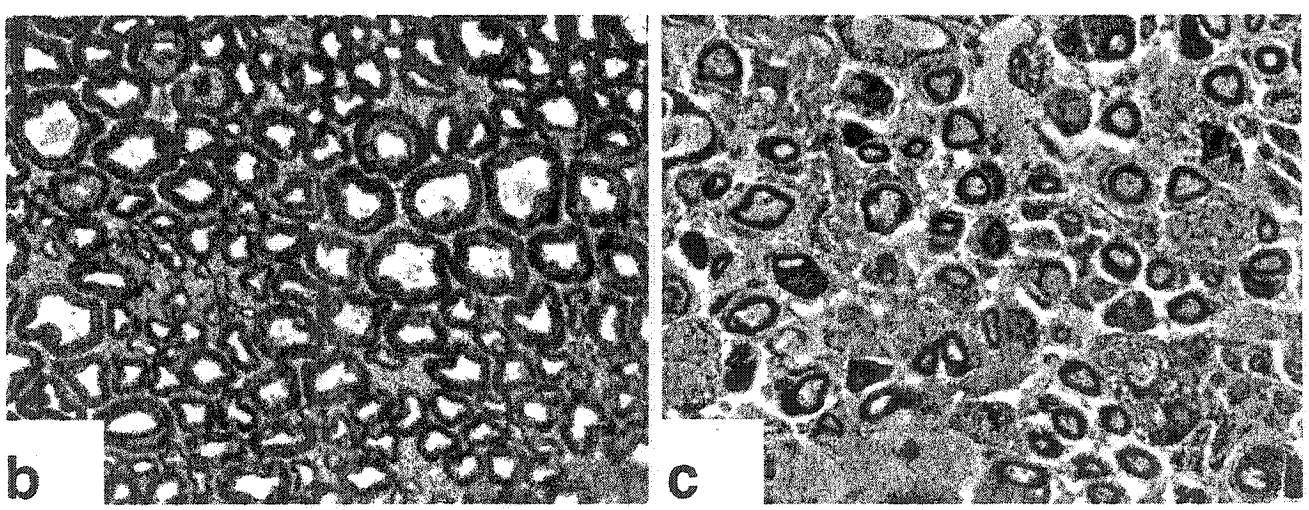
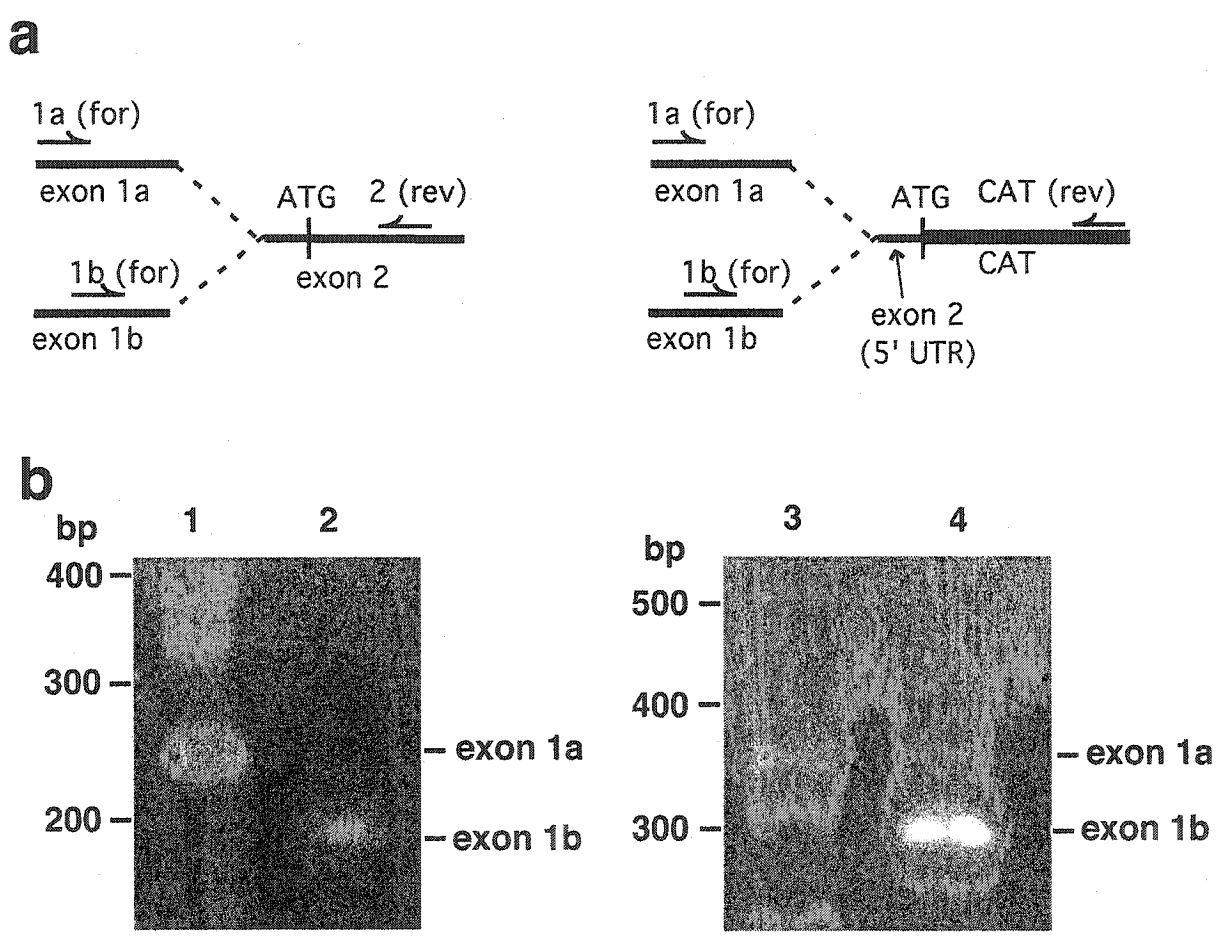

b
c

Figure 3-6.

Adult sciatic nerves from 8.5 kb *Pmp22* promoter transgenic mice preferentially produce mRNA containing the exon 1b transcript (panel c) as compared to the endogenous PMP22 mRNA (panel b). The design of the RT PCR is depicted in panel a. PCR was performed on reverse transcribed RNA derived from the sciatic nerves of the 8.5 kb *Pmp22*/CAT/*LacZ* mice. Semi-quantitative PCR was performed using primers specific for the endogenous PMP22 transcript (primer "2 (rev)' left, panel a; results in panel b) or for the CAT containing transcript (primer "CAT (rev)" right, panel a; results in panel c). Note that the forward exon 1a and exon 1b specific primers are identical.

Figure 3-6


a
1a (for)
exon 1a
ATG
2 (rev)
1b (for)
exon 2
exon 1b
1a (for)
exon 1a
ATG
CAT (rev)
1b (for)
CAT
exon 2
(5' UTR)
exon 1b
b
bp
1
2
400
300
200
exon 1a
exon 1b
3
4
bp
500
400
300
exon 1a
exon 1b

Chapter 4: The activity of 21 kb 5` *Pmp22* promoter constructs in transgenic mice.

In the previous chapter, I demonstrated that -8.5 kb of rat *Pmp22* promoter is capable of targeting the expression of *LacZ* and CAT reporter genes to the peripheral nervous system. As expected for a myelin gene promoter, this expression is upregulated during development (albeit moderately) as myelination ensues; it is down-regulated during axon degeneration as myelin is eliminated; but, it is not responsive to axonal signals associated with nerve regeneration and concomitant remyelination. Overall, the level of expression driven by the –8.5 kb promoter is low, and, as suggested by the RT-PCR studies in the previous chapter, the pattern of expression is consistent with activity of the P2 (exon 1B-containing) promoter alone. The most likely explanation for the apparent inactivity of the P1 promoter in the –8.5 kb promoter/reporter construct is that necessary *cis*-acting regulatory elements are absent. It is, of course, possible that *cis*-elements required for P1 promoter activity are present, but cannot be expressed for other reasons. For example, the integration of exogenous DNA into the genome of transgenic mice generated by standard pronuclear injection techniques often results in insertions of multiple copies of the foreign DNA into almost random sites throughout the mouse genome. Thus, enhancers and repressors from DNA surrounding the transgene insertion site might regulate expression from the transgene. The establishment and analysis of multiple independent lines of transgenic mice, presumably carrying different sites of insertion, minimize the untoward effects of genomic context. Thus, on the assumption that -8.5 kb of promoter sequence is insufficient to capture the necessary elements for the regulation of P1 promoter of PMP22 gene, I carefully considered the possibilities as to where the required elements might be located. As discussed in the introduction, functional analysis of other genes indicates a myriad of possibilities. Even among myelin structural genes, far upstream (5`) elements have been identified in the myelin basic protein gene, and important regulatory elements 3` to the first coding exon have been identified in the myelin protein zero (*Mpz*) gene (Feltri et al., 1999). Thus, for

Pmp22 the necessary elements could be a) further 5` to the –8.5 kb fragment used, b) further 3`, or c) further 5` and 3`. *A priori*, these possibilities appear equally likely.

In order to evaluate these possibilities and to further identify the elements that confer additional components necessary for the regulation for myelination, Schwann cell expression, nerve regeneration, and axon/glial interactions, I wanted to test and evaluate the activity of upstream and downstream DNA relative to the -8.5 kb discussed in the previous chapter for their ability to promote higher levels of expression, demonstrate responsiveness to axon regeneration, and drive significant expression from promoter P1. Thus, I isolated 3 BAC clones carrying mouse genomic *Pmp22*. As discussed previously, BACs carry DNA inserts of up to 300 kb, which should be sufficient to contain most of the elements necessary for most gene control regions. The BAC genomic library used for these studies was derived from DNA isolated from murine 129/SvJ ES cell lines. Note that our previous studies on the –8.5 kb promoter used rat genomic DNA. I switched to the study of murine *Pmp22* promoter for several reasons: 1) I anticipate that regulatory elements required for myelination will be evolutionarily conserved (this will be examined by interspecies sequence comparisons in Appendix 1, 2) if not, the murine *Pmp22* should be a better substrate for functional studies in transgenic mice, and 3) since the murine BAC library is constructed from genomic DNA isolated from 129/SvJ mice, the DNA can be more readily used for homologous recombination strategies using 129/SvJ-derived ES cells, 4) rat BAC genomic libraries are not widely available, 5) sequence information should be more readily forthcoming for the murine *Pmp22* gene compared to rat gene from the various genome projects. Indeed, I now have significant sequence information from the human, rat and mouse PMP22 gene control regions that will allow a direct comparison of their conserved regions. This issue will be discussed more fully in Appendix 1 and Chapter 6. Briefly, sequence comparisons performed on the -8.5 kb of rat DNA comparing it to mouse showed a great homology to a level that would give a high level of confidence that I can compare results obtained from transgenic mice using rat and mouse DNA.

Cloning Bacterial artificial chromosomes carrying the mouse Pmp22 genomic DNA

Using a PCR-based strategy, I isolated three clones from a mouse genomic BAC library (Genome Systems inc., St Louis, MO), using two different sets of PCR primers; one set specific for the 5` of *Pmp22*, and the other set specific for the 3` of *Pmp22* (see the methods section Chapter 2, see also figure 2-2). The clones were preliminarily characterized and sized by restriction enzyme digestion and agarose gel electrophoresis. As shown in Figure 2-2, BAC clone 138C18 contains the entire mouse *Pmp22* gene including -21 kb 5` of the *Pmp22* translation start site. A more detailed characterization of these *Pmp22*-containing BAC clones is discussed in the methods section in Chapter 2.

I hypothesized that there are important *cis*-acting DNA elements required for *Pmp22* promoter P1 expression in addition to those present in the –8.5 kb that I characterized in Chapter 3. Thus, I designed an experiment to check the regulation conferred either by cis-elements further upstream and/or downstream by analyzing transgenic mice expressing two separate transgenes: 1) the first transgene will incorporate 21 kb upstream of the *Pmp22* translation start site (derived from BAC clone 138C18) to promote *LacZ* expression. This transgene will assess the importance of regulatory elements in the 5' portion of the *Pmp22* control region. The second transgene will contain the entirety of mouse genomic DNA contained in BAC clone 138C18 (80 kb total, 20 kb upstream, 60 kb downstream of the *Pmp22* translation start site) that encompasses the entire coding region of the *Pmp22* gene driving the *LacZ* reporter gene inserted into the translation start site of the *Pmp22* gene. Since the only differences between these two transgenes is the addition of the 3` portion of the *Pmp22* gene in the second transgene (the 5` sequence of second transgene is identical to the first transgene), analysis of these two transgenes together will interrogate the importance of *cis*-elements 3` to the *Pmp22* translation start site on the expression of the reporter gene. In this chapter, I will discuss the expression of -21 kb *Pmp22* promoter construct driving the bacterial *LacZ* reporter gene.

4-1 Production and Characterization of transgenic mice

As noted above, the first BAC-derived construct contains contiguous mouse genomic DNA 21 kb upstream of the translation start codon of *Pmp22*. This fragment of putative *Pmp22* control region is used to drive the *LacZ* reporter gene (figure 4-1d). As discussed in the methods section (Chapter 2), the transgene was constructed in a bacterial artificial chromosome (BAC) as the cloning vector because BACs can handle a large insert of the size with higher fidelity than plasmid and cosmid vectors. The subcloning and assembly of the construct is discussed in detail in the methods section (Chapter 2, section 2). Subsequently, the *Xho* I linearized –21 kb *Pmp22/LacZ* construct was injected into fertilized ova for the production of transgenic mice. Five founder animals (designated BM5, BM10, BM18, BM63, and BM68) were generated and characterized. The mice were partially characterized by Southern blot analysis for the for the presence and approximate copy number of the 21 kb *Pmp22* promoter construct on *Eco*R I digested genomic DNA derived from the transgenic mice. The Southern blots were probed with a 421 bp *Sma* I ^{32}P-labeled fragment representing the region immediately 5` to exon 2 of *PMP22* gene. As shown in figure 4-1b, the probe recognizes a single band of 3 kb corresponding to a copy number of 8 for BM5, over 40 copies for BM10, 2 copies for BM18, one copy for line BM63, and over 12 copies for line BM68 after quantitative analysis by phosphoimaging.

4-2 Different patterns of expression

All transgenic lines were analyzed using β-galactosidase histochemistry. The most striking finding using the –21 kb *Pmp22/LacZ* transgene is the marked variability between the pattern of reporter gene expression among the different lines. In general, the expression profiles in peripheral nerve fell into one of two patterns. As will be discussed more fully below, one pattern (in one line, BM10) showed marked β-galactosidase expression in myelinating Schwann cells (as expected for a myelin structural gene), while the other favored expression in dorsal root ganglia (as observed with the –8.5 kb rat *Pmp22* promoter construct as described in Chapter 3). These two expression patterns

tended to be exclusive. The first mouse (F2 progeny) tested from transgenic line BM10 exhibited significant activity for promoting expression from the *LacZ* gene within the peripheral nerve fibers. Expression of β-galactosidase in the peripheral nerves in line BM10 was very high as assessed by the intensity of the BluoGal and X-Gal staining. Furthermore, the pattern of expression of the transgene in line BM10 as revealed by BluoGal staining resembled the endogenous *Pmp22* gene in terms of relative expression in different tissues, clear down-regulation in response to focal axon injury (crush, see below), and high levels of expression in mature peripheral nerves. In contrast to the mice expressing the rat –8.5 kb *Pmp22* promoter/*LacZ*/CAT transgene (see previous chapter) and other lines expressing the –21 kb *Pmp22/LacZ* construct (see below), the dorsal root ganglia of BM10 mice did not show any reactivity for β-galactosidase expression by either BluoGal or X-gal histochemical staining. On the other hand, the other 4 lines derived from the murine -21 kb *Pmp22* construct, BM5, BM18, BM63, BM68 demonstrated very low levels of β-galactosidase expression in their peripheral (sciatic) nerve fibers. This latter pattern of expression from the –21 kb *Pmp22/LacZ* transgene was qualitatively and quantitatively similar to the transgenic mouse lines carrying the rat –8.5 kb promoter/*LacZ*/CAT transgene. Also, like the rat –8.5 kb *Pmp22* promoter transgenic mice, lines BM5, BM18, BM63, BM68 demonstrated high level of expression in the dorsal root ganglia.

Although the line derived from BM10 expressed well in the PNS in early generations (F2, and F3), the expression in peripheral nerves gradually diminished and was then lost over the next few generations. The latter generations barely expressed β-galactosidase in the peripheral nerve fibers as assessed by BluoGal and X-gal histochemistry. Interestingly, as the β-galactosidase staining of the nerves was lost with successive generations, β-galactosidase staining became increasingly evident in the dorsal root ganglia of these animals. Ultimately, line BM10 came to express in a similar fashion to the other lines that carry the same –21 kb *Pmp22/LacZ* construct (lines BM5, BM18, BM63, and BM68). Initially, I thought that the disappearance of the expression could be due to insertional factors, i.e. having more than one site of insertion that was

segregated through breeding. This possibility was ruled out by a Southern blot that showed that the founder, expressing, and non-expressing mice shared the same insertion pattern. I also was unable to segregate a highly expressing line by back crossing the BM10 line to B6 and C3H separately and after 15 generations of backcrossing the expression pattern remained to be persistent in the dorsal root ganglia in both the B6 and the C3H back cross. In general, later generations of the BM10 mice were relatively consistent in showing staining in the DRGs and very weak, if any, staining in some Schwann cells. It is noteworthy that high level of expression in the sciatic nerve fascicles was never associated with significant expression of the transgene in the dorsal root ganglia in these animals. Interestingly, however, after over 20 generations, the β-galactosidase histochemical (BluoGal or X-gal) reactivity was restored in peripheral nerve fibers while concomitantly the staining in the DRG was lost in some litters. This restoration of fascicular staining did not last for more than a single generation before it started to disappear again. Thus, the changes in the pattern of gene activity through successive generations of the BM10 mice resembles one that might be expected from silencing mechanisms, such as methylation (see discussion). Thus, a cardinal feature of the BM10 line is that while the expression of successive generations of the BM10 line was presumably silenced, it was possible to have a sporadic mouse that would react similar to the first generation. None of the successive generations derived from the other transgenic mouse lines using the -21 kb *Pmp22/LacZ* construct ever showed a pattern of expression in myelinated fibers that resembled the early generations of the BM10 line.

4-3 Targeting to Schwann cells

As outlined above, only one transgenic line (BM10) showed high levels of expression in the myelinated fibers of the peripheral nervous system in some of its generations. All other lines showed barely detectable levels of expression in the nerve fibers, similar to the rat –8.5 kb *Pmp22* promoter/CAT /*LacZ* construct. Nonetheless, the transgene expression was targeted to the PNS in these animals, since lines BM5, BM18, BM63, and BM68 showed high level of expression in the dorsal root ganglia as assessed by BluoGal and X-gal histochemistry, comparable to the expression in the rat -8.5 kb

Pmp22/CAT/*LacZ* transgenic lines. Figure 4-2 shows whole mount images of spinal cord, spinal roots and sensory ganglia (panel a), and light micrographs of a longitudinal (panel b) and cross section (panel c) of a sciatic nerve from an adult mouse, line BM10, stained for β-galactosidase expression using X-gal. The reaction product appears to co-localize with Schwann cell cytoplasm in the perikaryon of the Schwann cells surrounding intact myelinated axons in one-micron plastic sections of peripheral nerve (see figure 4-2d). The other four –21 kb *Pmp22/LacZ* lines (BM5, BM18, BM63, and BM68) showed little expression in myelinated nerve fibers, and the reaction product that was found to be restricted to very few Schwann cells in a section.

4-4 Developmental regulation

One of the important features of endogenous *Pmp*22 gene expression is the marked developmental regulation that occurs during postnatal development, as myelination proceeds. For the –21 kb *Pmp22/LacZ* constructs, it was obviously difficult to demonstrate any developmental regulation conferred by this transgene. Analysis of line BM10 was complicated by presumed silencing which made it very difficult to determine the significance of negative staining for β-galactosidase in a nerve from a developing BM10 mouse. Analysis of the other –21 kb *Pmp22/LacZ* lines exhibited very low levels of expression in myelinating Schwann cells. Nonetheless, analysis of a relatively large number of animals revealed that low levels of BluoGal staining in whole mount sciatic nerve is detectable in some mice of line BM10 as early as 5 days postnatal, but not before (data not shown). This suggests that, under permissive conditions (lack of silencing?), the –21 kb construct in line BM10 is developmentally regulated. The level of expression of β-galactosidase in sciatic nerve bundles in the remainder lines was below the detectable threshold of the histochemical β-galactosidase assay (Chapter 2-9).

4-5 Regeneration

I was able to analyze the response of the –21 kb *Pmp22* promoter/*LacZ* construct during nerve regeneration in the focal crush injury model of Wallerian degeneration/regeneration in line BM10. These experiments were feasible because

positive expression from the -21 kb *Pmp22/LacZ* construct could be established for each individual mouse through analysis of β-galactosidase activity in contralateral and proximal nerve segments. Focal crush injury to sciatic nerves was applied using a #5 jeweler forceps at the mid thigh level, as described in detail in the methods section. I found that the β-galactosidase staining in the distal nerve stump of focally crushed sciatic nerves was significantly diminished within 4 days after crush injury compared to the intact contralateral control nerves in 4 β-galactosidase positive mice. By twenty-one days post-injury, β-galactosidase histochemistry revealed re-activation of the transgene activity during the remyelination that accompanies nerve regeneration. Compared to the rat -8.5 kb *Pmp22* promoter/*LacZ*/CAT construct, this -21 kb *Pmp22/LacZ* construct (in the BM10 line) is capable of functioning in response to nerve regeneration, suggesting that (an) enhancer element(s) responsible for regeneration could lie within the stretch between –8.5 and –21 of the *Pmp22* promoter. It was not possible to perform similar experiments on the other lines that carry the –21 kb *Pmp22/LacZ* transgene because of the low level of expression of the transgene in the myelinating fibers in the sciatic nerve of these mice.

4-6 Utilization of 1A vs. 1B promoters

As discussed earlier, the ratio of the exon 1A-containing PMP22 mRNA transcript to the exon 1B-containing PMP22 mRNA transcript reflects which promoter, P1 or P2, is preferentially activated in the transgene. This, in turn, can be used to determine the activation status of each of these promoters and can be used as a functional assay of specific elements that activate the myelin-associated *Pmp22* promoter, P1. Similar to the case of the – 8.5 kb *Pmp22/LacZ*/CAT mice, it is possible that the only difference between the two expression patterns observed between line BM10 and the other lines of the –21 kb *Pmp22/LacZ* mice is whether or not P1 is activated. While it is not practical to analyze the 1A/1B ratios corresponding to the two BM10 phenotypes (expression in sciatic nerve vs. DRG), we did compare the 1A/1B ratios of BM68 (expression in DRG) to that of the endogenous mouse sciatic nerve *Pmp22*. A pool of sciatic nerves from two month old transgenic mice was collected and homogenized as described in the methods

section (chapter 2). Two sets of RT-PCR reactions were performed in triplicate to determine the 1A/1B ratios for both –21 kb *Pmp22/LacZ* mRNA and endogenous PMP22 mRNA in line BM68. For *Pmp22/LacZ*, I used a forward primer specific for exon 1A and in a parallel PCR reaction a forward primer that recognizes exon 1B. Both primers were individually paired with a common reverse primer specific for the *LacZ* gene. The average 1A/1B ratio from the *Pmp22/LacZ* mRNA (BM68) was compared to the ratio observed in parallel samples from the endogenous PMP22 transcript RT-PCR using the same forward primers but with a reverse primer specific for exon 2. The products of these reactions were analyzed as the ratio of transgene 1A: 1B transcripts compared to endogenous 1A: 1B transcripts. The ratio of the endogenous 1A: 1B was 3:1 (figure 4-3), which, fortuitously, is comparable to values previously obtained by northern blot analysis. This is fortuitous because the 1A and 1B primers would appear to have similar priming efficiencies in order to give a result that accurately reflects the absolute concentrations of endogenous mRNA species. The transcript ratio 1A: 1B was 1:3 for the PMP22/*LacZ* transgene (figure 4-3). Thus, in line BM68, expression from the –21 kb *Pmp22/LacZ* transgene appears to be associated with preferential activation of promoter P2 rather than the P1 as seen in the endogenous gene. This comparison is possible because the ratios of the 1A: 1B transcripts (using identical forward primers) should be independent of the priming efficiency of the reverse primer.

4-7 Expression of 21 kb Pmp22 promoter/LacZ reporter construct at the hprt locus

Based on analyses of this small number of lines, it is impossible to determine which pattern of expression, the peripheral nerve expression of early generations of BM10, or the DRG expression of the later generations of line BM10 and lines BM5, BM18, BM63, and BM68, truly represents the intrinsic properties of the transgene. One approach to this problem would be to generate additional lines of conventional transgenic mice and seek a consensus for the pattern of expression. However, it is unclear how many lines it would take to be convincing. A second approach is to try to

control the site of insertion and copy number. We chose to do this by using homologous recombination to target transgenes to the *hprt* locus into ES cells. This system exploits ES cells that are partially deleted for the *hprt* locus on the X chromosome. Targeted recombination at the *hprt* locus is achieved by electroporating *hprt*-deficient ES cells with constructs containing the test transgene of interest flanked by *hprt* sequence that upon homologous recombination, incorporates a single copy of the transgene and functionally reconstitutes the *hprt* locus allowing for positive selection of recombinant ES cells in hypoxanthine/aminopterin/thymidine (HAT) containing media.

In collaboration with Dr A. Peterson, we assembled a test myelin gene promoter, incorporating –3.1 kb of the myelin basic protein (MBP) gene driving the *LacZ* reporter to create a targeting construct for the *hprt* locus to test the proof of principle that a defined site (the *hprt* locus) could targeted and that it would be permissive for expression of a chosen promoter transgene/reporter. Following electroporation into ES cells, incorporation into blastocysts, and ultimately transgenic mice, the –3.1 kb MBP/*LacZ* construct gave high levels of β-galactosidase expression in myelinating cells, oligodendrocytes and Schwann cells. The *hprt* locus, therefore, seemed to be permissive to short myelin basic protein MBP promoter (3.1 kb) function in myelinating cells and was capable of reproducing results obtained using traditional pronuclear transgenesis. Thus, targeting to the *hprt* locus represents a viable approach to reproducibly study myelin gene promoter analysis *in vivo*. To further pursue this approach, I modified the -21 kb *Pmp22/LacZ* construct by adding the appropriate *hprt* recombination arms so that I could target the homologous recombination of one copy of the –21 kb *Pmp22/LacZ* transgene to the *hprt* locus in partially *hprt*-deficient ES cells (see figure 2-5b).

Transgenic chimeras were generated by the procedures provided in the methods section in Chapter 2. Since the *hprt* locus is on the X-chromosome, male mice were analyzed. Whole mount BluoGal histochemistry for β-galactosidase showed expression of the -21 kb *Pmp22/LacZ* transgene only in the dorsal root ganglia in the PNS, exactly like the

other four/five -21 kb PMP22 lines derived by pronuclear injection as well as the four lines using the -8.5 kb *Pmp22* rat promoter. Subsequent microscopic histochemical analysis of peripheral nerves in adult mice using X-Gal confirmed that –21 kb *Pmp22* promoted expression of β-galactosidase from the *hprt* locus is restricted to the dorsal root ganglion in the –21 kb *Pmp22/LacZ/hprt* mouse (see figure 4-4, panel b).

In conclusion, analysis of the –21 kb *Pmp22/LacZ* construct is somewhat equivocal. The transgene appears to give one of two expression patterns in the PNS: one line, BM10, is capable of strong expression in myelinating Schwann cells; while the others, exemplified by the lines BM5, BM18, BM63, and BM68 as well as the –21 kb *Pmp22/LacZ/Hprt* mouse line show expression predominantly in the DRGs. The problem of which pattern of expression is truly representative of the –21 kb *Pmp22* promoter is further compounded by the fact that whereas four out of the five lines have comparable patterns of expression that do not resemble the expression expected from the endogenous PMP22 locus, it is difficult to discount line BM10 which has an expression pattern that closely resembles that expected for the endogenous *Pmp22* gene, albeit inconsistently across generations. We also cannot rule out the effect of the copy number, since line BM10 has the highest copy number of the transgene, nor the effect of the site of insertion, since presumably the insertion sites are random, as additional factors influencing the transcriptional regulation of the transgene.

If copy number and site of insertion are major confounding factors for expression of the -21 kb *Pmp22* promoter fragment, analysis of the –21 kb construct in the *hprt* locus should produce a result independent of these factors. If we assume that the *hprt* locus is neutral to the *Pmp22* promoter (as it appears to be for the MBP promoter), we can conclude that the pattern of expression determined by –21 kb *Pmp22/LacZ/hprt* transgenic mice (as well as lines BM5, BM18, BM63, and BM68, and a number of generations of the BM10 line) is more representative of the inherent regulatory properties of the –21 kb stretch of the *Pmp22* control region. In this scenario, the –21 kb promoter construct lacks *cis*-regulatory elements required to fully recapitulate the

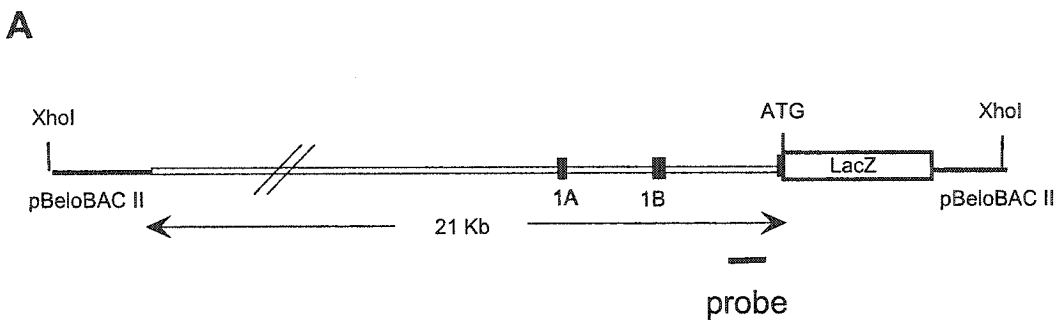
expression pattern of the endogenous *Pmp22* gene. Line BM10, while also lacking these same elements, may be functionally complemented for *Pmp22* expression by factors related to site of insertion, perhaps adjacent to another gene regulated during myelination. Alternatively, the transgene in the BM10 line may be insulated from negative influences of surrounding genes as a result of its high copy number and contiguous multiple insertions. In conclusion, we can say, 1) the –21 kb *Pmp22* promoter can target to the peripheral nervous system, 2) in contrast to the –8.5 kb *Pmp22* promoter, the –21 kb *Pmp22* construct appears to contain elements that are responsive to remyelination during nerve regeneration, and 3) with caveat, that most likely the 21 kb mouse *Pmp22* promoter does not contain the elements necessary to autonomously direct the proper expression pattern for *Pmp22*. It remains possible, however, that the regulatory elements of the *Pmp22* gene are very sensitive to the site of insertion for proper functioning or for protection from silencing.

In the next chapter, I will show that the site of insertion can be overcome as a variable for *Pmp22* gene expression. Furthermore, I will provide evidence that the pattern of expression of *LacZ* by the various *Pmp22* promoter constructs, which appear to provide conflicting results with regards to expression in peripheral nerves or dorsal root ganglia, may relate to activation of different promoter elements during development.

Figure 4-1

Construction of the –21kb *Pmp22* promoter/reporter transgene (A). The transgene consists of -21 kb of the mouse *Pmp22* control region relative to the translation start site driving the bacterial β-galactosidase (*LacZ*) gene. Also you can see the relative site of the probe used for the Southern blot of *Eco*R I digested genomic DNA from each mouse line (B) used to determine the copy number of each line. Lane 1 : BMH(-21 kb promoter reporter construct at the *Hprt* locus), 2: BM10, 3:BM5, 4: BM18, 5: BM63, 6: BM68, 7: wild type.

Figure 4-1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ATG
XhoI
LacZ
pBeloBAC II
1A
1B
pBeloBAC II
21 Kb
probe

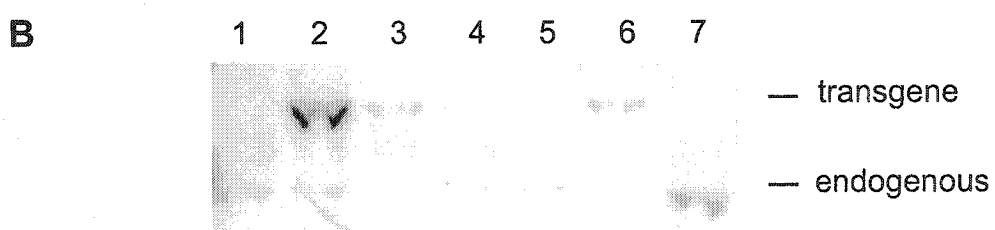

B
1
2
3
4
5
6
7
— transgene
— endogenous

Figure 4-2

Whole mount (a) and histological (b,c,d) preparations for β-galactosidase activity stained with Bluo-gal (a,d) and X-gal (b,c,d). Panel a shows Bluo-gal staining in peripheral nerve and not in dorsal root ganglia (arrow) of F2 transgenic mouse from line BM10. Histologic longitudinal sections of sciatic nerve, panel b, the arrow indicates a node of Ranvier. While panel c is a cross section of sciatic nerve of the same transgenic animal. Notice in panels b and c how the staining is confined to the cytoplasm of Schwann cells. Panel d is a 1 micron cross section of sciatic nerve showing the presence of X-gal crystals in the cytoplasm of Schwann cell.

Figure 4-2

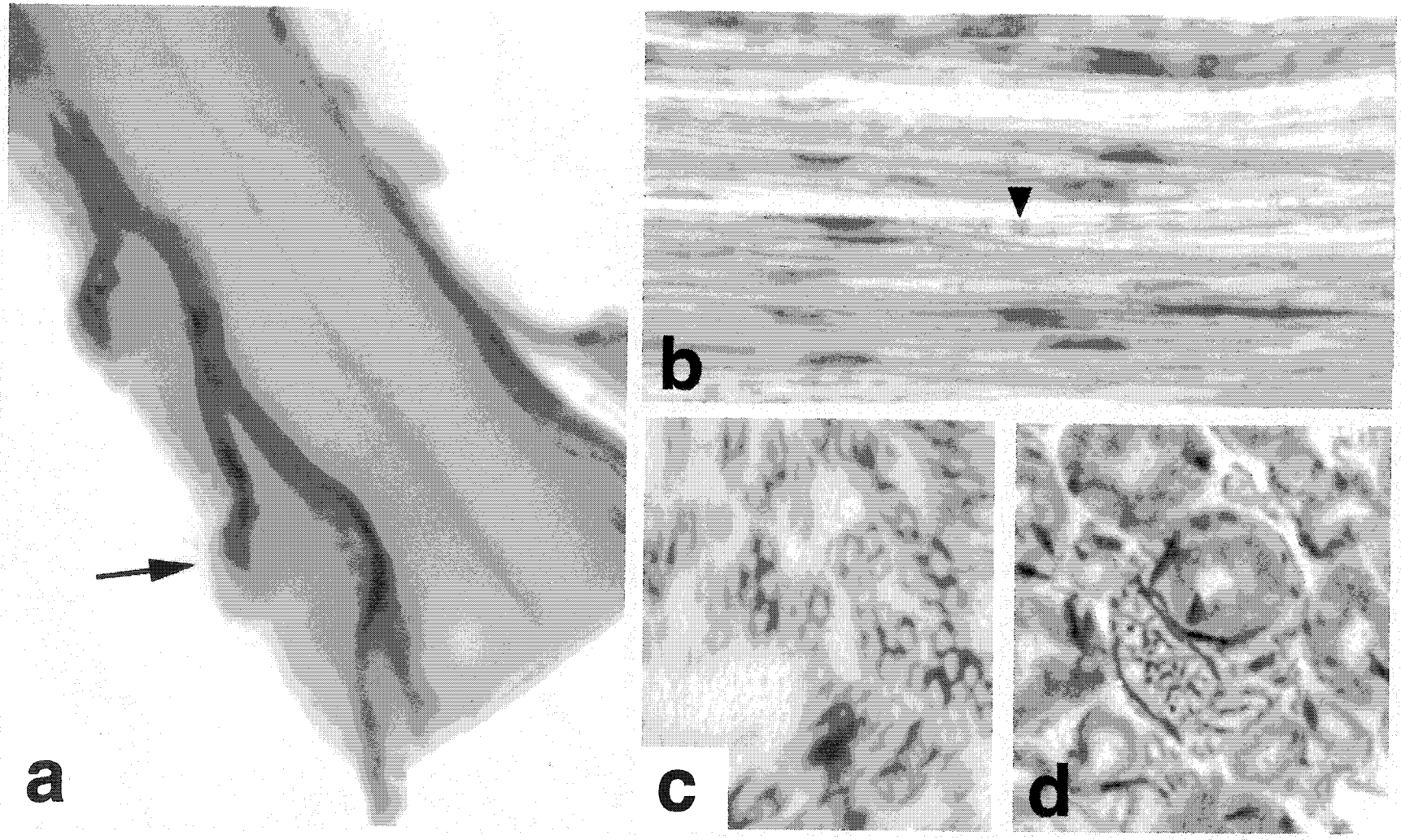
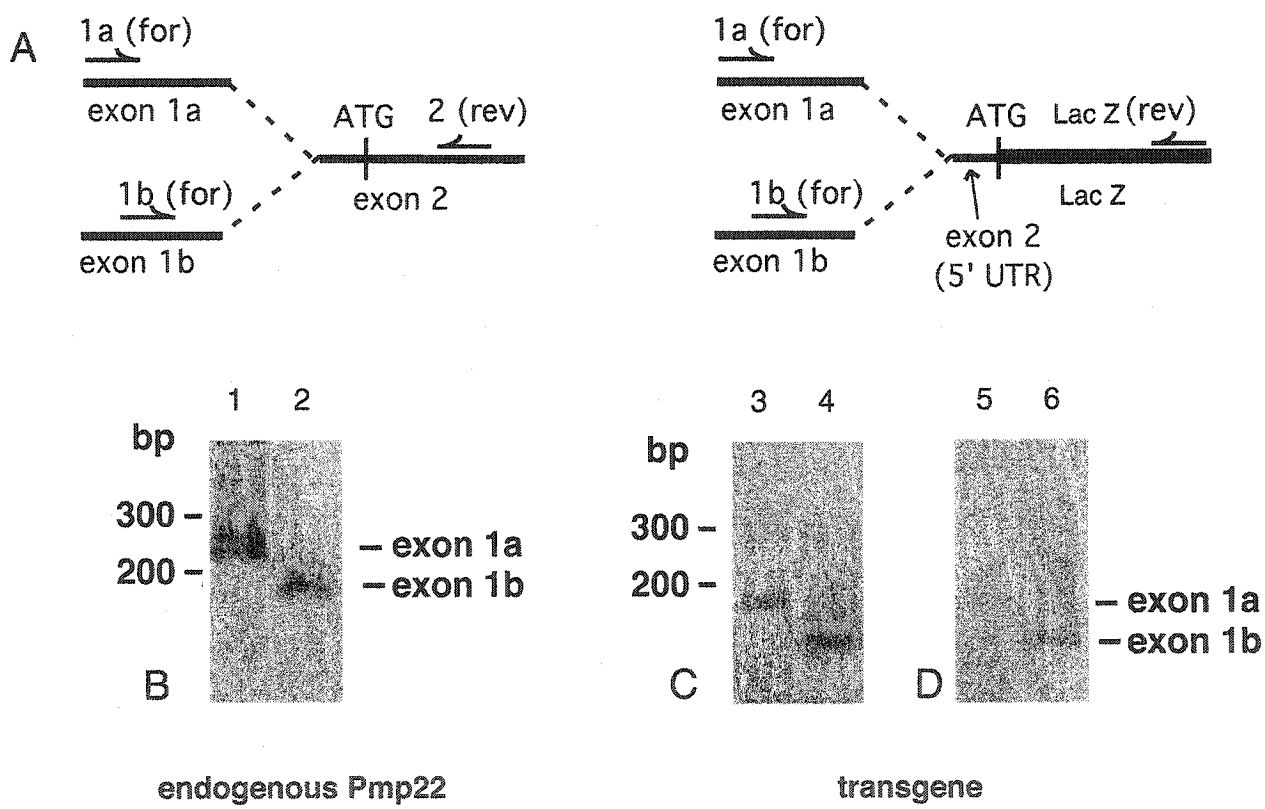


Figure 4-3.

Adult sciatic nerves from -21 kb *Pmp22* promoter transgenic mice preferentially produce mRNA containing the exon 1b transcript whether they are produced by pronuclear injection (panel D), or by targeting one copy of the transgene to the *Hprt* locus (panel C), as compared to the endogenous PMP22 mRNA (panel B). The design of the RT PCR is depicted in panel A. PCR was performed on reverse transcribed RNA derived from the sciatic nerves of the -21 kb *Pmp22/LacZ* mice. Semi-quantitative PCR was performed using primers specific for the endogenous PMP22 transcript (primer "2 (rev)' left, panel A; results in panel B) or for the *LacZ* containing transcript (primer "LacZ (rev)" right, panel A; results in panel C and D). Note that the forward exon 1a and exon 1b specific primers are identical. Percentages shown represent the average of three independent PCR reactions for the percent of total ethidium bromide staining for exons 1A and 1B in each experiment. Lanes 1, 3 and 5 show RT-PCR results using primers specific for 1A transcript; while lanes 2, 4, and 6 show RT-PCR results using primers specific for 1B transcript.

Figure 4-3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2 (rev)
1b (for)
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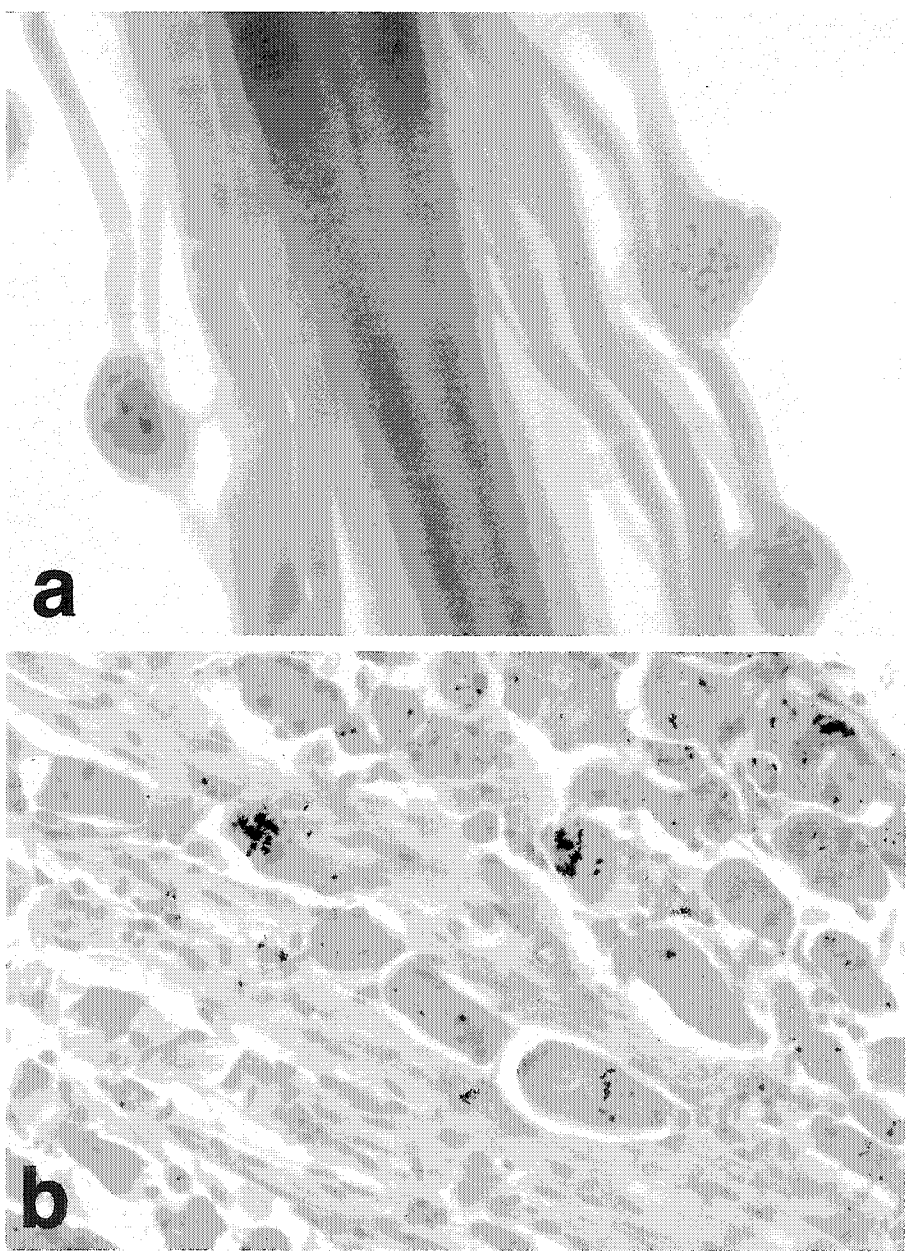
exon 2
1a (for)
exon 1a
ATG
Lac Z (rev)
1b (for)
exon 1b
Lac Z
exon 2
(5' UTR)
1 2
bp
300 –
200 –
– exon 1a
– exon 1b
B
3 4
bp
300 –
200 –
C
5 6
– exon 1a
– exon 1b
D
endogenous Pmp22
transgene

Figure 4-4

Panel a shows a whole mount of 9 weeks old male stained with punctate staining in the dorsal root ganglia. Frozen section of dorsal root ganglion is panel b, showing bluogal crystals in the cytoplasm of DRG neurons.

Figure 4-4



Chapter 5: The expression of a 100 kb *PMP22/LacZ* construct in transgenic mice

The location of enhancer/repressor elements varies from gene to gene. Most genes have their essential control conveyed by a proximal promoter sequence within the first 2-3 kilobases upstream of the translation initiation site. As discussed in the introduction (Chapter 1), several genes have control elements either far upstream or downstream of the translation start site. For PMP22, as I demonstrated in Chapter 2, -8.5 kb was able to target expression to the PNS predominantly through the P2 promoter activity, while promoter P1 did not show comparable activation. When I incorporated an additional 12 kb 5` to the original –8.5 kb construct, there seemed to be better regulation of P1 based on the expression of the reporter gene and assay of the transcript using RT-PCR techniques, but I hypothesized that major factors responsible for reproducibly boosting the expression of the transgene to the high levels as expected for the endogenous *Pmp22* gene activity were missing. Again, the missing elements could either be 5` or 3` or both. In chapter 4, I evaluated the ability of 21 kb upstream of the *Pmp22* translation start site to confer endogenous-PMP22-like regulation both by conventional pronuclear transgenesis and by direct insertion into the *hprt* locus and obtained equivocal results. So, at this juncture, I decided to use the -21 kb promoter driving *LacZ* and, in addition, to include an additional 80 kb (encompassing the entire coding region of *Pmp22*) of 3` sequence of *Pmp22* (Figure 5-1). I developed a novel methodology to enable insertion of a reporter gene (*LacZ*) at the start of the transcription of *Pmp22* embedded in a 100 kb BAC (clone 132C18) that carries the full *Pmp22* gene. I used homologous recombination in *E. coli* to create the desired BAC transgene. Briefly (for more details see the methods section, Chapter 2), a targeting vector containing two recombination arms derived from the *Pmp22* gene were used to target the insertion of the *LacZ* cassette, a poly adenylation signal and kanamycin resistance gene in frame with the start of translation of *Pmp22* in clone 132C18. The 5` arm consisted of approximately 2 kb of *Pmp22* DNA upstream of the start of translation, while the 3` arm extended approximately 1.5 kb downstream of the start of translation. The start codon of *Pmp22*

was modified to be in frame with the translation start codon, ATG, of *LacZ* after recombination. The targeting plasmid was electroporated into recombinase-positive *E. coli* (BJ5183) that had been stably transformed with BAC clone 132C18 and made electrocompetent. The insertion of the kanamycin resistance gene into the target was identified by the positive selection in the presence of kanamycin and chloramphenicol. The recombinant clones containing the *LacZ* gene were confirmed by restriction digestion and by Southern blotting (see figure 2-4). Several clones were identified that essentially retained the restriction digestion pattern of BAC clone 132C18 but also showed the expected rearrangement on Southern blot.

5-1 Production and Characterization of 100 kb Pmp22/LacZ transgenic mice

The BAC carrying the *Pmp22* gene with the *LacZ* gene at the translation start site was linearized using cosN Lambda terminase, ran on 0.4% agarose gel at 35 V for over 24 hours then gel purified and used for pronuclear injection in mouse fertilized ova. Six founder mice, BMW413, BMW418, BMW419, BMW420, BMW422, BMW428, were generated and the presence of the *LacZ* gene was confirmed by PCR as described in the methods section (Chapter 2). The presence of the transgene in the mice was further confirmed and relative copy numbers were determined by Southern blot. Based on the intensity of hybridization to the ^{32}P-labeled 421 bp *Sma* I probe (see chapters 2,3, and 4), the copy numbers of transgene insertion were estimated to be: 2 for lines BMW413, 419, and 422, while line BMW 428 carries 6 copies (Figure 5-1). Initial *LacZ* whole mount histochemical staining of sciatic nerves using BluoGal revealed strong blue staining in peripheral nerves of lines BMW413, BMW419, BMW422, BMW428 while lines BMW418, BMW420, expressed very poorly if at all. Clear differences in the staining intensities could be seen in X-gal stained sections of sciatic nerves from the different lines. By X-gal staining, line BMW428 is the strongest expresser, and BMW422 is the weakest, while BMW413 and BMW419 are intermediate. Qualitatively, however, the four expressing transgenic lines exhibit a similar pattern of expression.

5-2 The 100 kb Pmp22/LacZ transgene targets expression to Schwann cells in adult mice.

Sciatic nerves from the transgenic mice lines carrying the 100 kb *Pmp22/LacZ* transgene were stained as whole mounts in staining buffer containing X-gal (see the methods section, Chapter 2). Four lines carrying the 100 kb *Pmp22/LacZ* construct showed strong β-galactosidase expression in the peripheral nervous system (the nerves stained within 45 minutes after immersion in staining buffer). Sixteen-micron thick frozen sections from sciatic nerves showed high levels of β-galactosidase staining in Schwann cells. This is best shown by a representative cross-section of an adult sciatic nerve from transgenic mouse line BMW428 stained for β-galactosidase activity. The blue X-gal staining pattern recapitulates the toroidal shape of the Schwann cell cytoplasm around myelin and axons. Essentially all Schwann cells in the cross section stain for β-galactosidase. Since the majority of perikarya in a cross section of sciatic nerve are Schwann cells, it is evident that the transgene must be expressed by Schwann cells. These studies, however, do not rule out the possibility that endoneurial fibroblasts also contribute to the β-galactosidase activity. β-galactosidase histochemistry of longitudinal sections and of teased nerve preparations from adult sciatic nerves of transgenic mouse also strongly indicate that the 100 kb *Pmp22/LacZ* construct targets expression to Schwann cells. As shown in figure 5-2, cross sections of sciatic nerves from the 100 kb *Pmp22/LacZ* mice show dark blue reaction product in the cytoplasm of myelinating Schwann cells.

The dorsal root ganglia of the 100 kb *Pmp22/LacZ* mice also demonstrated β-galactosidase activity. As shown in Figure 5-2, the staining in the DRG at the light microscopic level is observed mainly in nerve sheath cells, presumably Schwann cells. Little is the staining in the neuronal cell bodies of the DRG of adult mice, and of the anterior horn of the spinal cord. No staining was detected in the brain, but motor cranial nerves were positive for the histochemical detection for β-galactosidase expression. No expression of the transgene is detected in the brain of adult animals, while some cell

bodies in the anterior horn of the spinal cord showed low reactivity to β-galactosidase detection. On the other hand, frozen sections of the vibrissae roots in adult mice showed considerable staining in the innervation for the roots. I detected no extra-neural expression of the 100 kb transgene construct in adult mice in the four expressing transgenic mouse lines.

5-3 Prenatal expression of the 100kb Pmp22/LacZ transgene

The expression of the 100 kb *Pmp22/LacZ* transgene was robust and stable, unlike the –21 kb *Pmp22/LacZ* transgene; thus, it was possible to undertake a detailed study of the temporal and spatial expression patterns of the 100 kb transgene during embryonic development. In previous studies using *in situ* mRNA hybridization, endogenous PMP22 mRNA (Parmantier et al., 1997; see the introduction) was detected in the mouse embryo as early as embryonic day E9.5 in the neural tube and dorsal root ganglia. Subsequently, it is expressed in the capsules of visceral organs (e.g. liver), the skin, and pre-cartilaginous tissue. By E17.5, however, PMP22 mRNA expression in these tissues was extinguished and expression in peripheral nerve had not yet begun. Since one of our goals is to generate a transgene that recapitulates the expression pattern of the endogenous *Pmp22*, it was of considerable interest to determine the expression pattern of the 100 kb *Pmp22/LacZ* transgene during embryonic development.

Analysis of the prenatal expression of the 100 kb *Pmp22/LacZ* mice by Bluo-gal histochemistry showed that as early as E 8.5 (Fig 5-3), β-galactosidase activity could be detected in the neural tube. At E9.5, the staining pattern in the nervous system extended to include the dorsal root ganglia and the medulla. By E12.5-E13.5, the *LacZ* expression becomes evident in precartilagenous tissue, and midbrain. Then, by E14.5-E15.5, the capsule of the liver and the skin of the embryo show significant β-galactosidase activity as well (Fig 5-4). Almost all the histochemical reactivity for β-galactosidase disappears by E17.5-E18.5 in the 100 kb/*LacZ* mice only to reappear after birth in peripheral nerves. Thus, the results from analysis of the 100 kb *Pmp22/LacZ*

transgene are qualitatively similar to the results of the *in situ* PMP22 mRNA expression studies.

5-4 Developmental regulation

Pools of sciatic nerves from transgenic mice aged 3, 7, 14, and 21days were homogenized in Reporter Lysis Buffer (Promega) using polytron tissue homogenizer (see the methods section, Chapter 2). The homogenate is analyzed using ONPG at 37 C for 30 minutes. It was noticed that the activity of ß-galactosidase determined by the assay have increased about 40 times between the age of 3 days and the age of 21 days in a pattern similar to the one noticed before (Suter et al., 1994) when the endogenous PMP22 was analyzed at the level of mRNA. Even though the increase then was about 200 times, the levels here are normalized to the total protein content of the homogenate. Figure 5-5 presents the results of a triplicate experiment of ß-galactosidase level in the sciatic nerves of transgenic animals at the ages of 3, 7, 14, and 21, and shows the increase of the level of the transgene product during development.

5-5 Regeneration

As discussed in the previous chapters, there is a stereotyped expression pattern for myelin genes that are down-regulated during Wallerian degeneration and reactivated during the nerve regeneration and remyelination that occurs after focal crush injury. As we have seen in Chapter 3, the –8.5 kb *Pmp22*/CAT/*LacZ* construct is down-regulated after focal crush injury, but it is not reactivated during remyelination. Analysis of the –21 kb *Pmp22*/*LacZ* construct in line BM10 in Chapter 4, on the other hand, revealed that this construct can respond to signals that turn off myelin gene expression during Wallerian degeneration and can be activated during the remyelination that occurs during nerve regeneration. As part of the characterization of the 100 kb *Pmp22*/*LacZ* construct, I performed a focal crush injury on the right sciatic nerves of several adult transgenic mice. As assessed by Bluo-Gal histochemistry and shown in Fig 5-6, β-galactosidase activity was almost completely lost over the five days following nerve crush compared to unoperated contralateral nerve controls. Some individual nerve fibers did retain some

β-galactosidase activity. By 21 days post crush, however, the transgene was reactivated and the staining reaction was significantly increased relative to the levels observed at 5 days post crush and when compared to the staining of contralateral control nerves. Thus, the 100 kb *Pmp22/LacZ* transgene like the endogenous *Pmp22* gene contains elements that are responsive to denervation and reinnervation.

5-6 Promoter utilization

Analysis of the –8.5 kb *Pmp22*/CAT /*LacZ* and –21 kb *Pmp22/LacZ* mice suggested that the ability of these transgenes to recapitulate the expression pattern of the endogenous *Pmp22* gene, particularly the high expression levels in myelinating Schwann cells, might reside in the ability to activate transcription from promoter P1, upstream of the myelin-associated exon 1A. I investigated whether the strong expression of the 100 kb *Pmp22/LacZ* transgene in peripheral nerves was associated with preferential activation of the exon 1A-containing transcript using an RT-PCR strategy analogous to that employed in chapters 3 and 4. The reverse transcription/PCR utilized the same primers described for the -21 kb *Pmp22/LacZ* promoter construct (see Chapters 2, 3, and 4). Figure 5-7 shows one of the samples of the RT-PCR. Triplicate experiments were done and quantified using ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA), and then averaged. By semi-quantitative PCR, the ratio of the 1A: 1B transcripts using primer pairs specific for the endogenous gene was 3:1, while the 1A: 1B ratio for the transgene was 3:2. Recall that this ratio for the transgene is reversed from the –8.5 kb *Pmp22*/CAT/*LacZ* and –21 kb *Pmp22/LacZ* transgenes, which do not show high levels of expression of the transgene in peripheral nerves. This result shows that the 100 kb *Pmp22/LacZ* transgene has elements that are capable of upregulating the exon 1A-containing PMP22 mRNA transcript in peripheral nerve in a comparable fashion to the endogenous *Pmp22* gene.

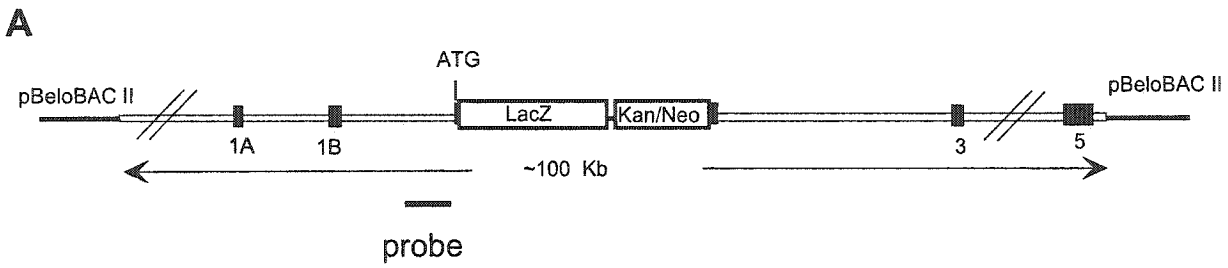
In summary, the expression from the 100 kb *Pmp22*/LacZ transgene is temporally and spatially comparable to the endogenous *Pmp22* expression. If we put this together with

the results we obtained from the *Pmp22* constructs mentioned in chapters 3 and 4 we can appreciate the effect of the 3` elements.

Figure 5-1

Structure of the 100 kb *Pmp22* BAC transgene (A). The transgene consists of -21 kb of the mouse *Pmp22* promoter region relative to the translation start site driving the bacterial β-galactosidase (*LacZ*) gene and the 3` the complete mouse *Pmp22* gene. Also you can see the relative site of the probe used for the southern blot (B) of *Eco*R I digested genomic DNA from each mouse line used to determine the copy number of each line. Lane 1 : line BMW413, 2: BMW419, 3:BMW422, 4: BMW428, 5: wild type.

Figure 5-1


A
ATG
pBeloBAC II
LacZ
Kan/Neo
pBeloBAC II
1A
1B
3
5
~100 Kb
probe

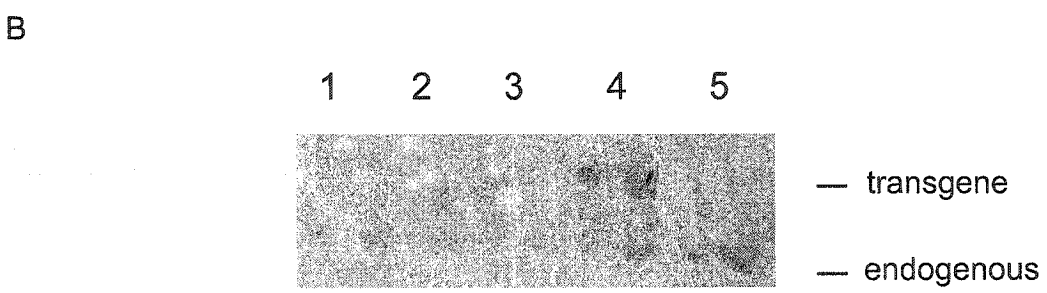

B
1
2
3
4
5
— transgene
— endogenous

Figure 5-2

Whole mount (a) and histological (b,c) preparations for β-galactosidase activity stained with Bluo-gal (a) and X-gal (b,c) for an adult mouse from line BMW428. Panel a shows intense staining in the peripheral nerve roots emerging from the spinal cord. Panel b, 16 micron frozen section of DRG, the strong X-gal staining is present in ensheathing cells with punctate X-gal staining in DRG neurons. Panel c is a cross section of a peripheral nerve from the 100 kb *Pmp22/LacZ* . The X-gal staining show a characteristic staining for Schwann cell cytoplasm surrounding myelinated fibers.

Figure 5-2

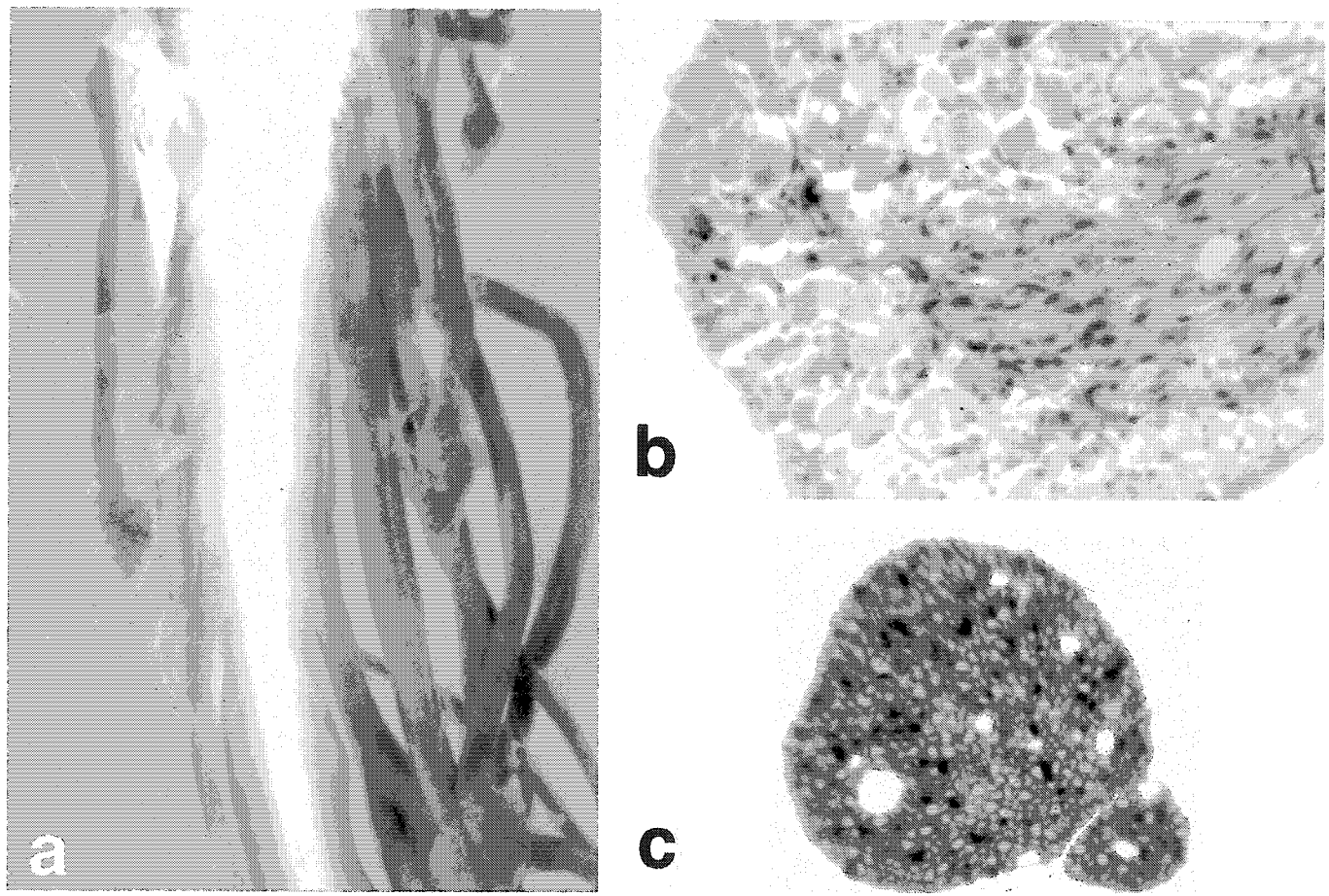


Figure 5-3

Prenatal developmental regulation of the 100 kb *Pmp22/LacZ* transgenic embryos carrying the full *Pmp22* gene and *LacZ* reporter gene. Staining is detected as early as E8.5 in the neural tube (panel a), then extends to the hindbrain by E10.5 (panel b) when early DRGS start expressing the transgene. Further staining of the developing nervous system is evident at E12.5. The *LacZ* expression gets downregulated before birth.

Figure 5-3

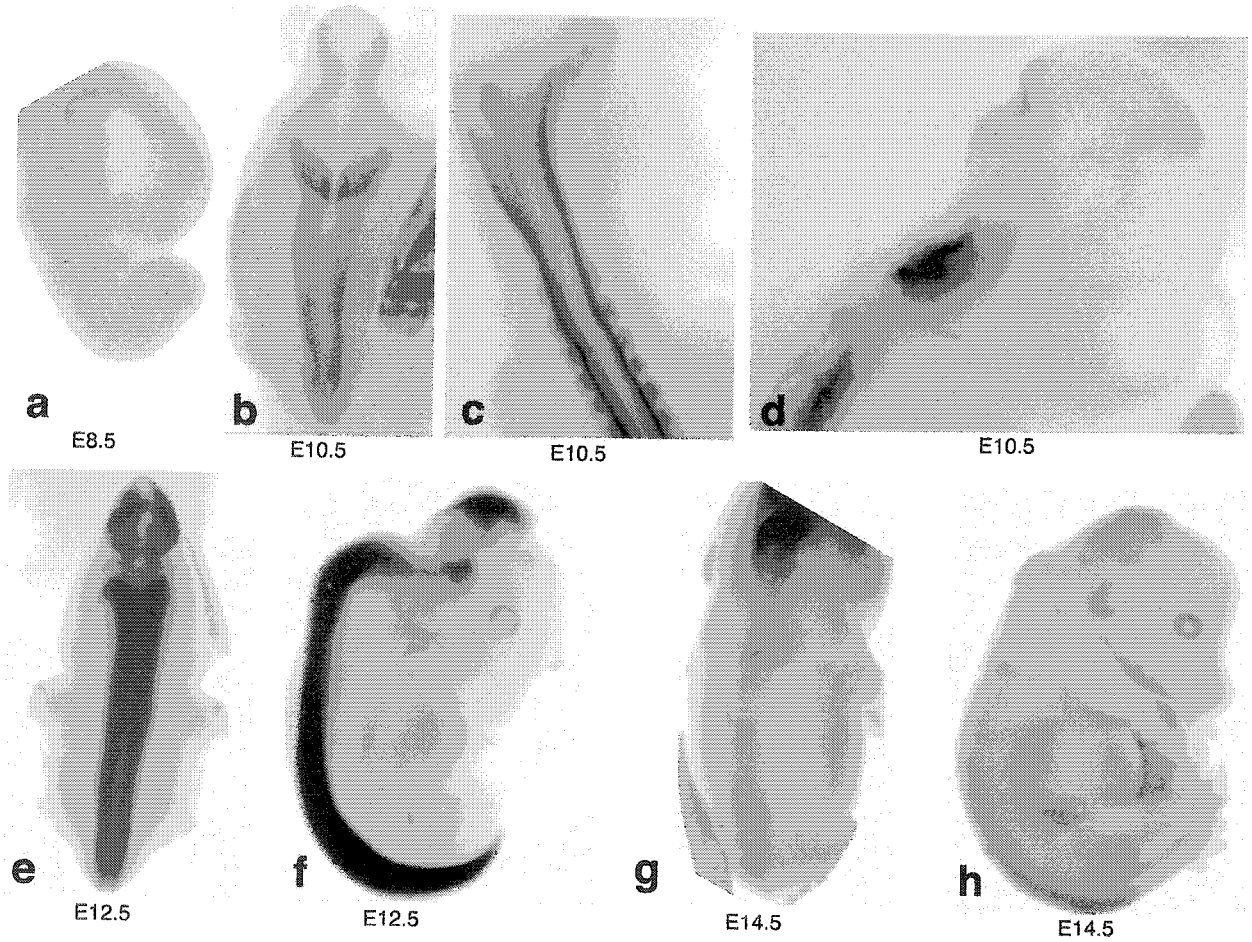


Figure 5-4

Panel a is a whole mount staining of E16.5 100 kb *Pmp22/LacZ* transgenic mouse and it is wt litter mate. At this age the expression is very intense in the skin. Panel b is a section in the skin showing expression in the basal layer of the epidermis. Further below the skin is a thin layer of X-gal reactive cells in the surface of the liver. Panel c shows X-gal staining in a sensory organ in the skin.

Figure 5-4

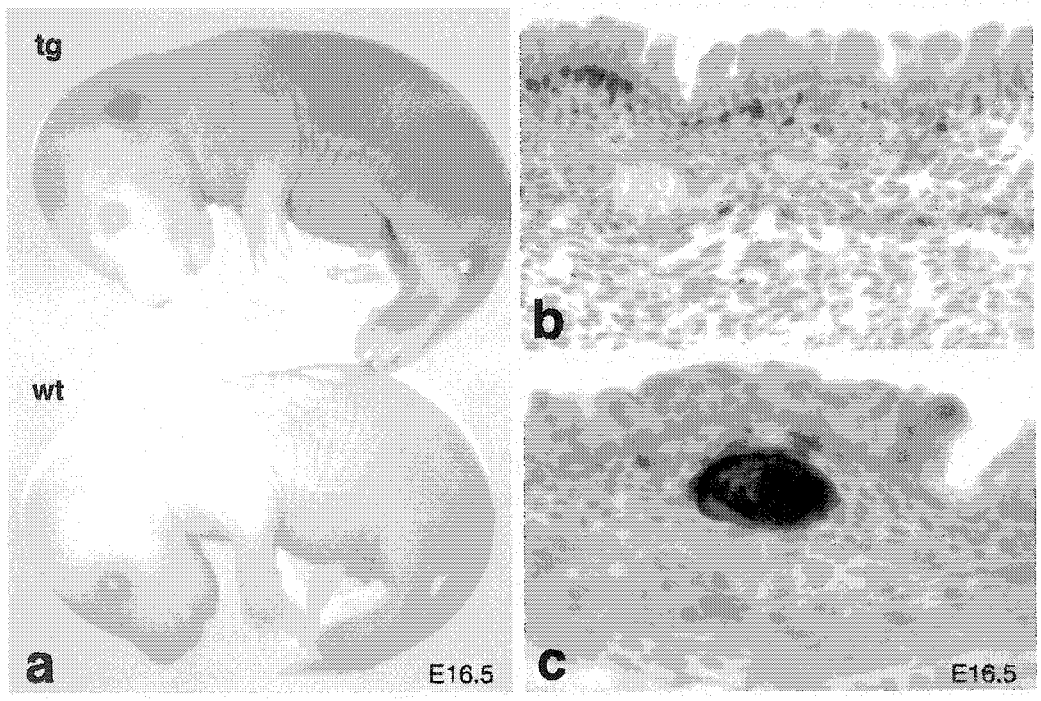


Figure 5-5.

Developmental regulation of the 100 kb *Pmp22*/LacZ transgenic mice. Sciatic nerves were collected from 3, 7, 14, and 21 day old mice. β-galactosidase assays were performed on lysates from the pools of sciatic nerves, and normalized to the total content of protein in each lysate. The bars indicate standard deviation based on analysis of triplicate samples.

Figure 5-5

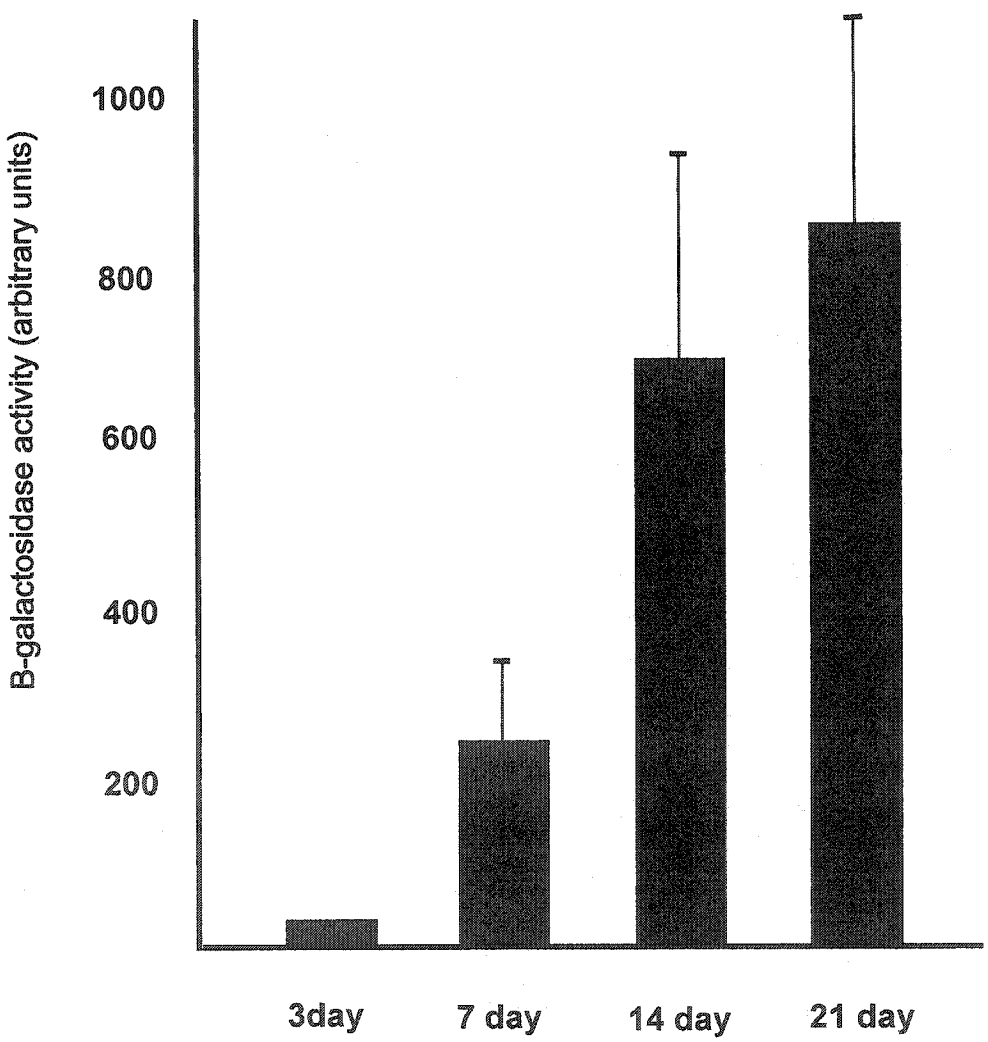

B-galactosidase activity (arbitrary units)
1000
800
600
400
200
3day
7 day
14 day
21 day

Figure 5-6

Panel a is a whole mount staining of focally crushed sciatic nerve (the arrow indicates the site of crush) 4 days post crush injury and the intact contralateral sciatic nerve. The staining has almost disappeared 4 days post injury, and in panel b illustrates how it is restored 21 days post injury. Note the decrease in Bluogal staining distal to the site of crush injury (arrow). Panel b shows result of a similar experiment 21 days after focal nerve crush. Note that the bluogal reaction product distal to the site of crush injury is similar to uncrushed contralateral nerve on the right.

Figure 5-6

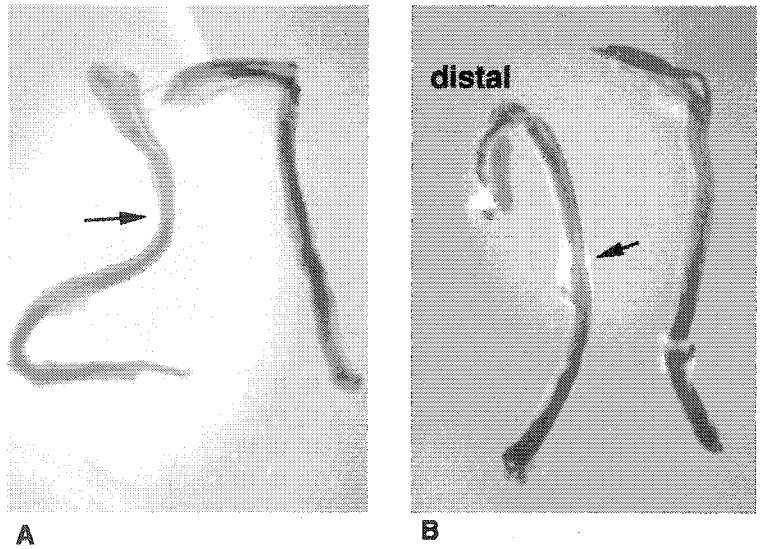
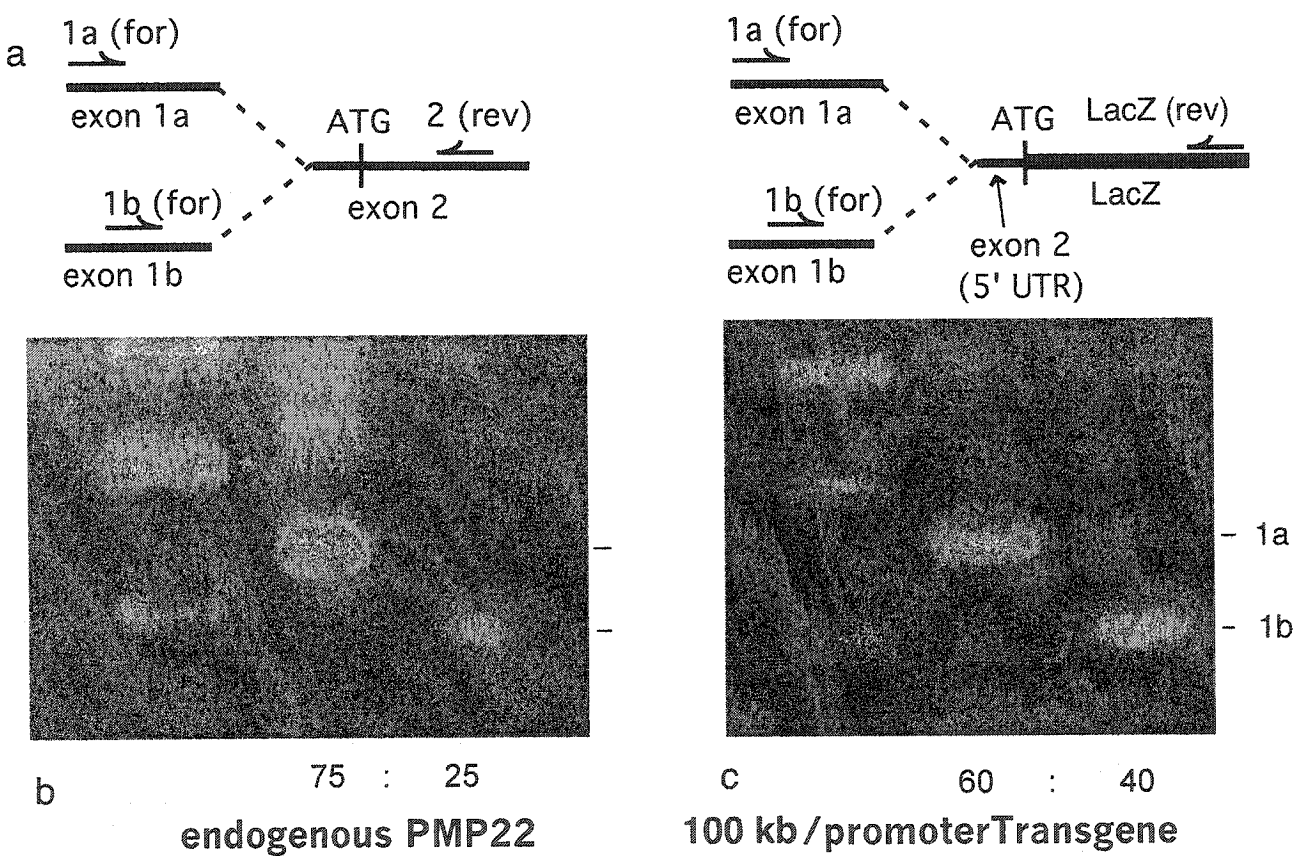


Figure 5-7

Adult sciatic nerves from 100 kb *Pmp22* BAC transgenic mice preferentially produce mRNA containing the exon 1a transcript (panel c) similar to the endogenous PMP22 mRNA (panel b). The design of the RT PCR is depicted in panel a. PCR was performed on reverse transcribed RNA derived from the sciatic nerves of the 100 kb *Pmp22/LacZ* mice. Semi-quantitative PCR was performed using primers specific for the endogenous PMP22 transcript (primer "2 (rev)' left, panel a; results in panel b) or for the LacZ containing transcript (primer "LacZ (rev)" right, panel a; results in panel c). Note that the forward exon 1a and exon 1b specific primers are identical. Percentages shown represent the average of three independent PCR reactions for the percent of total ethidium bromide staining for exons 1a and 1b in each experiment.

Figure 5-7


a
1a (for)
exon 1a
ATG
2 (rev)
1b (for)
exon 2
exon 1b
1a (for)
exon 1a
ATG
LacZ (rev)
1b (for)
LacZ
exon 2
(5' UTR)
exon 1b
1a
1b
b
75 : 25
endogenous PMP22
c
60 : 40
100 kb/promoterTransgene

Chapter 6: Discussion

By way of review, the *Pmp22* gene is organized into two 5' noncoding exons (exons 1a and 1b) upstream of the first of four coding exons (exons 2-5). There are two major transcription start sites that have been mapped upstream of exons 1A and 1B. In addition, there may be a third transcription start site corresponding to the beginning of exon 2. Although the putative third PMP22 mRNA transcript (1C) was described during the initial characterization of the human PMP22 gene (Patel et al., 1992), it was interpreted as an artifact because the transcription start site corresponded exactly to the beginning of exon2. More recent studies have questioned this interpretation (Huehne 2001) although the contribution of this transcript to the overall expression pattern of the PMP22 gene remains to be determined. In any event, PMP22 promoter P1 is upstream of exon 1A and its activation correlates with myelination. PMP22 promoter P2 is upstream of exon 1B (downstream of exon 1A) and its activation correlates somewhat with myelination, but more importantly, activation of P2 is associated with widespread, albeit low levels, PMP22 mRNA expression. The presence of these two PMP22 promoters is one way of regulating the level of expression of the PMP22 gene in different tissues.

Structurally, PMP22 promoter P1 contains a consensus "TATA-box" that is evolutionarily conserved from humans to rodents, while PMP22 promoter P2 is characterized by being GC rich. What does this mean and what are the implications of this difference in the regulation of each promoter? Smale (2001) reviewed the different types of core promoters as follows: In *Drosophila*, there are two kinds of promoters: 1) a TATA box combined with initiator (Inr) element; and, 2) an Inr element combined with a downstream promoter element, DPE (Kutach and Kadonaga, 2000; Burke et al., 1998). All these elements serve as recognition sites for transcription factor IID (TFIID). In mammals, 1) a smaller percentage of core promoters contain TATA boxes, 2) a smaller percentage still of these TATA boxes are paired with Inr elements, 3) DPE

elements are present, but are very difficult to identify, and 4) many promoters, including a number of promoters within CpG islands, appear to lack all these three core elements.

Hypermethylation of CpG islands has emerged as a common mechanism for tissue-specific silencing of gene expression (for a recent review, see Attwood et al., 2002). Several CpG islands have been identified in the *Pmp22* promoter region, specifically upstream of exons 1b and 2. Van de Watering (1999) hypothesized that PMP22 promoter P2 is a "housekeeping" promoter that is regulated by methylation, but was unable to prove this because the ubiquitous expression of PMP22 from promoter P2 prevented the identification of a control tissue that did not express the exon 1b-containing PMP22 mRNA transcripts. Huehne et al. (2001), using methylation-specific PCR, however, were able to demonstrate hypermethylation of the PMP22 P2 promoter in a glioblastoma cell line (SF763) that correlates with silencing of PMP22 exon 1b-containing mRNA transcript. No significant methylation upstream of either exon 1b or 2 was observed in peripheral nerves, which clearly expresses PMP22 transcripts, nor in leukocytes, which do not express PMP22 transcripts. In conclusion, methylation may play a role in the regulation of PMP22 expression from promoter P2, but its significance *in vivo* remains to be established.

There are several possibilities that may serve to explain the existence of different types of core promoters. One possibility is that during evolution they developed with interchangeable and functionally equivalent functions to serve as landing sites for specific recognition factors (O'Shea-Greenfield et al., 1992). A second possibility is that they are not functionally equivalent, but are recognized by different proteins or protein complexes. The most intriguing possibility is that the diversity of promoter elements makes an important contribution to combinatorial gene regulation (Butler et al., 2001) such that the two types of the Drosophila promoters exist, at least in part, to limit enhancer effects to a specific subset of promoters. Also, Mack et al. (1993) showed that promoters containing TATA boxes were potently repressed by p53 while initiator (Inr) promoters were resistant to repression. NC2 (a transcription factor) is the

best example for core-promoter selectivity; it is an inhibitor for TATA-Inr promoters and activator for Inr-DPE promoters (Willy et al., 2000; Maldonado et al., 1999).

TATA containing promoters are more efficient in transcription re-initiation than TATA-less promoters (Yean and Gralla, 1997). For PMP22, P1 probably depends on efficient initiation and re-initiation of transcription. Thus, it is reasonable that P1 would contain a TATA box rather than P2 which is responsible for sustained low levels of expression and does not need much fine-tuning or modulation. The effect of TATA and TATA-less promoters on gene activity, however, remains to be established.

In the next part of this chapter, I will discuss the results from each of the transgenic constructs individually, and then consider the implications of these results together.

-8.5 kb rat *Pmp22*/CAT/*LacZ* construct.

The -8.5 kb fragment of the *Pmp22* control region included in this study extends from –4 kb of P1 upstream of the transcription start site for exon 1A, and includes P2, exon 1B, intron 1b/2 and exon 2 up to the translation start site. I demonstrated that 8.5 kb upstream of the translation start site targets expression to peripheral nerve in a weakly developmentally regulated fashion. In addition, the –8.5 kb construct contains elements that down-regulate *Pmp22* expression in response to the loss of axonal contact secondary to focal axonal lesion in the crush injury model. The -8.5 fragment of *Pmp22*, however, appears to lack elements that are responsible for significant upregulation during development, and response to remyelination during nerve regeneration. Most significantly, this -8.5 kb construct appears to lack elements necessary for high levels of expression in peripheral nerve, which correlates with its ability to transcribe mRNA significantly from the non myelin-associated promoter, P2.

Our results are in substantial agreement with results obtained from transient transfection studies using *Pmp22* promoter constructs demonstrating that elements up to 3 kb upstream of the transcription start site of *Pmp22* can target PMP22-like expression to

Schwann cell lines, but not lines derived from other cell types (Saberan-Djoneidi et al., 2000). This suggests that a construct from 0 to –3 kb can target expression specifically to Schwann cells. Indeed, our construct, which extents up to –4 kb from the transcription start site for exon 1A, also targets expression to peripheral nerves, most significantly to sensory neurons in the dorsal root ganglion (as shown by *LacZ* histochemistry), but also to peripheral nerve (as shown by CAT activity) Schwann cells. Hai et al. (2001) concluded, based on RNase protection assays on a variety of transiently transfected cell lines, that their construct containing up to –3.5 kb of the human *PMP22* gene was not capable of significantly activating the promoter, P1. They proposed that this was most likely due to lack of axonal contact and stimulation of myelin formation in their cultured cells. Our results using up to –4 kb to drive reporter gene expression in transgenic mice that demonstrate relatively low levels of transgene expression in peripheral nerve bundles taken together with low level of activation of the transgene by P1, led us to suggest that an additional explanation would be that the –3.5 (or –4) kb constructs do not have the necessary *cis*-regulatory elements required for significant activation of P1. Examination of other peripheral nerve myelin gene promoters in transgenic mice reveal a major Schwann cell enhancer 9.6 kb upstream of the myelin basic protein transcription start site (Forghani et al., 2001) whereas regulatory elements important for myelination have been identified 3' to the first exon of the myelin protein zero (*MPZ*) gene (Feltri et al., 1999). Based on analysis of the –8.5 kb *Pmp22* promoter alone, it remains an open question as to whether these additional elements are to be found further 5' or 3' to the 8.5 kb region that I have analyzed in this study. In sequence comparison studies, I have identified significant regions of homology between the rat and mouse *Pmp22* noncoding regions both upstream of our construct and downstream of exon 2 (see appendix 1). In addition, Maier et al. (2002) have recently published that –10 kb of the murine *Pmp22* promoter can drive high levels of β-galactosidase expression to myelinating Schwann cells. These results strongly indicate that additional *cis*-regulatory elements important for promoter P1 expression exist between –8.5 and –10 kb of the *Pmp22* promoter. Given the importance of these elements for activation of P1 in myelination, it will be important to further characterize these elements responsible

for significant P1 activity. As we will see (*vide infra*), we have also obtained evidence that there are additional functional elements 3' to the translation start site.

The finding that the -8.5 kb *Pmp22* construct targets transgene to sensory neurons in post-natal DRG was somewhat unexpected. By *in situ* hybridization, Hagedorn et al. (1999), however, have noted significant expression of PMP22 mRNA in DRG neurons during embryonic development. It is not clear whether the expression of the –8.5 kb *Pmp22* promoted transgene in postnatal DRG neurons reflects normal persistent activation of P2 (but at levels far below that of myelinating Schwann cells) or whether it reflects the inability of the transgene to respond to a switching mechanism that changes PMP22 expression from a developmental pattern that emphasizes expression in DRG neurons to a more mature pattern that emphasizes expression in Schwann cells.

Reporter gene expression from the -8.5 kb *Pmp22* construct is strongly down-regulated in distal nerve stumps undergoing Wallerian degeneration following a focal crush injury indicating the presence of *cis*-regulatory elements within this region responsive to loss of axonal contact. In previous RNase protection studies of PMP22 mRNA species following nerve crush, down-regulation of both the exon 1A and exon 1B containing transcripts was noted (Suter et al., 1994). Thus, even though activation of P1 does not appear to contribute significantly to the expression of our transgene, it is reasonable to observe P2-like down-regulation of our reporter gene during Wallerian degeneration. In tissue culture, forskolin activation of cAMP responsive pathways has been hypothesized to mimic the effects of axonal contact on myelin gene expression (see Cheng and Mudge, 1996). Saberan-Djoneidi et al. (2000) have identified a region between –1699 and –1731 of human *PMP22* containing putative cAMP responsive elements, including CREB and CRE-BP1, that when deleted increase activity of reporter gene constructs suggesting that they may act as transcriptional silencers. We have sequenced through this region of the rat *Pmp22* gene. Sequence comparisons of the human, rat, and mouse PMP22 genes aligned by PIPMAKER (Schwartz et al., 2000) and scanned by TRANSFAC (Heinemeyer et al, 1998) for putative transcription factor binding sites

fails to demonstrate evolutionary conservation for the position of these putative CREB and CRE-BP1 binding sites in either the rat or mouse genomes, although a number of other putative CREB and/or CRE-BP1 sites were identified elsewhere within the -4 kb upstream of the transcription start site (data not shown). Thus, it will be difficult to test whether these regions are responsive to perturbations of axon glial interactions in myelinating Schwann cells using rat or mouse *Pmp22* promoted reporter genes in transgenic mice. This raises the question of whether the regulatory elements of PMP22 are evolutionarily conserved. Since overexpression of the human *PMP22* gene results in a CMT like phenotype in mice (Norreel et al., 2001) and overexpression of the mouse *Pmp22* results in a CMT like phenotype in rats (Sereda et al., 1996), we conclude that the major *cis*-acting regulatory elements for PMP22 expression are conserved between humans and rodents. This is further supported by our interspecies sequence comparisons of the human, rat, and mouse PMP22 control regions (see Appendix) in which we observe approximately 85% sequence conservation over much of the region from –10 to +5 kb relative to the translation start site.

We have demonstrated, by semi-quantitative RT-PCR, that expression from the -8.5 kb rat *Pmp22* promoted construct results in activation of P2 more than the myelination-associated PMP22 promoter, P1. Nonetheless, there are still differences between the expression pattern of this transgene and expression from the endogenous P2 promoter. In our previous characterization of rat P2 expression, in contrast to the –8.5 kb *Pmp22/LacZ* transgene, we demonstrated significant upregulation and expression of the exon 1b containing PMP22 mRNA transcript during development and at 21 days following focal nerve crush in a nerve regeneration model (Suter et al., 1994). Thus, while this construct does not appear to respond strongly to signals for myelination either during development or during nerve regeneration, it does have elements that respond to the loss of axonal contact during Wallerian degeneration. Thus, we hypothesize that recognizing the loss of axonal contact and responding to signals for myelination represent two independent molecular mechanisms of axon-Schwann cell interactions that operate during nerve regeneration.

As outlined in the introduction, it is important to understand the regulation of the PMP22 gene for the development of treatments for CMT and HNPP and for an understanding of the axon-Schwann cell interactions that regulate myelination. We have begun to investigate the regulation of the PMP22 control region in transgenic mice and have confirmed transient transfection studies indicating that –4 kb of the *Pmp22* promoter contains elements responsible for increased expression in Schwann cells. Transient transfection paradigms have identified putative regulatory elements at the single nucleotide level of the PMP22 promoter that regulate its expression. Transient transfection paradigms, however, are limited in their ability to recapitulate the axon-Schwann cell interactions that are critical for myelination. Based on our results and the results of Maier et al. (2002), important regions critical for myelination remain to be investigated by transient transfection. In practice, this may prove difficult since it is difficult to control for transfection efficiency when evaluating large constructs by transient transfection. Transgenic approaches, though well suited for interrogating the effects of axon-Schwann cell interactions and for the evaluation of large constructs, are laborious and are often limited by qualitative and quantitative differences between experimental animals because of the effects of random and often multiple insertions in the mouse genome. As we have seen and as will be discussed more fully below, these insertional effects can be minimized by targeted transgenesis. Thus, transient transfection and transgenic approaches to promoter analysis should prove complementary for the elucidation of PMP22 promoter activity.

In summary, the -8.5 kb of *Pmp22* DNA is able to direct reporter gene expression to peripheral nerves. The peripheral nerve expression of the *Pmp22* transgene shows modest developmental regulation, and is responsive to axonal signals during Wallerian degeneration, but not to nerve regeneration in adult animals. Unlike the endogenous gene, however, the –8.5 kb transgene shows very low overall level of expression, is not strongly developmentally regulated, and is not responsive to remyelination signals in adult animals. The RT-PCR studies suggest that the -8.5 kb *Pmp22* transgene lacks the

necessary elements to activate significant expression from promoter P1, the major determinant of *Pmp22* expression during myelination in peripheral nerve, in response to axonal contact. Thus, while important elements for PMP22-like expression are present in the -8.5 kb of the rat *Pmp22* promoter, it is probably premature to design strategies aimed at modifying specific regulatory elements within this region until their overall contribution to *Pmp22* gene expression in the context of myelinating Schwann cells is established. Further studies are underway to try to isolate and identify these additional *cis*-regulatory elements in the PMP22 gene that are responsible for myelination.

-21 kb *Pmp22/LacZ* construct.

The results from the analysis of transgenic mouse lines produced from the –21 kb *Pmp22/LacZ* construct are very difficult to interpret. Four out of five lines derived from the –21 kb *Pmp22/LacZ* construct exhibited almost identical patterns of β-galactosidase expression. Specifically, in adult animals, the transgene expression was targeted to the PNS predominantly in the dorsal root ganglia, although there was a modicum of staining in individual Schwann cells in the PNS. Thus, four out of the five –21 kb *Pmp22/LacZ* lines had β-galactosidase expression patterns that closely resembled those obtained from the –8.5 kb rat *Pmp22*/CAT/*LacZ* lines. In contrast, one –21 kb *Pmp22/LacZ* line, line BM10, directed strong β-galactosidase expression to myelinating Schwann cells in a manner that would be expected for the endogenous *Pmp22* gene. Analysis of line BM10 was further complicated by a presumed silencing mechanism in which successive generations exhibited highly variable expression of the transgene. At one extreme were generations of the BM10 line in which the β-galactosidase expression in myelinating Schwann cells was very low and the expression of the transgene in DRG was relatively high like we observed in the –8.5 kb *Pmp22*/CAT/*LacZ* mice and the remaining four out of five –21 kb *Pmp22/LacZ* mice. At the other extreme were generations of the BM10 line that showed high β-galactosidase expression in adult myelinating Schwann cells. One interpretation of these results is that there are two patterns of *Pmp22/LacZ* expression: (1) an expression pattern driven predominantly by *Pmp22* promoter P2 that gives relatively high levels of expression in DRG neurons, but low levels of expression

in myelinating Schwann cells. This "P2" pattern of expression is shared by the –8.5 kb *Pmp22*/CAT/*LacZ*, four out of five independent lines of the –21 kb *Pmp22/LacZ* mice, and in successive generations of line BM10. This hypothesis is partially supported by the RT-PCR results indicating that the –8.5 kb *Pmp22/LacZ* transgene is regulated predominantly from the *Pmp22* promoter, P2. (2) The second pattern of transgene expression is driven predominantly by *Pmp22* promoter P1 that gives high levels of β-galactosidase expression in adult myelinating Schwann cells, but low levels of expression in DRG neurons. This "P1" pattern of expression is shared by some generations of line BM10 prepared from the –21 kb *Pmp22/LacZ* transgene and by the endogenous *Pmp22* gene. Unfortunately, the variability of the expression of the transgene in line BM10 precluded my attempts to correlate patterns of β-galactosidase expression with alternative promoter usage by RT-PCR.

Based on the results I have presented so far, the preponderance of evidence suggests that the –21 kb *Pmp22/LacZ* construct, like the –8.5 kb *Pmp22*/CAT/*LacZ* construct, has a "P2" PMP22 promoter expression pattern. We hypothesized that line BM10 might represent an anomalous result due to effects of random insertion of the transgene. In order to verify that the"P2" PMP22 promoter pattern was the "true" expression pattern for the –21 kb *Pmp22* promoter elements, I inserted the –21 kb *Pmp22* promoter/reporter construct into the *hprt* locus in ES cells and subsequently into transgenic mice in an effort to control for transgene insertional effects. Results from the –21 kb *Pmp22/LacZ* construct in the *hprt* locus revealed an expression pattern that basically resembled all of the other lines with a "P2" PMP22 promoter expression pattern. If we assume that the *hprt* locus is neutral for the *Pmp22* elements and is permissive for a true expression of the inserted gene, we would conclude that the four out of five lines of the –21 kb *Pmp22/LacZ* construct represent the activity of *cis*-acting –21 kb *Pmp22* promoter elements in transgenic animals. We would then conclude that either 1) the –21 kb *Pmp22* promoter construct lacks important regulatory elements for *Pmp22*-like expression, or 2) that the "P1" PMP22 promoter activity which may be present in the –21 kb *Pmp22* construct is masked by negative regulatory elements. As

we will see, both of these possibilities are likely. We also cannot completely exclude the possibility that the transgenes were not transmitted in their entirety. This is less likely for the –21 kb *Pmp22/LacZ* transgene inserted into the *hprt* locus since sequence flanking the intact transgene is required for successful recombination and selection of recombinant ES cells.

While analyzing our data, Maier et al. (2002) published a study showing that -10.5 kb of mouse *Pmp22* promoter is capable of directing the expression of the β-galactosidase reporter gene to myelinating Schwann cells in sciatic nerves of transgenic mice. Expression driven by this construct (-10 kb *Pmp22/LacZ*) is spatially regulated like the endogenous *Pmp22* gene. That is, it exhibits relatively strong expression in adult myelinating Schwann cells in a "P1" PMP22 promoter-like expression pattern. There are several small differences and one potentially large difference between the expression pattern of the –10 kb *Pmp22/LacZ* gene and the endogenous *Pmp22* gene. The small differences relate to the expression patterns during prenatal development and the relative lack of expression of the transgene in non-neural tissues. The potentially important difference is that the expression from the –10 kb *Pmp22/LacZ* transgene in myelinating Schwann cells is delayed compared to the endogenous *Pmp22* gene by approximately 5-7 days.

So, how do the results from the –10 kb *Pmp22/LacZ* mouse change our interpretation of our results so far? First, there appear to be important positive regulatory elements for *Pmp22* expression in myelinating Schwann cells that are located between –8.5 and –10 kb of the *Pmp22* promoter. Secondly, we hypothesize that there are negative regulatory elements between –10 and –21 kb of the *Pmp22* promoter that act specifically on the PMP22 P1 promoter to explain the relative lack of expression of the –21 kb *Pmp22/LacZ* transgene in myelinating Schwann cells. Third, the delay in onset of expression driven by the –10 kb *Pmp22/LacZ* transgene as compared to the temporal expression pattern of the endogenous *Pmp22* gene in myelinating Schwann cells

suggests that there might be additional regulatory elements, perhaps 3' to the translation start site.

The 100 kb *Pmp22/LacZ* transgene

In Chapter 5, I demonstrated that the 100 kb *Pmp22/LacZ* construct (–21 kb *Pmp22/LacZ* and the 3' part of the gene extending to +80) very strongly targets expression to the PNS including Schwann cells and dorsal root ganglia. Temporally, the β-galactosidase reporter from this construct can be detected in the PNS within a few days postnatal and is up-regulated approximately 40-fold by 3 weeks of age. Interestingly, cranial nerves originating from motor nuclei express the reporter gene. In addition, the temporal and spatial expression pattern of the 100 kb *Pmp22/LacZ* transgene appears to recapitulate the endogenous *Pmp22* mRNA expression pattern obtained by mRNA *in situ* hybridization studies which demonstrate PMP22 mRNA expression in the neural tube, neural crest, and peripheral organs, such as skin, during embryonic development from E8.5 to birth.

Since the only difference between the –21 kb *Pmp22/LacZ* and the 100 kb *Pmp22/LacZ* transgenes resides in the inclusion of the portion of the *Pmp22* gene 3' to exon 2, we can attribute differences in the expression patterns obtained by the two transgenes to elements in the 3` portion of the *Pmp22* gene. If we assume that the –21 kb *Pmp22/LacZ* transgene has a "P2" pattern of expression, meaning that it shows poor expression in myelinating Schwann cells, we can conclude that the 3' elements are very important for high levels of *Pmp22* expression required for myelin production. Furthermore, it would appear that these elements could override the putative repressors that are hypothesized to exist to explain the different expression patterns observed using the –10.5 *Pmp22/LacZ* and the -21 kb *Pmp22/LacZ* transgenes. If, on the other hand, we assume that the –21 kb *Pmp22/LacZ* transgene, exemplified by transgenic mouse line BM10, with its "P1-like" pattern of expression reflects the true activity of the –21 kb *Pmp22* fragment, then the activity of the –21 kb *Pmp22* promoter fragment resembles that of the –10.5 kb *Pmp22* promoter fragment; and, there is no reason to hypothesize

negative regulatory elements between –10 and –21 kb. Even so, the delayed onset of β-galactosidase expression driven by the –10.5 kb *Pmp22/LacZ* transgene compared to the 100 kb *Pmp22/LacZ* transgene suggests that there are important elements 3' to the *Pmp22* translation start site required for early onset and perhaps intensity of expression. Thus, in either scenario, it appears likely that the 3' portion of the *Pmp22* gene contributes substantially to the regulation of *Pmp22* expression.

Significance and future directions

This study is an attempt to understand the regulation of the PMP22 gene. I am able to conclude that the 3` region of the gene is important for the level of expression and the temporal aspect of the initiation of expression of PMP22. And also, the likelihood of existence of silencers or repressors in the –21 –8.5 region that work negatively on the enhancers located in –10.5 and –8.5 kb. And I was able to conclude that the elements that control remyelination during nerve regeneration are not those of the initial myelination during development. And that the two processes are different from each other. Further more, these elements are most likely located in between –8.5 and –21 kb of the *Pmp22* promoter. The next step of experiments in this line should include more transgenic animals carrying smaller stretches of the 3` region of *Pmp22* and the –21 kb 5` to determine the whereabout is these elements, and then try to find out whether they can be functional alone or with the assistance of a hetrologous promoter. The methodology of targeting to the *hprt* locus will make it easier to compare the expression of transgenic construct as they will, in this context, share the site of insertion and the number of copies (one copy).

1) Establishment of *in vivo* models

All these transgenic animals are useful not only in studying regulation but also in studying the activity of promoter regions in the *in vivo* situation where prenatal and postnatal development takes place and can be quantified. Furthermore, these animals serve as valuable tools to study the interaction of transcription factors with myelin genes. This can be achieved by using dominant negative mutations of transcription

factors in transgenic animals and directly crossing them with promoter/reporter gene transgenic animals.

2) Anticipate that mutations in promoter will cause disease (HNPP).

Recently a CMT case was found to be due to a mutation in the promoter of the Periaxin gene. Respectively, HNPP, caused by reduction of PMP22 expression could be caused by mutations in the promoter region of PMP22 that could reduce the activity of that copy of the gene and result in the HNPP phenotype. And from the results of this study, it seems that the 3` region of *PMP22* could be primarily responsible for the high level of expression of PMP22, and as a consequence, all efforts to find a 5` mutation in unexplained HNPP cases were fruitless.

APPENDIX 1 Sequence comparison

Regulatory sequences constitute a small percentage of the expansive non-coding sequence of higher mammalian genome, but they are extremely important as they determine the spatial and temporal pattern of expression of a gene. Despite their importance, our ability to identify them is limited. Nowadays, the genomes of many species (e.g. human, rat, mouse, viruses, bacteria, yeasts, etc) are being sequenced. The availability of these sequences can contribute a great deal in the identification of regulatory sequences through intergene and interspecies comparisions.

The search for DNA regulatory elements has involved relatively laborious approaches. These included several strategies, such as: A) the generation of deletion constructs to determine the regions required for gene expression in transient transfection assays. B) DNAse hypersensitivity studies to identify changes in chomosome conformation C) DNA footprinting and gel shifts to identify and characterize DNA binding proteins. D) Screening for regulatory elements have been carried out in transgenic rodents. E) many others.

And now, as the DNA sequence of several species is available (and others are being sequenced), computational sequence analysis is possible using specialized software that can recognize putative consensus DNA binding sites, or determine regions of sequence homology between different species that were important enough during evolution to conserve. Such conserved non-coding sequences have several possible functions, including, chromosomal assembly, replication, and gene regulation. Computational sequence analysis provides three different approaches to identify possible or predicted sequences that play in the regulation in gene expression.1) interspecies sequence comparisons. 2) Sequence analysis of co-regulated genes within a species. 3) Use of databases for known transcription factors binding sites.

Examples: Human-mouse sequence comparisons for ~1 Mb of human chromosome region 5q31 (including 5 interleukins and 18 other genes) was compared to the orthologous mouse chromosome 11. 90 conserved regions were identified. One of them that was located in the 15 kb interval between IL4 and IL13 (a ~400 bp with ~87% homology) was further studied and shown to be important for the regulation of IL4, IL5 and IL13, that are separated by approximately 120 kb distance.

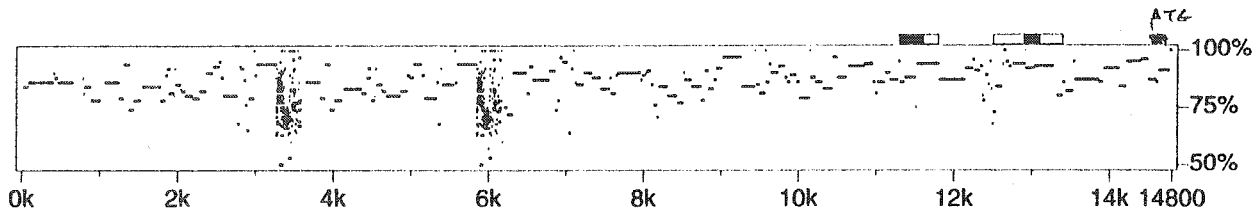
The downside of this methodology include that not having significant conserved sequences does not reflect the level of importance in regulation. Another caveat is that several characterized regulatory regions failed to show significant inter-species homology and still they were very significant regulatory regions. Another approach is to look for known DNA motifs that bind to known transcription factors. This strategy, however, can generate a large number of false positive returns. Refinements to this approach include the use pattern recognition algoriths that search for the correct context for individual transcription factor binging sites.

For PMP22, previously (Suter et al., 1994) analysis of the promoter predicted the presence of a TATA box and SP-1 site upstream of exon 1A and a GC sequence upstream of 1B. Recently, Saberan-Djoneidi et al. (2000) predicted the presence of a CREB site upstream of 1A and a steroid responsive element and tested it in tissue culture.

Here, I will present the comparison between mouse rat and human PMP22 promoter sequence, and because we saw that the 3` region of the gene is important for its transcription, the 3` region will be included in the comparison. In brief, these studies show that the PMP22 gene is highly conserved between human, rat, and mouse from –12 kb to well beyond exon 3. This agrees with our results suggesting that both 5' and 3' regions of the PMP22 gene may be involved in its regulation. For this comparison we used Pipmaker (Schwartz, et al., 2000) provided at the following website: (http://bio.cse.psu.edu)

-Mouse Pmp22 vs. rat Pmp22

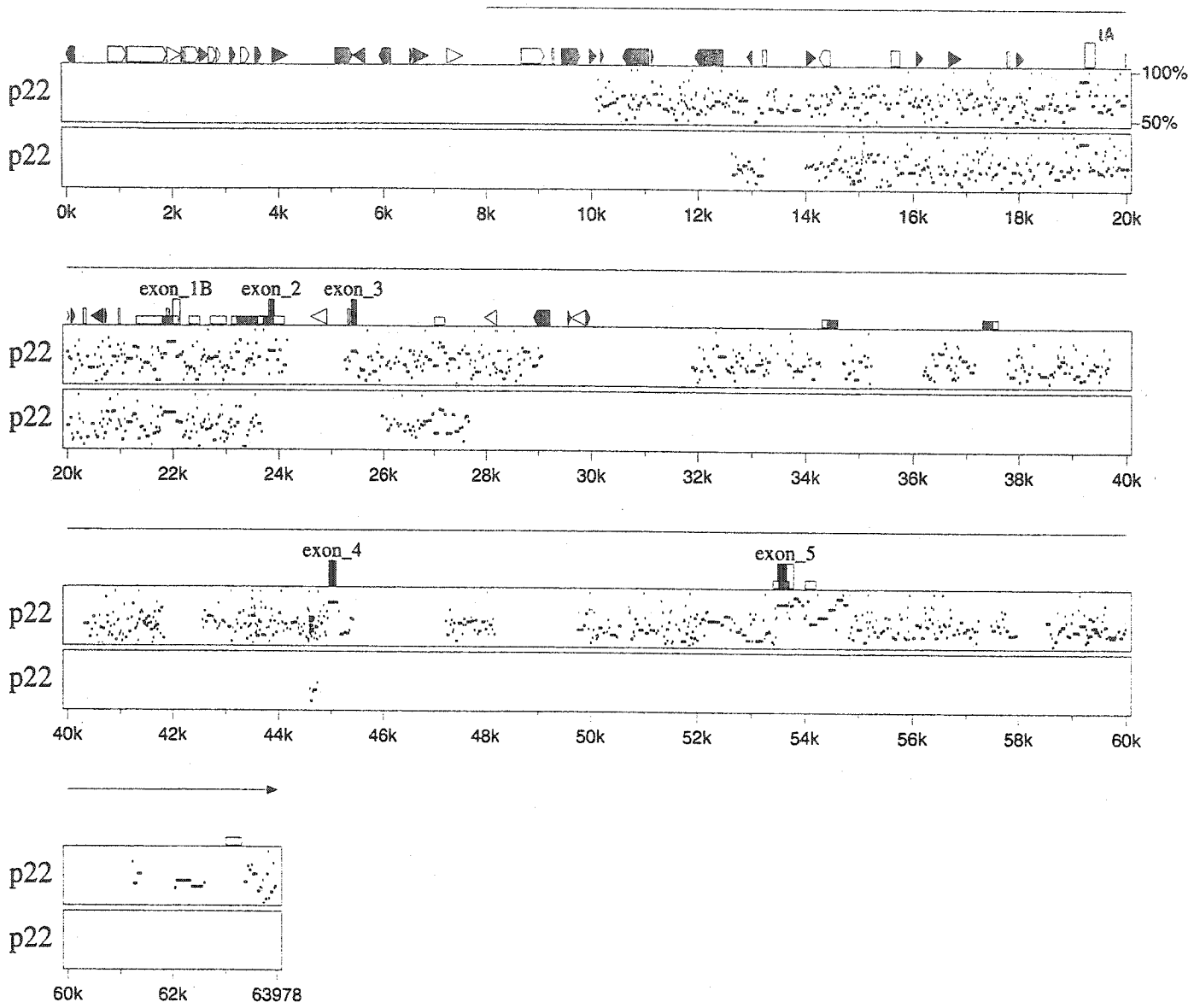
As depicted in the following alignment report there is significant homology between mouse and rat *Pmp22* noncoding sequence over the –16 kb compared in this plot. In general, the homology exceeds 75% and probably averages around 85% over a large amount of the sequence. This comparison covers the region used for the transgenic mouse lines generated and discussed in Chapters 3 and 6 (-8.5 kb PMP22/CAT/*LacZ* and –21 kb *Pmp22/LacZ*).



Gene
Exon
UTR
RNA
Simple
MIR
Other SINE
LINE1
LINE2
LTR
Other repeat
CpG/GpC≥0.60
CpG/GpC≥0.75

-Human, mouse, and rat PMP22 gene comparison.

In this comparison, I extended the analysis to the 3` to include the rest of human and mouse Pmp22 gene. It is clear that there is significant homology between the human and mouse sequence for up to –14 kb relative to the translation start site in exon 2. Also note two regions of high homology between the distal portions of the Pmp22 gene: 3` to exon 3 and 3` to exon 5. The significance of these regions of homology remains to be determined.


1A
p22
100%
50%
p22
0k
2k
4k
6k
8k
10k
12k
14k
16k
18k
20k
exon_1B
exon_2
exon_3
p22
p22
20k
22k
24k
26k
28k
30k
32k
34k
36k
38k
40k
exon_4
exon_5
p22
p22
40k
42k
44k
46k
48k
50k
52k
54k
56k
58k
60k
p22
p22
60k
62k
63978

Appendix 2 Animal use compliance certificate

4. RESEARCH PERSONNEL:

Name	Department	Check Appropriate Classification				
		Investigator	Technician/ Research Assistant	Student		Fellow
				Undergrad.	Graduate	
k Snipes	Neurol.& Neurosurg	X				
ert Nicholson	Pathology					x
el Orfali	Neurology Neurosurgery				X	

EMERGENCY: Person(s) designated to handle emergencies

e: Jack Snipes Phone #: Work: 398-5319 Home: 735-3318

e: Phil Barker Phone #: Work: 398-3064 Home:

MARY OBJECTIVE(S) OF THE STUDY: (In lay terminology) The overall goal of this earch is to investigate the molecular mechanisms by which mutations in Peripheral lin Protein-22 gene cause peripheral nerve disease. We will generate animal models recreate the disease, test hypotheses generated during in vitro experiments, and ign therapies.

NIMALS:

nimal Species	Strain	Age	Sex	Weight	# needed at one time	Total	#/cage
t	Sprague-Dawley	60 days	either	>250 gm	20	100	4
use	CDI	45 days	either		5	20 + progeny	6

ecial cages	no	X	yes		specify
ecial diet	no	X	yes		specify
ecial handling	no	X	yes		specify

7. ANIMALS (cont'd)

Animal housing location desired: MNI Animal Facilities

:e area(s) where animal use procedures l be conducted:	Building:	MNI	Room #:	8th Floor
the number of animals proposed the minimal number ssary to achieve valid results?	Yes	X	No	
consideration been given to the use of alternative ods which do not involve live animals, eg. tissue ure?	Yes	X	No	

POSED PROCEDURES WITH ANIMALS: (provide details of surgical procedures, unizations, behavioural tests, immobilization, food/water deprivation, requirements post operative care, sample collection, substance administration, etc.)

Sciatic nerve regeneration experiments.

Rats will be deeply anaesthetized with metofane, then pentobarbital.Care will be en to ensure that the animals are unresponsive to pain before beginning the cedure. The animals are positioned in such a way that the bony protuberances of the ur and the hip are landmarks bounding the site where a small incision can be made der sterile conditions)to expose the muscle which is bluntly dissected gitudinally to reveal the sciatic nerve. The sciatic nerve is then crushed with 'eler's forceps until translucent (about 5 sec). The nerve is replaced, muscle pposed and the skin is sutured (or stapled shut). The animals will be allowed to over (with gentle heating, if appropriate), before returning to the animal care ility. To relieve post-operative discomfort, the rats will receive buprenorphrine t 0.1-0.5 mg/kg SC). The animals will be checked periodically (at least daily) to ck for wound infections, self mutilation, or obvious discomfort. Sutures (or clips) l be removed at the end of the first week. If the animals show any signs of fering, they will be euthanized immediately.The animals will be housed for up to 6 ks following the nerve injury. At the end of the experiment, the animals will be hanized by CO2 inhalation.

Production and characterization of transgenic mice.
nsgenic mice will be generated by established procedures in collaboration with Dr. n Peterson at the RVH. Procedures involved in the generation of transgenic mice will performed in his facility under his supervision. Several different lines of nsgenic mice will be engineered. In the first group, the animals will be expressing arker gene under the control of heterologous promoter elements. Since this should alter the phenotype of the mice (except by mistake) we anticipate that these mice l require no extraordinary care. In the second group, we will be trying to recreate uman disease phenotype which is known not to cause pain pain in humans, but we may an unexpected phenotype in these animals. We will carefully monitor the behaviour the founder mice and their progeny.We will seek appropriate consultation if the nsgenic mice show abnormal behaviours, such as those described above for the nerve jury. For subsequent analysis, the animals will the euthanized by pentobarbital erdose.

9.

PAIN AND DISCOMFORT: categorize pain and discomfort level in accordance with the UACC elines which accompany this application.

[ ] C [ ] D [X] E [ ]

. appropriate anaesthetic, analgesic or tranquilizer used to alleviate pain or distress? Yes X No ____

lo, why not? ____

: the drug(s) proposed for anaesthesia, analgesia or tranquilization, indicate the age in mg/kg body weight and route of administration:1) Metofane, to effect: 2) :obarbital (Somnitol): 50 mg/kg IP, SC, or IM; 3) buprenorphrine (Temgesic), post-:atively: 0.05-0.1 mg/kg mouse or 0.1-0.5 mg/kg rat.

rojects involving surgery, will these experiments be carried out ccordance with UACC Policy for Surgical Research? Yes X No ____

:ify person(s) who will provide quate post operative care: Dr. Snipes and staff (to be named).

GER AND HAZARD TO STAFF AND TO ANIMAL POPULATION:
l the project involve use of any if the following animals?

ioisotope [N] Carcinogens [N] Infectious agents [N] Transplantable tumors [ ]

cify agent(s) to be used:

unt involved per animal per experiment: ____

te of inistration: oral ____ injection ____ Other ____ (specify) ____

ber of animals olved ____

gth of in vivo procedures er administration: < 8h ____ 8h-24h ____ 24h ____

er administration, animals will be sed in: ____ the animal care facility

____ the laboratory under supervision of laboratory personnel

: cages will be labelled with biohazard or liation labels Yes ____ No ____

scribe measures that will be used to reduce risk to animal facility personnel:

HOD OF EUTHANASIA:

SPECIES	
se	anesthetic overdose, agent pentobarbital route IP
	exsanguination with anesthesia
se	cervical dislocation
	decapitation ___ with anesthesia, specify _____ ___ without anesthesia
	CO2 chamber
	other (specify)
	not applicable (explain)

Appendix 3 Radioactive material use compliance certificate

Canadian Nuclear Safety Commission | Commission canadienne de sûreté nucléaire

01187-2-03.0

License Number / Numéro de permis

NUCLEAR SUBSTANCES AND RADIATION DEVICES LICENCE | PERMIS PORTANT SUR LES SUBSTANCES NUCLÉAIRES ET LES APPAREILS À RAYONNEMENT

I) LICENSEE

Pursuant to section 24(2) of the Nuclear Safety and Control Act, this licence is issued to:

Montreal Neurological Hospital & Institute/Institut et Hôpital neurologiques de Montréal
3801 University Street
Montreal, QC
H3A 2B4
Canada

hereinafter «the licensee».

II) PERIOD

This licence is valid from: May 1 2001 to April 30 2003.

III) LICENSED ACTIVITIES

This licence authorizes the licensee to possess, transfer, import, export, use and store the nuclear substances listed in section IV) of this licence at the locations specified in section V) of this licence.

This licence is issued for: laboratory studies: 10 or more laboratories where radioisotopes are used or handled (B36)

IV) NUCLEAR SUBSTANCES AND PRESCRIBED EQUIPMENT

ITEM	NUCLEAR SUBSTANCE	UNSEALED SOURCE MAXIMUM QUANTITY	SEALED SOURCE MAXIMUM QUANTITY	EQUIPMENT MAKE AND MODEL
1	Carbon 14	11 GBq	n/a	n/a
2	Calcium 45	800 MBq	n/a	n/a
3	Chromium 51	2 GBq	n/a	n/a
4	Fluorine 18	2 GBq	n/a	n/a
5	Iron 55	100 MBq	n/a	n/a
6	Gallium 68	400 MBq	n/a	n/a
7	Hydrogen 3	33 GBq	n/a	n/a
8	Iodine 125	4 GBq	n/a	n/a
9	Iodine 131	2 GBq	n/a	n/a
10	Krypton 85	74 GBq	n/a	n/a
11	Phosphorus 32	11 GBq	n/a	n/a
12	Phosphorus 33	2 GBq	n/a	n/a
13	Sulfur 35	22 GBq	n/a	n/a
14	Technetium 99m	11 GBq	n/a	n/a

The total quantity of an unsealed nuclear substance in possession shall not exceed the corresponding listed unsealed source maximum quantity. The total quantity of nuclear substance per sealed source shall not exceed its corresponding listed sealed source maximum quantity. Sealed sources shall only be used in the corresponding listed equipment.

V) LOCATION(S) OF LICENSED ACTIVITIES

used or stored at:
3801 University Street
Montreal, QC

VI) CONDITIONS

1. Prohibition of Human Use

This licence does not authorize the use of nuclear substances in or on human beings.
(2696-0)

2. Area Classification
The licensee shall classify each room, area or enclosure where more than one exemption quantity of an unsealed nuclear substance is used at a single time as:

(a) basic-level if the quantity does not exceed 5 ALI,
(b) intermediate-level if the quantity used does not exceed 50 ALI,
(c) high-level if the quantity does not exceed 500 ALI,
(d) containment-level if the quantity exceeds 500 ALI; or
(e) special purpose if approved in writing by the Commission or a person authorized by the Commission.

Except for the basic-level classification, the licensee shall not use unsealed nuclear substances in these rooms, areas or enclosures without written approval of the Commission or a person authorized by the Commission.
(2108-1)

3. Laboratory Lists
The licensee shall maintain a list of all areas, rooms and enclosures in which more than one exemption quantity of a nuclear substance is used or stored.
(2569-1)

4. Laboratory Procedures
The licensee shall post and keep posted, in a readily visible location in areas, rooms or enclosures where nuclear substances are handled, a radioisotope safety poster approved by the Commission or a person authorized by the Commission, which corresponds to the classification of the area, room or enclosure.
(2570-1)

5. Thyroid Monitoring
Every person who
(a) uses at a single time a quantity of volatile iodine-125 or iodine-131 exceeding:
(i) 5 MBq in an open room;
(ii) 50 MBq in a fume hood;
(iii) 500 MBq in a glove box;
(iv) any other quantity in other containment approved in writing by the Commission or a person authorized by the Commission; or
(b) is involved in a spill of greater than 5 MBq of volatile iodine-125 or iodine-131;
(c) or on whom iodine-125 or iodine-131 external contamination is detected; and shall, undergo thyroid screening within five days following the exposure to iodine-125 or iodine-131.
(2046-5)

6. Thyroid Screening
Screening for internal iodine-125 and iodine-131 shall be performed using:
(a) a direct measurement of the thyroid with an instrument that can detect 1 kBq of iodine-125 or iodine-131; or
(b) a bioassay procedure approved by the Commission or a person authorized by the Commission.
(2600-1)

7. Thyroid Bioassay
If direct screening detects more than 10 kBq of iodine-125 or iodine-131 in the thyroid, then a bioassay approved by the Commission or a person authorized by the Commission shall be performed within 24 hours.
(2601-1)

8. Extremity Dosimetry
The licensee shall ensure that any person who handles a container which contains more than 50 MBq of phosphorus 32, strontium 89, yttrium 90, samarium 153 or rhenium 186 wears a ring dosimeter. The dosimeters must be supplied and read by a dosimetry service licenced

by the Commission.
(2578-0)

9. Contamination Criteria
The licensee shall ensure that for nuclear substances listed in the licence application guide table titled "Classification of Radionuclides";
(a) non-fixed contamination in all areas, rooms or enclosures where unsealed nuclear substances are used or stored does not exceed:
(i) 3 becquerels per square centimetre for all Class A radionuclides;
(ii) 30 becquerels per square centimetre for all Class B radionuclides; or
(iii) 300 becquerels per square centimetre for all Class C radionuclides;
averaged over an area not exceeding 100 square centimetres; and
(b) non-fixed contamination in all other areas does not exceed:
(i) 0.3 becquerels per square centimetre for all Class A radionuclides;
(ii) 3 becquerels per square centimetre for all Class B radionuclides; or
(iii) 30 becquerels per square centimetre for all Class C radionuclides;
averaged over an area not exceeding 100 square centimetres.
(2642-1)

10. Decommissioning
The licensee shall ensure that prior to decommissioning any area, room or enclosure where the licensed activity has been conducted;
(a) the non-fixed contamination for nuclear substances listed in the licence application guide table titled "Classification of Radionuclides" does not exceed:
(i) 0.3 becquerels per square centimetre for all Class A radionuclides;
(ii) 3 becquerels per square centimetre for all Class B radionuclides; and
(iii) 30 becquerels per square centimetre for all Class C radionuclides;
averaged over an area not exceeding 100 square centimetres;
(b) the release of any area, room or enclosure containing fixed contamination, is approved in writing by the Commission or person authorized by the Commission;
(c) all nuclear substances and radiation devices have been transferred in accordance with the conditions of this licence; and
(d) all radiation warning signs have been removed or defaced.
(2571-2)

11. Storage
The licensee shall:
(a) ensure that when in storage radioactive nuclear substances or radiation devices are accessible only to persons authorized by the licensee;
(b) ensure that the dose rate at any occupied location outside the storage area, room or enclosure resulting from the substances or devices in storage does not exceed 2.5 microSv/h; and
(c) have measures in place to ensure that the dose limits in the Radiation Protection Regulations are not exceeded as a result of the substances or devices in storage.
(2575-0)

12. Disposal (Laboratories)
When disposing of unsealed nuclear substances to municipal garbage or sewer systems, the licensee shall ensure that the following limits are not exceeded:

COLUMN 1	COLUMN 2(a)	COLUMN 3(b)
-	LIMITS	LIMITS
Nuclear Substance	solids to municipal garbage system (quantity per kilogram)	liquids(water soluble)to municipal sewer system (quantity per year)

References

Adlkofer, K., R. Frei, D. H. Neuberg, J. Zielasek, K. V. Toyka and U. Suter (1997). "Heterozygous peripheral myelin protein 22-deficient mice are affected by a progressive demyelinating tomaculous neuropathy." J Neurosci **17**: 4662-71.

Adlkofer, K., R. Martini, A. Aguzzi, J. Zielasek, K. V. Toyka and U. Suter (1995). "Hypermyelination and demyelinating peripheral neuropathy in Pmp22-deficient mice." Nat Genet **11**: 274-80.

Attwood, J.T., Yung, R.L., and B.C. Richardson (2002). DNA methylation and the regulation of gene transcription. Cell Mol Life Sci **59**: 241-57

Baechner, D., T. Liehr, H. Hameister, H. Altenberger, H. Grehl, U. Suter and B. Rautenstrauss (1995). "Widespread expression of the peripheral myelin protein-22 gene (PMP22) in neural and non-neural tissues during murine development." J Neurosci Res **42**: 733-41.

Baulieu, E. E., M. Schumacher, H. Koenig, I. Jung-Testas and Y. Akwa (1996). "Progesterone as a neurosteroid: actions within the nervous system." Cell Mol Neurobiol **16**: 143-54.

Ben Othmane, K., F. Hentati, F. Lennon, C. Ben Hamida, S. Blel, A. D. Roses, M. A. Pericak-Vance, M. Ben Hamida and J. M. Vance (1993). "Linkage of a locus (CMT4A) for autosomal recessive Charcot-Marie-Tooth disease to chromosome 8q." Hum Mol Genet **2**: 1625-8.

Ben Othmane, K. J., E., Menold, M., Graham, F. L., Ben Hamida, M., Hasegawa, O., Rogala, A. D., Ohnishi, A., Pericak-Vance, M., Hentati, F., Vance, J. M. (1999). "Identification of a new locus for autosomal recessive Charcot-Marie-Tooth disease with focally folded myelin on chromosome 11p15." Genomics **62**: 344-9.

Boerkoel, C. F., H. Takashima, P. Stankiewicz, C. A. Garcia, S. M. Leber, L. Rhee-Morris and J. R. Lupski (2001). "Periaxin mutations cause recessive Dejerine-Sottas neuropathy." Am J Hum Genet **68**: 325-33.

Bolino, A., V. Brancolini, F. Bono, A. Bruni, A. Gambardella, G. Romeo, A. Quattrone and M. Devoto (1996). "Localization of a gene responsible for autosomal recessive demyelinating neuropathy with focally folded myelin sheaths to chromosome 11q23 by homozygosity mapping and haplotype sharing." Hum Mol Genet **5**: 1051-4.

Bolino, A., M. Muglia, F. L. Conforti, E. LeGuern, M. A. Salih, D. M. Georgiou, K. Christodoulou, I. Hausmanowa-Petrusewicz, P. Mandich, A. Schenone, A. Gambardella, F. Bono, A. Quattrone, M. Devoto and A. P. Monaco (2000). "Charcot-

Marie-Tooth type 4B is caused by mutations in the gene encoding myotubularin-related protein-2." Nat Genet **25**: 17-9.

Brady, S. T., A. S. Witt, L. L. Kirkpatrick, S. M. de Waegh, C. Readhead, P. H. Tu and V. M. Lee (1999). "Formation of compact myelin is required for maturation of the axonal cytoskeleton." J Neurosci **19**: 7278-88.

Bronson, S., E. G. Plaehn, K. D. Cluckman, J. R. Hagaman, N. Maeda and O. Smithies (1996). "Single-copy transgenic mice with chosen-site integration." Proc Natl Acad Sci USA **93**: 9067-72.

Brooks, A. R., B. D. Blackhart, K. Haubold and B. Levy-Wilson (1991). "Characterization of tissue-specific enhancer elements in the second intron of the human apolipoprotein B gene." J Biol Chem **266**: 7848-59.

Brown, A. M. and G. Lemke (1997). "Multiple regulatory elements control transcription of the peripheral myelin protein zero gene." J Biol Chem **272**: 28939-47.

Burke, T.W., Willy, P.J., Kutach, A.K., Butler, J.E., Kadonaga, J.T. (1998). The DPE, a conserved downstream core promoter element that is functionally analogous to the TATA box. Cold Spr Harb Symp Quant Biol. **63**:75-82.

Butler, J. E. F., and and J. T. Kadonaga (2001). "Enhancer-promoter specificity mediated by DPE or TATA core promoter motifs." Genes Dev **15**: 2515-19.

Campagnoni, A. T., J. M. Verdi, A. N. Verity and S. Amur-Umarjee (1990). "Posttranscriptional events in the expression of myelin protein genes." Ann N Y Acad Sci **605**: 270-9.

Chance, P. F. and D. Pleasure (1993). "Charcot-Marie-Tooth syndrome." Arch Neurol **50**: 1180-4.

Charcot, J. M. and P. Marie (1886.). "Sur une forme particuliere d'atrophie musculaire progressive souvent famillíale debutant par les pieds et les jambes et atteignant plus tard les mains." Rev Med **6**: 97-136.

Cheng, L., Mudge, AW (1996). "Cultured Schwann cells constitutively express the myelin protein P0." Neuron **16**: 309-19.

Cremers, F. P. M. P., R. A., van de Pol, T. J. R.; Hofker, M. H.; Kruse, T. A., Wieringa, B., Ropers, H. H. (1987). "An interstitial duplication of the X chromosome in a male allows physical fine mapping of probes from the Xq13-q22 region." Hum Genet **77**: 23-7.

Cvetkovic, B., B. Yang, R. A. Williamxon and C. D. Sigmund (2000). "Appropriate tissue- and cell-specific expression of a single copy human angiotensinogen transgene specifically targeted upstream of the HPRT locus by homologous recombination." J Biol Chem **275**: 1073-8.

de Waegh, S. M., V. M. Lee and S. T. Brady (1992). "Local modulation of neurofilament phosphorylation, axonal caliber, and slow axonal transport by myelinating Schwann cells." Cell **68**: 451-63.

Delague, V., C. Bareil, S. Tuffery, P. Bouvagnet, E. Chouery, S. Koussa, T. Maisonobe, J. Loiselet, A. Megarbane and M. Claustres (2000). "Mapping of a new locus for autosomal recessive demyelinating Charcot-Marie-Tooth disease to 19q13.1-13.3 in a large consanguineous Lebanese family: exclusion of MAG as a candidate gene." Am J Hum Genet **67**: 236-43.

Desarnaud, F., S. Bidichandani, P. I. Patel, E. E. Baulieu and M. Schumacher (2000). "Glucocorticosteroids stimulate the activity of the promoters of peripheral myelin protein-22 and protein zero genes in Schwann cells." Brain Res **865**: 12-6.

Desarnaud, F., A. N. Do Thi, A. M. Brown, G. Lemke, U. Suter, E. E. Baulieu and M. Schumacher (1998). "Progesterone stimulates the activity of the promoters of peripheral myelin protein-22 and protein zero genes in Schwann cells." J Neurochem **71**: 1765-8.

Doyle, J. P. and D. R. Colman (1993). "Glial-neuron interactions and the regulation of myelin formation." Curr Opin Cell Biol **5**: 779-85.

Fabbretti, E., P. Edomi, C. Brancolini and C. Schneider (1995). "Apoptotic phenotype induced by overexpression of wild-type gas3/PMP22: its relation to the demyelinating peripheral neuropathy CMT1A." Genes Dev **9**: 1846-56.

Farhadi, H. F., R. Forghani and A. C. Peterson (1999). "MBP-lacZ transgene expression in juvenile and adult Trembler-J mice." Ann N Y Acad Sci **883**: 538-9.

Feltri, M. L., M. D'Antonio, A. Quattrini, R. Numerato, M. Arona, S. Previtali, S. Y. Chiu, A. Messing and L. Wrabetz (1999). "A novel P0 glycoprotein transgene activates expression of lacZ in myelin-forming Schwann cells." Eur J Neurosci **11**: 1577-86.

Foran, D. R. and A. C. Peterson (1992). "Myelin acquisition in the central nervous system of the mouse revealed by an MBP-Lac Z transgene." J Neurosci **12**: 4890-7.

Forghani, R., Garofalo, L., Foran, DR, Farhadi HF, Lepage P, Hudson TJ, Tretjakoff I, Valera P, Peterson A (2001). "A distal upstream enhancer from the myelin basic protein gene regulates expression in myelin-forming schwann cells." J Neurosci **21**: 3780-7.

Garcia, C. A., R. E. Malamut, J. D. England, G. S. Parry, P. Liu and J. R. Lupski (1995). "Clinical variability in two pairs of identical twins with the Charcot-Marie-Tooth disease type 1A duplication." Neurol **45**: 2090-3.

Ghislain, J., C. Desmarquet-Trin-Dinh, M. Jaegle, D. Meijer, P. Charnay and M. Frain (2002). "Characterisation of cis-acting sequences reveals a biphasic, axon-dependent regulation of Krox20 during Schwann cell development." Dev **129**: 155-66.

Gow, A., V. L. Friedrich, Jr. and R. A. Lazzarini (1992). "Myelin basic protein gene contains separate enhancers for oligodendrocyte and Schwann cell expression." J Cell Biol **119**: 605-16.

Hagedorn, L., Suter, U., Sommer, L. (1999). "P0 and PMP22 mark a multipotent neural crest-derived cell type that displays community effects in response to TGF-beta family factors." Dev **126**: 3781-94.

Hai M, B. S., Patel PI (2001). "Identification of a positive regulatory element in the myelin-specific promoter of the PMP22 gene." J Neurosci Res **65**: 508-19.

Haney, C., G. J. Snipes, E. M. Shooter, U. Suter, C. Garcia, J. W. Griffin and B. D. Trapp (1996). "Ultrastructural distribution of PMP22 in Charcot-Marie-Tooth disease type 1A." J Neuropathol Exp Neurol **55**: 290-9.

He, X., R. Gerrero, D. M. Simmons, R. E. Park, C. J. Lin, L. W. Swanson and M. G. Rosenfeld (1991). "Tst-1, a member of the POU domain gene family, binds the promoter of the gene encoding the cell surface adhesion molecule P0." Mol Cell Biol **11**: 1739-44.

Heinemeyer, T., Wingender, E., Reuter, I., Hermjakob H, Kel AE, Kel OV, Ignatieva EV, Ananko EA, Podkolodnaya OA, Kolpakov FA, Podkolodny NL, Kolchanov NA (1998). "Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL." Nucleic Acid Res **26**: 362-7.

Huehne, K., Rautenstrauss, B. (2001). "Transcriptional startpoints and methylation patterns in the PMP22 promoters of peripheral nerve, leukocytes and tumor cell lines." Internat J Mole Med **7**: 669-75.

Huhne, K., O. Park, T. Liehr and B. Rautenstrauss (1999). "Expression analysis of the PMP22 gene in glioma and osteogenic sarcoma cell lines." J Neurosci Res **58**: 624-31.

Huxley, C., E. Passage, A. Manson, G. Putzu, D. Figarella-Branger, J. F. Pellissier and M. Fontes (1996). "Construction of a mouse model of Charcot-Marie-Tooth disease type 1A by pronuclear injection of human YAC DNA." Hum Mol Genet **5**: 563-9.

Huxley, C., E. Passage, A. M. Robertson, B. Youl, S. Huston, A. Manson, D. Saberan-Djoniedi, D. Figarella-Branger, J. F. Pellissier, P. K. Thomas and M. Fontes (1998). "Correlation between varying levels of PMP22 expression and the degree of demyelination and reduction in nerve conduction velocity in transgenic mice." Hum Mol Genet **7**: 449-58.

Inoue, K., Y. Tanabe and J. R. Lupski (1999). "Myelin deficiencies in both the central and the peripheral nervous systems associated with a SOX10 mutation." Ann Neurol **46**: 313-8.

Ionasescu, V. V., C. C. Searby, R. Ionasescu, R. Reisin, V. Ruggieri and C. Arberas (1997). "Severe Charcot-Marie-Tooth neuropathy type 1A with 1-base pair deletion and frameshift mutation in the peripheral myelin protein 22 gene." Muscle Nerve **20**: 1308-10.

Jaegle, M. and D. Meijer (1998). "Role of Oct-6 in Schwann cell differentiation." Microsc Res Tech **41**: 372-8.

Kalaydjieva, L., D. Gresham, R. Gooding, L. Heather, F. Baas, R. de Jonge, K. Blechschmidt, D. Angelicheva, D. Chandler, P. Worsley, A. Rosenthal, R. H. King and P. K. Thomas (2000). "N-myc downstream-regulated gene 1 is mutated in hereditary motor and sensory neuropathy-Lom." Am J Hum Genet **67**: 47-58.

Kalaydjieva, L., J. Hallmayer, D. Chandler, A. Savov, A. Nikolova, D. Angelicheva, R. H. King, B. Ishpekova, K. Honeyman, F. Calafell, A. Shmarov, J. Petrova, I. Turnev, A. Hristova, M. Moskov, S. Stancheva, I. Petkova, A. H. Bittles, V. Georgieva, L. Middleton and P. K. Thomas (1996). "Gene mapping in Gypsies identifies a novel demyelinating neuropathy on chromosome 8q24." Nat Genet **14**: 214-7.

Koenig, H. L., M. Schumacher, B. Ferzaz, A. N. Thi, A. Ressouches, R. Guennoun, I. Jung-Testas, P. Robel, Y. Akwa and E. E. Baulieu (1995). "Progesterone synthesis and myelin formation by Schwann cells." Sci **268**: 1500-3.

Kuhlbrodt, K., B. Herbarth, E. Sock, I. Hermans-Borgmeyer and M. Wegner (1998). "Sox10, a novel transcriptional modulator in glial cells." J Neurosci **18**: 237-50.

Kutach, A.K., and Kadonaga, J. T. (2000). The downstream promoter element DPE appears to be as widely used as the TATA box in Drosophila core promoters. Mol Cel Biol **20**:4754-64.

LeBlanc, A. C., J. Pringle, J. Lemieux, J. F. Poduslo and C. Mezei (1992a). "Regulation of 2',3'-cyclic nucleotide phosphodiesterase gene expression in experimental peripheral neuropathies." Brain Res Mol Brain Res **15**: 40-6.

LeBlanc, A. C., A. J. Windebank and J. F. Poduslo (1992b). "P0 gene expression in Schwann cells is modulated by an increase of cAMP which is dependent on the presence of axons." Brain Res Mol Brain Res **12**: 31-8.

LeGuern, E., A. Guilbot, M. Kessali, N. Ravise, J. Tassin, T. Maisonobe, D. Grid and A. Brice (1996). "Homozygosity mapping of an autosomal recessive form of demyelinating Charcot-Marie-Tooth disease to chromosome 5q23-q33." Hum Mol Genet **5**: 1685-8.

Lemke, G. and M. Chao (1988). "Axons regulate Schwann cell expression of the major myelin and NGF receptor genes." Dev **102**: 499-504.

Levy-Wilson, B., B. Paulweber, B. P. Nagy, E. H. Ludwig and A. R. Brooks (1992). "Nuclease-hypersensitive sites define a region with enhancer activity in the third intron of the human apolipoprotein B gene." J Biol Chem **267**: 18735-43.

Li, X., L. Wrabetz, Y. Cheng and J. Kamholz (1994). "A novel cyclic AMP response element, CACTTGATC, mediates forskolin induction of the myelin basic protein promoter in the rat Schwannoma line, D6P2T." J Neurochem **63**: 28-40.

Lupski, J. R., P. F. Chance and C. A. Garcia (1993). "Inherited primary peripheral neuropathies. Molecular genetics and clinical implications of CMT1A and HNPP." JAMA **270**: 2326-30.

Lupski, J. R., L. R. de O, S. Slaugenhaupt, L. Pentao, V. Guzzetta, B. J. Trask and e. al. (1991). "DNA duplication associated with Charcot-Marie-Tooth disease type 1A." Cell **66**: 219-32.

Mack, D. H., J. Vartikar, J. M. Pipas and L. A. Laimins (1993). "Specific repression of TATA-mediated but not initiator-mediated transcription by wild type p53." Nat **363**: 281-3.

Magyar, J. P., R. Martini, T. Ruelicke, A. Aguzzi, K. Adlkofer, Z. Dembic, J. Zielasek, K. V. Toyka and U. Suter (1996). "Impaired differentiation of Schwann cells in transgenic mice with increased PMP22 gene dosage." J Neurosci **16**: 5351-60.

Maier, M., P. Berger, K. A. Nave and U. Suter (2002). "Identification of the regulatory region of the peripheral myelin protein 22 (PMP22) gene that directs temporal and spatial expression in development and regeneration of peripheral nerves." Mol Cell Neurosci **20**: 93-109.

Maldonado, E., M. Hampsey and D. Reinberg (1999). "Repression: Targetting the heart of the matter." Cell **99**:455-8.

Mandemakers, W., R. Zwart, M. Jaegle, E. Walbeehm, P. Visser, F. Grosveld and D. Meijer (2000). "A distal Schwann cell-specific enhancer mediates axonal regulation of the Oct-6 transcription factor during peripheral nerve development and regeneration." EMBO J **19**: 2992-3003.

Matsunami, N., B. Smith, L. Ballard, M. W. Lensch, M. Robertson, H. Albertsen, C. O. Hanemann, H. W. Muller, T. D. Bird, R. White and et al. (1992). "Peripheral myelin protein-22 gene maps in the duplication in chromosome 17p11.2 associated with Charcot-Marie-Tooth 1A." Nat Genet **1**: 176-9.

McCormick, S. P. and L. B. Nielsen (1998). "Expression of large genomic clones in transgenic mice: new insights into apolipoprotein B structure, function and regulation." Curr Opin Lipidol **9**: 103-11.

Meerovitch K, Nicholson R, Sonenberg N. (1991). "In vitro mutational analysis of cis-acting RNA translational elements within the poliovirus type 2 5' untranslated region". J Virol. **65**:5895-901.

Meijer, D., A. Graus and G. Grosveld (1992). "Mapping the transactivation domain of the Oct-6 POU transcription factor." Nucleic Acids Res **20**: 2241-7.

Melcangi, R. C., V. Magnaghi, I. Cavarretta, L. Martini and F. Piva (1998). "Age-induced decrease of glycoprotein Po and myelin basic protein gene expression in the rat sciatic nerve. Repair by steroid derivatives." Neurosci **85**: 569-78.

Melcangi, R. C., V. Magnaghi, I. Cavarretta, I. Zucchi, P. Bovolin, D. D'Urso and L. Martini (1999). "Progesterone derivatives are able to influence peripheral myelin protein 22 and P0 gene expression: possible mechanisms of action." J Neurosci Res **56**: 349-57.

Melcangi, R. C., V. Magnaghi and L. Martini (1999). "Steroid metabolism and effects in central and peripheral glial cells." J Neurobiol **40**: 471-83.

Mersiyanova, I. V., A. V. Perepelov, A. V. Polyakov, V. F. Sitnikov, E. L. Dadali, R. B. Oparin, A. N. Petrin and O. V. Evgrafov (2000). "A new variant of Charcot-Marie-Tooth disease type 2 is probably the result of a mutation in the neurofilament-light gene." Am J Hum Genet **67**: 37-46.

Mirsky, R. and K. R. Jessen (1996). "Schwann cell development, differentiation and myelination." Curr Opin Neurobiol **6**: 89-96.

Monuki, E. S., R. Kuhn and G. Lemke (1993). "Cell-specific action and mutable structure of a transcription factor effector domain." Proc Natl Acad Sci USA **90**: 9978-82.

Monuki, E. S., R. Kuhn and G. Lemke (1993). "Repression of the myelin P0 gene by the POU transcription factor SCIP." Mech Dev **42**: 15-32.

Monuki, E. S., R. Kuhn, G. Weinmaster, B. D. Trapp and G. Lemke (1990). "Expression and activity of the POU transcription factor SCIP." Sci **249:** 1300-3.

Monuki, E. S., G. Weinmaster, R. Kuhn and G. Lemke (1989). "SCIP: a glial POU domain gene regulated by cyclic AMP." Neuron **3**: 783-93.

Nagamatsu, M., R. B. Jenkins, D. J. Schaid, D. M. Klein and P. J. Dyck (2000). "Hereditary motor and sensory neuropathy type 2C is genetically distinct from types 2B and 2D." Arch Neurol **57**: 669-72.

Nakao, J., J. Shinoda, Y. Nakai, S. Murase and K. Uyemura (1997). "Apoptosis regulates the number of Schwann cells at the premyelinating stage." J Neurochem **68**: 1853-62.

Navon, R., B. Seifried, N. S. Gal-On and M. Sadeh (1996). "A new point mutation affecting the fourth transmembrane domain of PMP22 results in severe de novo Charcot-Marie-Tooth disease." Hum Genet **97**: 685-7.

Nelis, E., P. De Jonghe, E. De Vriendt, P. I. Patel, J. J. Martin and C. Van Broeckhoven (1998). "Mutation analysis of the nerve specific promoter of the peripheral myelin protein 22 gene in CMT1 disease and HNPP." J Med Genet **35**: 590-3.

Nelis, E., N. Haites and C. Van Broeckhoven (1999a). "Mutations in the peripheral myelin genes and associated genes in inherited peripheral neuropathies." Hum Mutat **13**: 11-28.

Nelis, E., V. Timmerman, P. De Jonghe, C. Van Broeckhoven and B. Rautenstrauss (1999b). "Molecular genetics and biology of inherited peripheral neuropathies: a fast-moving field." Neurogenet **2**: 137-48.

Nelis, E., C. Van Broeckhoven, P. De Jonghe, A. Lofgren, A. Vandenberghe, P. Latour, E. Le Guern, A. Brice, M. L. Mostacciuolo, F. Schiavon, F. Palau, S. Bort, M. Upadhyaya, M. Rocchi, N. Archidiacono, P. Mandich, E. Bellone, K. Silander, M. L. Savontaus, R. Navon, H. Goldberg-Stern, X. Estivill, V. Volpini, W. Friedl, A. Gal and et al. (1996). "Estimation of the mutation frequencies in Charcot-Marie-Tooth disease type 1 and hereditary neuropathy with liability to pressure palsies: a European collaborative study." Eur J Hum Genet **4**: 25-33.

Nicholson, G. A., L. J. Valentijn, A. K. Cherryson, M. L. Kennerson, T. L. Bragg, R. M. DeKroon, D. A. Ross, J. D. Pollard, J. G. McLeod, P. A. Bolhuis and et al. (1994). "A frame shift mutation in the PMP22 gene in hereditary neuropathy with liability to pressure palsies." Nat Genet **6**: 263-6.

Nielsen, L. B., S. P. McCormick and S. G. Young (1999). "A new approach for studying gene regulation by distant DNA elements in transgenic mice." Scand J Clin Lab Invest Suppl **229**: 33-9.

Nielsen, L. B., M. Sullivan, T. Vanni-Reyes, I. J. Goldberg and S. G. Young (1999). "The DNA sequences required for apolipoprotein B expression in the heart are distinct from those required for expression in the intestine." J Mol Cell Cardiol **31**: 695-703.

Norreel, J. C., Jamon, M., Riviere G, Passage E, Fontes M, Clarac F (2001). "Behavioural profiling of a murine Charcot-Marie-Tooth disease type 1A model." Eur J Neurosci **13**: 1625-34.

Notterpek, L., E. M. Shooter and G. J. Snipes (1997). "Upregulation of the endosomal-lysosomal pathway in the trembler-J neuropathy." J Neurosci **17**: 4190-200.

Notterpek, L., G. J. Snipes and E. M. Shooter (1999). "Temporal expression pattern of peripheral myelin protein 22 during in vivo and in vitro myelination." Glia **25**: 358-69.

O'Shea-Greenfield, A., and and S. T. Smale (1992). "Roles of TATA and initiator elements in determining the start site location and direction of RNA polymerase II transcription." J Biol Chem **267**: 1391-402.

Paik, Y. K., D. J. Chang, C. A. Reardon, M. D. Walker, E. Taxman and J. M. Taylor (1988). "Identification and characterization of transcriptional regulatory regions associated with expression of the human apolipoprotein E gene." J Biol Chem **263**: 13340-9.

Pareek, S., L. Notterpek, G. J. Snipes, R. Naef, W. Sossin, J. Laliberte, S. Iacampo, U. Suter, E. M. Shooter and R. A. Murphy (1997). "Neurons promote the translocation of peripheral myelin protein 22 into myelin." J Neurosci **17**: 7754-62.

Parmantier, E., C. Braun, J. L. Thomas, F. Peyron, S. Martinez and B. Zalc (1997). "PMP-22 expression in the central nervous system of the embryonic mouse defines potential transverse segments and longitudinal columns." J Comp Neurol **378**: 159-72.

Parmantier, E., F. Cabon, C. Braun, D. D'Urso, H. W. Muller and B. Zalc (1995). "Peripheral myelin protein-22 is expressed in rat and mouse brain and spinal cord motoneurons." Eur J Neurosci **7**: 1080-8.

Patel, P. I., B. B. Roa, A. A. Welcher, R. Schoener-Scott, B. J. Trask, L. Pentao, G. J. Snipes, C. A. Garcia, U. Francke, E. M. Shooter and et al. (1992). "The gene for the peripheral myelin protein PMP-22 is a candidate for Charcot-Marie-Tooth disease type 1A." Nat Genet **1**: 159-65.

Peirano, R. I., D. E. Goerich, D. Riethmacher and M. Wegner (2000). "Protein zero gene expression is regulated by the glial transcription factor Sox10." Mol Cell Biol **20**: 3198-209.

Pelletier J, Flynn ME, Kaplan G, Racaniello V, Sonenberg N.(1988) "Mutational analysis of upstream AUG codons of poliovirus RNA." J Virol **62**:4486-92.

Pentao, L., C. A. Wise, A. C. Chinault, P. I. Patel and J. R. Lupski (1992). "Charcot-Marie-Tooth type 1A duplication appears to arise from recombination at repeat sequences flanking the 1.5 Mb monomer unit." Nat Genet **2**: 292-300.

Poduslo, J. F., R. S. Walikonis, M. C. Domec, C. T. Berg and C. J. Holtz-Heppelmann (1995). "The second messenger, cyclic AMP, is not sufficient for myelin gene induction in the peripheral nervous system." J Neurochem **65**: 149-59.

Raskind, W. H., C. A. Williams, L. D. Hudson and T. D. Bird (1991). "Complete deletion of the proteolipid protein gene (PLP) in a family with X-linked Pelizaeus-Merzbacher disease." Am J Hum Genet **49**: 1355-60.

Roa, B. B., P. J. Dyck, H. G. Marks, P. F. Chance and J. R. Lupski (1993). "Dejerine-Sottas syndrome associated with point mutation in the peripheral myelin protein 22 (PMP22) gene." Nat Genet **5**: 269-73.

Roa, B. B., C. A. Garcia, U. Suter, D. A. Kulpa, C. A. Wise, J. Mueller, A. A. Welcher, G. J. Snipes, E. M. Shooter, P. I. Patel and et al. (1993). "Charcot-Marie-Tooth disease type 1A. Association with a spontaneous point mutation in the PMP22 gene." N Engl J Med **329**: 96-101.

Robertson, A. M., C. Huxley, R. H. King and P. K. Thomas (1999). "Development of early postnatal peripheral nerve abnormalities in Trembler-J and PMP22 transgenic mice." J Anat **195**: 331-9.

Robertson, A. M., R. H. King, J. R. Muddle and P. K. Thomas (1997). "Abnormal Schwann cell/axon interactions in the Trembler-J mouse." J Anat **190**: 423-32.

Russo, M. W., B. R. Sevetson and J. Milbrandt (1995). "Identification of NAB1, a repressor of NGFI-A- and Krox20-mediated transcription." Proc Natl Acad Sci U S A **92**: 6873-7.

Saberan-Djoneidi, D., V. Sanguedolce, Z. Assouline, N. Levy, E. Passage and M. Fontes (2000). "Molecular dissection of the Schwann cell specific promoter of the PMP22 gene." Gene **248**: 223-31.

Sancho, S., J. P. Magyar, A. Aguzzi and U. Suter (1999). "Distal axonopathy in peripheral nerves of PMP22-mutant mice." Brain **122**: 1563-77.

Sarafanov, A. G., R. V. Kondratov, V. M. Zakhar'ev, V. S. Prasolov and V. Kozlov Iu (1999). "[Gene for the human tissue activator plasminogen: regulation of expression by signals, located in the 3'-untranslated region]." Mol Biol (Mosk) **33**: 413-22.

Scherer, S. S., D. Y. Wang, R. Kuhn, G. Lemke, L. Wrabetz and J. Kamholz (1994). "Axons regulate Schwann cell expression of the POU transcription factor SCIP." J Neurosci **14**: 1930-42.

Schwartz, S., Zhang, Z., Frazer KA, Smit A, Riemer C, Bouck J, Gibbs R, Hardison R, Miller W (2000). "PipMaker--a web server for aligning two genomic DNA sequences." Genome Res **10**: 577-86.

Sereda, M., I. Griffiths, A. Puhlhofer, H. Stewart, M. J. Rossner, F. Zimmerman, J. P. Magyar, A. Schneider, E. Hund, H. M. Meinck, U. Suter and K. A. Nave (1996). "A transgenic rat model of Charcot-Marie-Tooth disease." Neuron **16**: 1049-60.

Skre, H. (1974). "Genetic and clinical aspects of Charcot-Marie-Tooth's disease." Clin Genet **6**: 98-118.

Smale, S. T. (2001). "Core promoters: Active contributors to combinatorial gene regulation." Genes Dev **15**: 2503-8.

Snipes, G. J., U. Suter, A. A. Welcher and E. M. Shooter (1992). "Characterization of a novel peripheral nervous system myelin protein (PMP-22/SR13)." J Cell Biol **117**: 225-38.

Sonenberg, N. 1994. "mRNA translation: influence of the 5' and 3' untranslated regions." Curr Opin Genet Dev. **4**: 310-5.

Stahl, N., J. Harry and B. Popko (1990). "Quantitative analysis of myelin protein gene expression during development in the rat sciatic nerve." Brain Res Mol Brain Res **8**: 209-12.

Suter, U., J. J. Moskow, A. A. Welcher, G. J. Snipes, B. Kosaras, R. L. Sidman, A. M. Buchberg and E. M. Shooter (1992). "A leucine-to-proline mutation in the putative first transmembrane domain of the 22-kDa peripheral myelin protein in the trembler-J mouse." Proc Natl Acad Sci U S A **89**: 4382-6.

Suter, U. and P. I. Patel (1994). "Genetic basis of inherited peripheral neuropathies." Hum Mutat **3**: 95-102.

Suter, U. and G. J. Snipes (1995). "Biology and genetics of hereditary motor and sensory neuropathies." Annu Rev Neurosci **18**: 45-75.

Suter, U., G. J. Snipes, R. Schoener-Scott, A. A. Welcher, S. Pareek, J. R. Lupski, R. A. Murphy, E. M. Shooter and P. I. Patel (1994). "Regulation of tissue-specific expression of alternative peripheral myelin protein-22 (PMP22) gene transcripts by two promoters." J Biol Chem **269**: 25795-808.

Suter, U., A. A. Welcher, T. Ozcelik, G. J. Snipes, B. Kosaras, U. Francke, S. Billings-Gagliardi, R. L. Sidman and E. M. Shooter (1992). "Trembler mouse carries a point mutation in a myelin gene." Nat **356**: 241-4.

Svaren, J., B. R. Sevetson, T. Golda, J. J. Stanton, A. H. Swirnoff and J. Milbrandt (1998). "Novel mutants of NAB corepressors enhance activation by Egr transactivators." EMBO J **17**: 6010-9.

Swiatek, P. J. and T. Gridley (1993). "Perinatal lethality and defects in hindbrain development in mice homozygous for a targeted mutation of the zinc finger gene Krox20." Genes Dev **7**: 2071-84.

Taylor V, Zgraggen C, Naef R, Suter U. (2000).Membrane topology of peripheral myelin protein 22. J Neurosci Res **62**:15-27

Timmerman, V., E. Nelis, W. Van Hul, B. W. Nieuwenhuijsen, K. L. Chen, S. Wang, K. Ben Othman, B. Cullen, R. J. Leach, C. O. Hanemann and et al. (1992). "The peripheral myelin protein gene PMP-22 is contained within the Charcot-Marie-Tooth disease type 1A duplication." Nat Genet **1**: 171-5.

Topilko, P., S. Schneider-Maunoury, G. Levi, A. Baron-Van Evercooren, A. B. Chennoufi, T. Seitanidou, C. Babinet and P. Charnay (1994). "Krox-20 controls myelination in the peripheral nervous system." Nat **371**: 796-9.

van de Watering, R. A. C., A. A. W. M. Gabreels-Fasten, H. Kremer, V. Kalscheuer, F. J. M. Gabreels and E. C. Mariman (1999). "Regulation and expression of the murine Pmp22 gene." Mamalian Genome **10**: 419-22.

Verdi, J. M. and A. T. Campagnoni (1990). "Translational regulation by steroids. Identification of a steroid modulatory element in the 5'-untranslated region of the myelin basic protein messenger RNA." J Biol Chem **265**: 20314-20.

Warner, L. E., J. Svaren, J. Milbrandt and J. R. Lupski (1999). "Functional consequences of mutations in the early growth response 2 gene (EGR2) correlate with severity of human myelinopathies." Hum Mol Genet **8**: 1245-51.

Wegner, M. (2000). "Transcriptional control in myelinating glia: flavors and spices." Glia **31**: 1-14.

Welcher, A. A., M. De Leon, U. Suter, G. J. Snipes, S. O. Meakin and E. M. Shooter (1992). "Isolation of transcriptionally regulated sequences associated with neuronal and non-neuronal cell interactions." Prog Brain Res **94**: 163-76.

Welcher, A. A., U. Suter, M. De Leon, G. J. Snipes, and E. M. Shooter (1991). "A myelin protein is encoded by the homologue of a growth arrest-specific gene." Proc Natl Acad Sci, USA **88**:7195-9.

Willy, P. J., Kobayashi, R., Kadonaga JT (2000). "A basal transcription factor that activates or represses transcription." Sci **290**: 982-5.

Yean, D. and J. Gralla (1997). "Transcription initiation rate: A special role for the TATA box." Mol Cel Biol **17**: 3809-16.

Young, P., H. Wiebusch, F. Stogbauer, B. Ringelstein, G. Assmann and H. Funke (1997). "A novel frameshift mutation in PMP22 accounts for hereditary neuropathy with liability to pressure palsies." Neurol **48**: 450-2.

Zoidl, G., S. Blass-Kampmann, D. D'Urso, C. Schmalenbach and H. W. Muller (1995). "Retroviral-mediated gene transfer of the peripheral myelin protein PMP22 in Schwann cells: modulation of cell growth." EMBO J **14**: 1122-8.

Zoidl, G., D. D'Urso, S. Blass-Kampmann, C. Schmalenbach, R. Kuhn and H. W. Muller (1997). "Influence of elevated expression of rat wild-type PMP22 and its mutant PMP22Trembler on cell growth of NIH3T3 fibroblasts." Cell Tissue Res **287**: 459-70.