Progress Report

Moran Foundation Award 2004-2005

Title: Protein degradation in Charcot-Marie-Tooth disease.

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This is a progress report for the Moran Foundation Award for 2004-2005. Our proposal had two main aims. Aim 1 was to determine whether sequestration of calnexin (CNX) by mutant Pmp22Tr-J is a mechanism for the "gain of function" in Pmp22 neuropathies and Aim 2 was to determine whether inhibition of the proteasome is a mechanism for the Tr-J neuropathy. We have made significant progress on both aims in spite of the fact that we encountered unanticipated technical problems with the transgenic mice.

Background. We have been studying the Pmp22Tr-J mutation (Pmp22L16P, which causes a hereditary demyelinating neuropathy both in humans and in mice. We previously demonstrated that Pmp22Tr-J mutation is associated with retention of the mutant protein in the lumen of the ER. In addition, we demonstrated that the mutant protein, unlike wild-type Pmp22, is not incorporated into myelin in vivo. Comparison of the autosomal dominant Pmp22Tr-J neuropathy with heterozygous Pmp22 null mouse indicates that the Pmp22Tr-J mutation is a "gain of function" neuropathy. We hypothesize that the "gain of function" affects ER quality control functions, or post-ER degradation pathways. We previously identified that the association the ER chaperone protein, calnexin (CNX), with Pmp22Tr-J is prolonged relative to the association with wild-type Pmp22, and that, unlike the wild-type Pmp22-EGFP fusion protein, the Pmp22Tr-J-EGFP fusion protein associates with CNX in post-ER autophagic-like structures likely targeted for lysosomal degradation. These findings led to the hypothesis that Pmp22Tr-J might sequester CNX as a basis for the "gain of function".

During this award period, we have further examined the nature of Pmp22/CNX interactions. In pulse-chase experiments, we have found that newly-synthesized Pmp22Tr-J has a markedly prolonged half-life (3-5 hrs) relative to Pmp22wt (45 min) in HEK293 cells. In similar experiments, but using anti-CNX antibodies for immunoprecipitations, we find that Pmp22Tr-J remains associated with CNX for the majority of the time (T1/2 approx. 2hr) before Pmp22Tr-J is degraded. When Pmp22Tr-J and CNX are cotransfected into HEK293 cells, the presence of CNX further increases the half-life of Pmp22Tr-J (decreases the rate of degradation). Thus, not only does CNX overexpression not normalize the rate of degradation of Pmp22Tr-J, it potentiates its deviation from normal. It appears that CNX levels can have profound effects on the degradation of both Pmp22wt and Pmp22Tr-J. We proposed to generate transgenic mice that overexpress CNX in peripheral nerves to see whether increased CNX could ameliorate the Tr-J neuropathy. We generated a number of transgenic founder mice using a well-characterized Schwann cell enhancer (SCE), derived from the myelin basic protein promoter to drive expression of a bicistronic calnexin/EGFP expression cassette. We have analyzed 9 founder mice, but none express significant levels of the transgene as assessed by EGFP expression and by Northern blot. In an extension of previous work funded by the Moran Foundation, we have been studying the function of promoter elements from Myelin Protein zero (*Mpz*) and Pmp22. We have identified regions of the *Mpz* gene that efficiently target high levels of transgene expression into peripheral nerves. We will use this promoter to overexpress CNX in Schwann cells to determine the effects of CNX overexpression in vivo as part of a grant funded by the Muscular Dystrophy Association that was made possible by the Moran Foundation Award. We will also examine whether overexpression of CNX might reduce the efficiency of wild-type Pmp22 biosynthesis, which may provide a treatment target for hereditary neuropathies characterized by duplication of the PMP22 gene and PMP22 overexpression, as in the majority of cases of Charcot-Marie-Tooth disease.

In collaboration with Dr. Rick Sifers, we have tested the effects of other critical ER quality control proteins on the degradation of Pmp22Tr-J and found that overexpression of EDEM decreases the

association of Pmp22Tr-J with CNX, but does not affect the overall half-life of Pmp22Tr-J degradation. We also found that ER Mannosidase I has a small, but significant, effect on the overall rate of degradation of Pmp22Tr-J. Blocking ER ManI activity with kifunensine slightly prolongs the degradation of newly-synthesized Pmp22Tr-J, whereas overexpression of ER ManI slightly increases the rate of degradation of newly-synthesized Pmp22Tr-J. Thus, although ER Man I and EDEM affect the rate of degradation of Pmp22, they do not appear to be rate-limiting.

We also found that inhibition of the proteasome by lactacystin markedly prolongs the rate of degradation of Pmp22Tr-J, whereas lactacystin has no significant effect on the rate of degradation of newly-synthesized Pmp22wt. Blocking lysosomal degradation had no effect on the rate of degradation of either Pmp22wt or Pmp22Tr-J. Thus, mutant Pmp22Tr-J appears to be degraded exclusively via the proteasomal pathway, whereas the mode of degradation of Pmp22wt remains unknown. It has been hypothesized that one mechanism for the "gain of function" in protein conformation diseases caused by misfolding of wild-type or mutant proteins, is inhibition of proteasomal function by abnormal protein degradation by products (e.g. oligomers, toxic intermediates, etc). In order to evaluate the hypothesis that overexpression of mutant Pmp22Tr-J interferes with the function of the proteasome in vivo, we have obtained transgenic mice overexpressing a form of enhanced green fluorescence protein (EGFP) fused to mutant ubiquitin (UbG76V), UbG76V-GFP, as a reporter of proteasomal function in vivo (kindly provided by Dr. N. Dantuma, Karolinska Institut). The UbG76V-GFP, which is expressed from the CAG (CMV promoter/enhancer, chicken actin promoter) is rapidly degraded unless the function of the proteasome is blocked, which leads to the accumulation of fluorescent UbG76V-GFP. When we bred the Pmp22Tr-J mutation onto the UbG76V-GFP mice, we observed no accumulation of EGFP in peripheral nerves. Control experiments, however, established that while Ub-G76VGFP is expressed in almost all tissues in the UbG76V-GFP mice (as expected from the activity of the CAG promoter), we could find not UbG76V-GFP in peripheral nerves even after prolonged treatment with lactacystin to inhibit proteasomal function. In order to verify that the CAG promoter is competent to drive expression to myelinating Schwann cells, we obtained transgenic mice in which the CAG promoter drives EGFP expression. Analysis of the CAG/EGFP mice reveal that the CAG promoter drives reasonable levels of EGFP expression in Schwann cells. Thus, we will have to remake the UbG76V-GFP mice and screen for lines that express the reporter in Schwann cells. To this end, we have obtained the CAG promoter and the UbG76V-GFP construct for the reconstruction of the transgene.

In summary, the funds provided by the Moran Foundation have allowed us to identify and evaluate a number of possible mechanisms for the "gain of function" in the Tr-J neuropathy. We are working on a manuscript describing our work on ER quality control and degradation mechanisms in the Tr-J neuropathy and we have submitted an application to the NIH for further funding for this work. We have also published a manuscript based on previous work supported by the Moran Foundation on the regulation of the PMP22 gene (appended). In addition, the Moran Foundation Award helped us to generate preliminary data for an application that was funded by the Muscular Dystrophy Association on Protein Degradation in Charcot-Marie-Tooth disease. We greatly appreciate this award.

Studies on the role of CNX on Pmp22 degradation and on the role of protein degradation pathways are ongoing. We are in the process of obtaining CNX null mice and cell lines from Dr. John Bergeron at McGill University. We are continuing to evaluate the role of additional cellular molecules in the ER quality control and degradation of Pmp22wt and Pmp22Tr-J. Towards this goal, we have obtained

shRNA for p97 for the production of p97 expressing shRNA lentivirus, and we will be obtaining adenovirus expressing dominant-negative p97 protein from Dr. Wayne Lencer, Harvard Medical School to evaluate the role of p97 in Pmp22 degradation. p97 is a multifunctional ATPase that is involved in retrotranslocation of misfoled proteins from the ER and it apparently has chaperone function that may help to stabilize the highly hydrophobic mutant Pmp22 in the cytoplasm as it is targeted to the proteasome for degradation. In addition, we are working with Dr. Michael Mancini, Baylor College of Medicine to develop a high-throughput screen for molecules (drugs and shRNAs) that affect the degradation of Pmp22Tr-J-EGFP.

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An 8.5-kb Segment of the PMP22 Promoter Responds to Loss of Axon Signals During Wallerian Degeneration, But Does Not Respond to Specific Axonal Signals During Nerve Regeneration

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Altered expression of the *PMP22* gene causes Charcot-Marie-Tooth disease type 1A (CMT1A) and hereditary neuropathy with liability to pressure palsies (HNPP). We have examined the promoter activity of 8.5 kb upstream of the first coding exon of the rat peripheral myelin protein-22 (r*Pmp22*) gene in transgenic mice. We found that the −8.5 kb r*Pmp22*/chloramphenicol acetyl transferase (CAT)/β-galactosidase (*lacZ*) construct directs reporter gene expression in a weakly developmental and tissue-specific pattern, consistent with the expression pattern of the endogenous *Pmp22* gene. The −8.5 kb r*Pmp22*/CAT/*lacZ* transgene responds to loss of axonal signals during Wallerian degeneration but unlike the endogenous *Pmp22* gene, the transgene fails to respond to axonal signals during nerve regeneration after a sciatic nerve crush injury. In conclusion, the function of the −8.5 kb r*Pmp22*/CAT/*lacZ* transgene suggests that there are separable regulatory elements in the r*Pmp22* gene that respond differently to axonal signals received by Schwann cells during nerve development, and during remyelination.

Key words: peripheral nerve; myelin; neuropathy; gene regulation

Mutations affecting the peripheral myelin protein-22 (*PMP22*) gene cause Charcot-Marie-Tooth disease (CMT) and hereditary neuropathy with liability to pressure palsies (HNPP) (reviewed in Saifi et al., 2003). Most cases of CMT and HNPP are due to duplication or deletion, respectively, of a 1.5-Mb region at chromosome 17p11.2–12 that encompasses the entire *PMP22* gene. The high prevalence of these disorders (1/2,500; Skre, 1974) is explained by misalignment during meiosis of repetitive DNA elements (CMT-REPs) that flank the duplicated region, allowing crossover that duplicates or deletes the *PMP22* gene (Pentao et al., 1992). The effects of most *PMP22* mutations are reflected in abnormalities of the myelin sheath in the peripheral nervous system (PNS), which is produced by Schwann cells. Understanding the regulation of the *PMP22* gene has important implications in the design of treatment strategies aimed at normalizing *PMP22* expression.

The *PMP22* gene is strongly upregulated during development and during nerve regeneration as myelin is formed, and is strongly downregulated during Wallerian degeneration (Snipes et al., 1992). This strong dependence on axonal contact for myelin gene regulation underscores the importance of axon–Schwann cell interactions for the activation of signal transduction pathways that lead to myelin formation. Understanding regulation of the *PMP22* gene should also help provide important insights into molecular mechanisms of axon–Schwann cell interactions that are responsible for myelination.

Regulation of the *PMP22* gene is complex, in part because of its tissue-specific regulation by at least two promoters, but also because the positioning of its regulatory elements spans at least 10 kb, and probably further. The *PMP22* gene itself spans approximately 40 kb and consists of six exons (Patel et al., 1992). The first two exons, exons 1a and 1b, are noncoding and are regulated separately by different promoters, designated P1 and P2. The exon 1a-containing mRNA transcript is expressed at high levels specifically in myelin-forming Schwann cells, whereas the exon

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1b-containing transcript is expressed at low levels in a variety of cell types (Suter et al., 1994). A third *PMP22* transcript initiating at the start of exon 2 has also been described (Huehne and Rautenstrauss, 2001). Transient transfection of human *PMP22* gene/reporter constructs extending as far as 6 kb upstream of exon 1a into rat and mouse cell lines indicate that this portion of the *PMP22* gene contains both positive and negative regulatory elements (Saberan-Djoneidi et al., 2000; Hai et al., 2001). Elements sufficient for Schwann cell-specific expression are present in the proximal 300 base pairs (bp) upstream of the transcription start site (Saberan-Djoneidi et al., 2000). Interpretation of these transient transfection studies is hampered by the lack of axon–Schwann cell interactions, and the inability to reproduce the 200-fold upregulation of *PMP22* that characterizes its response to myelination signals (Snipes et al., 1992).

To explore the role of axon–Schwann cell interactions on *PMP22* gene expression, we have analyzed a transgene that extends 8.5 kb upstream of the translation start site (+1) in exon 2 driving a compound reporter construct containing chloramphenicol acetyl transferase (CAT) and the β-galactosidase (*lacZ*) genes. In characterizing the −8.5 kb r*Pmp22*/CAT/*lacZ* mice, we have confirmed a number of observations made by Maier et al. (2002, 2003) on −4 kb *Pmp22*/*lacZ* transgenic mice. Because we are analyzing regions of the *PMP22* gene that have weak promoter activity in vivo, the increased sensitivity afforded by the addition of CAT to our reporter construct has allowed us to extend their studies and identify a region of the r*Pmp22* promoter that is responsive to denervation, but unlike the endogenous *Pmp22* gene, it does not get reactivated during nerve regeneration. The inability of the r*Pmp22*/CAT/*lacZ* 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.

MATERIALS AND METHODS

Production of the −8.5 kb r*Pmp22*/CAT/*lacZ* 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 in exon 2, designated +1) driving a bicistronic reporter cassette containing CAT and *lacZ* genes separated by a 612-bp internal ribosomal entry site (IRES) from polio virus (Meerovitch et al., 1993). 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) kindly provided by Dr. A.A. Welcher (Amgen, Thousand Oaks, CA). The CAT and β-galactosidase open reading frame (ORF) was isolated from pSV2-CAT (Gorman et al., 1982) and pSV-β-Gal (Promega, Madison, WI), respectively. To assure that the CAT ORF was in frame with PMP22, both segments were modified by PCR overlap mutagenesis to create a novel *AgeI* site before ligating them together. The polio IRES (a 612-bp *Hin*dIII fragment of FYK612 plasmid, kindly provided by Dr. N. Sonenberg, McGill University) was subcloned between CAT and *lacZ* to give plasmid P1P2CPG (the transgene is designated −8.5 kb r*Pmp22*/CAT/*lacZ*). Additional details of the subcloning are available upon request.

Production of Transgenic Mice

All transgenic mice were produced under the guidelines of the Canadian Council for Animal Care. The McGill University Animal Care Committee approved all surgical procedures. B6/C3F2 fertilized ova were collected for pronuclear injection. 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 and 5 mM EDTA, pH 7.4). Lines (designated 8, 16A, 16B, and 18) were established from four transgenic founder mice.

PCR Genotyping

DNA was isolated from tail biopsies as described previously (Foran and Peterson, 1992). A 400-ng sample of DNA was used as a template for PCR 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). These primer pairs amplify an approximately 700-bp fragment of the bacterial *lacZ* gene.

Southern Blot Analysis

Genomic DNA was isolated from mouse tails as outlined above. Purified DNA was digested with *Eco*RI enzyme and resolved on a 1% agarose gel and transferred to a positively charged nylon membrane (Nylon Membranes; Roche Diagnostics GmbH, Indianapolis, IN). The blot was hybridized overnight at 42°C in hybridization buffer (0.5% skim milk powder, 4× SSPE [150 mM sodium chloride, 10 mM sodium phosphate, 1 mM EDTA], 50% deionized formamide, 1% SDS, and 0.1% dextran sulfate) with a 470-bp *Sma*I probe derived from the mouse *Pmp22* gene that was labeled with ^{32}P-dCTP (3,000 Ci/mmol) introduced by DNA polymerase extension of random hexamers (Highprime; Bochringer, Indianapolis, IN).

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). CAT activity was assayed using ^{14}C-chloramphenicol (ICN, Irvine, CA) and *n*-butyryl CoA (Promega) at 37°C for 12 hr using the CAT Enzyme Assay System (Promega) according to the manufacturer's instructions. CAT activity was normalized to total protein content as determined by BCA protein assay (Pierce, Rockford, IL).

Detection of β-Galactosidase Activity

Whole Mount. Adult animals were perfused transcardially with 0.5% paraformaldehyde (PFA) and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), and then dissected and postfixed for 1 hr at 4°C, washed with ice-cold 0.1 M phosphate buffer (pH 7.4) twice, and stained overnight at 37°C with 0.4 mg/ml BluoGal (Invitrogen, Carlsbad, CA) as described Forghani et al. (2001).

Tissue Sections. Animals were perfused with fixative as above, dissected, and then postfixed by immersion for 1 hr at

4°C. The tissue was equilibrated with aqueous 30% sucrose overnight at 4°C, and then embedded in Tissue Tek O.C.T. compound (Sakura Finetechnical, Tokyo, Japan) and frozen in isobutanol/dry ice (−50°C). Serial 10–16-μm frozen sections were stained in 0.4 mg/ml X-gal (Invitrogen) as described previously (Forghani et al., 2001). Selected sections were counterstained with nuclear fast red.

Sciatic Nerve Crush Injury

Mice were anesthetized with freshly prepared Avertin (0.066 M 2,2,2-tribromoethanol in 2-methyl-2-butanol, 0.1 ml/20 g body weight, intraperitoneally; Sigma-Aldrich, St. Louis, MO). The right sciatic nerve was exposed at the midthigh level under aseptic conditions. Single focal crush injury was administered by manual pressure applied through #5 jeweler's forceps twice for 15 sec as described previously (Snipes et al., 1987). The skin was closed using metal clips. Focally crushed sciatic nerves and contralateral control nerves were taken at selected time points.

Reverse Transcriptase-PCR

For all reverse transcriptase (RT) experiments, total RNA was isolated from pooled sciatic nerves of 30-day-old transgenic animals using TRIZOL (Invitrogen) according to the manufacturer's instructions.

Detection of Endogenous PMP22 Transcripts. RT-PCR was carried out on 1 μg random hexamer (Roche)-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 exon 2 (Rev) as a reverse primer (using Taq polymerase; Promega) at 94°C for 30 sec, 54°C for 45 sec, and 72°C for 45 sec, 25 cycles.). The PCR primers are as follows: 1a forward (1Afor), 5′-CTC CGA GTC TGG TCT GCT GTG; 1b forward (1Bfor), 5′-ACC CGA GTT TGT GCC TGA GGC; and exon 2 reverse (Exon2Rev), 5′-TGG CTG ACG ATG GTG GAG ACG. The reverse primer (Exon2-Rev) is common to both 1a- and 1b-containing transcripts; 1AFor and Exon2Rev reaction gives a product of 220 bp, whereas 1BFor and Exon2Rev primers amplify a 170-bp product.

Detection of the Transgene Transcripts. Because of the low abundance of transgene mRNA, reverse transcription was primed with a CAT-specific primer (CAT [Rev], 5′-TCC CAT ATC ACC AGC TCA CCG) instead of random hexamers. Semiquantitative PCR was carried out under identical conditions for the detection of endogenous *Pmp22* transcripts except that the CAT (Rev) primer was used in place of Exon2Rev reverse primer. As above, the reverse primer (CAT [Rev]) is common to both 1a- and 1b-containing transcripts, but is specific for the transgene. The PCR product for the transgene is predicted to be 340 and 290 bp using the 1a and 1b forward primers, respectively. PCR products were resolved on 2% agarose gels stained with SYBR Gold (Molecular Probes, Eugene, OR) and quantitated by imaging on a Storm Phosphorimager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software (Molecular Dynamics).

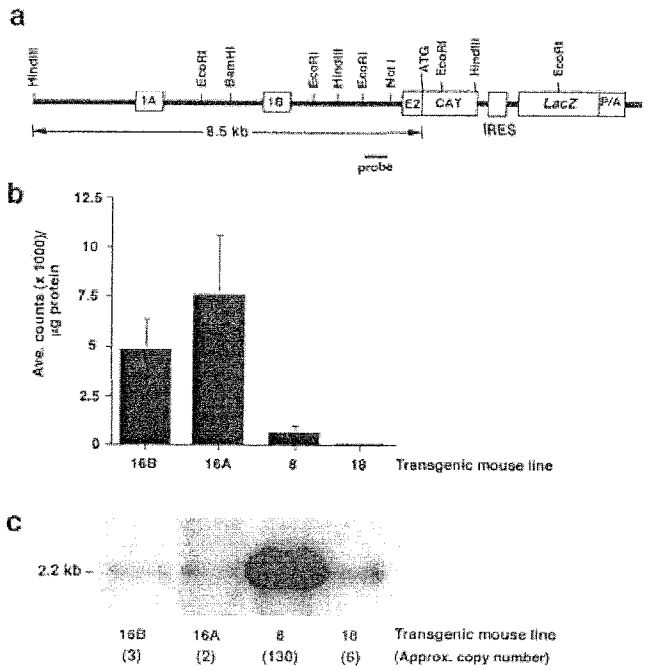

Fig. 1. Construction and expression of the r*Pmp22* reporter transgene. **a:** The r*Pmp22* CAT/*lacZ* construct used for this study consists of 8.5 kb of the rat *Pmp22* promoter relative to the translation start site (−4.5 kb relative to the transcription start site for exon 1a). We created a compound reporter cassette that encodes for a bicistronic mRNA containing CAT, an internal ribosome entry site (IRES) derived from poliovirus, and the bacterial β-galactosidase (*lacZ*) gene with polyadenylation signal. The construct is designed so that the open reading frame for the CAT is in frame with the start codon for PMP22. The bottom of a shows the approximate position of the *Sma*I hybridization probe derived from the *Pmp22* gene that was used for Southern blot analysis (**c**). **b:** The relative expression CAT activity (average counts × 1,000/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 a. Each transgenic line was analyzed in triplicate from independent pools of approximately nine nerves each from 6-week-old mice. Error bars represent standard deviation. c: Southern blot analysis of *Eco*RI-digested genomic DNA from each of the indicated transgenic mouse lines probed with the *Sma*I fragment indicated in a. The relative copy number of the transgene was determined on a Storm Phosphorimager using ImageQuant software and is given in parentheses.

RESULTS

Production and Characterization of Transgenic Mice

As shown schematically in Figure 1a, the design of our 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 the CAT gene to the start codon of PMP22. 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 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* gene derives from our observation that P1 and P2 are coregulated during development and remyelination after nerve injury and therefore their regulation may not be completely independent.

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 precipitation into COS-7 cells. We observed that the level of expression of CAT and β-galactosidase were comparable to the level of expression of these genes after parallel transfections of the parental plasmids, pSV2CAT and pSV-β-galactosidase control vector, (both utilizing the SV40 promoter) under identical conditions (data not shown).

Having validated the reporter cassette, the −8.5 kb r*Pmp22*/CAT/*lacZ* construct (from plasmid 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, three of four 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 8. Line 18 demonstrated the lowest r*Pmp22*-promoted expression of CAT.

Southern blot analysis was carried out on *Eco*RI-digested genomic DNA from lines 16A, 16B, 18, and 8 using a ^{32}P-labeled 421-bp *Sma*I fragment (from murine *Pmp22* gene but homologous to rat *Pmp22* located approximately 200 bp 5′ to the start codon of rat *Pmp22*; see Fig. 1a) as a hybridization probe. The Southern blots from all lines showed the expected size of the hybridization signal for the wild-type (not shown) and transgene *Pmp22* (Fig. 1c). The copy number of the transgene was estimated based on quantitation of the Southern blot by phosphorimaging (Storm Phosphorimager; Molecular Dynamics) using ImageQuant (Molecular Dynamics) software. The 8.5-kb *Pmp22* transgene copy number was estimated as 2 copies for line 16A, 3 copies for line 16B, 130 copies for line 8, and 6 copies for line 18. Clearly, the transgene copy number does not correlate with the relative CAT activity measured in the sciatic nerves from each line of transgenic mice indicating that, like many promoter fragments, the 0 to −8.5-kb fragment of the r*Pmp22* gene does not have locus control activity.

8.5 kb of 5′ Flanking *Pmp22* Promoter Targets Reporter Expression Preferentially to Peripheral Nerve and Schwann Cells

Our previous RNase protection studies demonstrated that the expression from the endogenous r*Pmp22* gene is very high in sciatic nerves, but can be detected at much lower levels in many nonneural tissues (Suter et al., 1994). To test whether the −8.5 kb r*Pmp22*/CAT/*lacZ* transgene also exhibited a similar tissue-restricted pattern of expression, we assayed CAT activity in homogenates from a variety of tissues from line 16B of the r*Pmp22*/CAT/*lacZ* mice, as triplicate pools, and compared to similar samples from control mice (same strain). As shown in Figure 2, maximal levels of CAT activity were detected readily 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 nontransgenic controls. Similar results were obtained from transgenic mouse lines 16A and 18 (not shown). The peripheral nerve sampled in the transgenic mice consisted only of the distal portion of the sciatic nerve, without dorsal root ganglia (DRG; see below) or spinal roots. Because 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 r*Pmp22* promoter contains at least some of the regulatory elements that confer tissue-preferred expression of PMP22 in peripheral nerves.

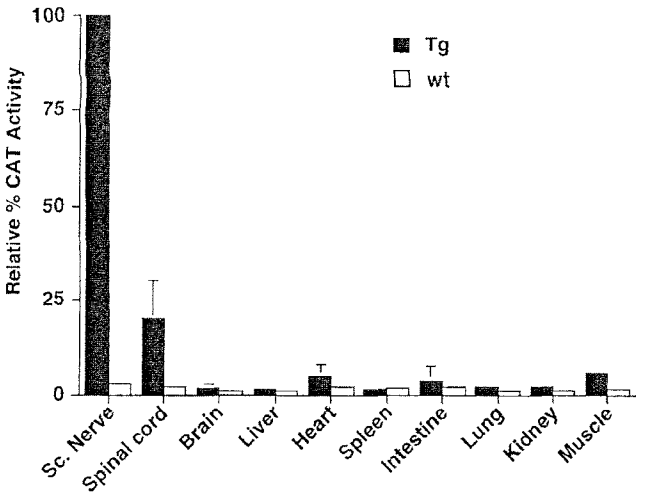

Fig. 2. The 8.5 kb of the *Pmp22* control region targets expression to peripheral nerve (Sc, sciatic). Lysates from representative tissues of transgenic mice (Tg) and control (wt) mice were assayed for CAT activity. The CAT activity/μg of protein in the different tissues is expressed in arbitrary units relative to sciatic nerve (100%). The depicted values represent the averages of three independent pools of tissues from five or six adult mice/pool from transgenic mouse line 16B. Standard deviations are shown as error bars.

We stained transgenic mouse tissue for β-galactosidase expression using BluoGal on whole mounts and observed moderate staining in the DRGs and punctate staining in peripheral nerve (Fig. 3a,b, respectively). Frozen sections of DRGs and sciatic nerve were stained with BluoGal and X-Gal. The BluoGal reaction product was readily apparent in sensory neurons of DRGs of lines 16B (Fig. 3c), 16A, and to a lesser extent in line 18 (not shown), but was barely detectable in peripheral nerves, observable in only a subset of myelinating Schwann cells (see Fig. 3d for line 16B). The

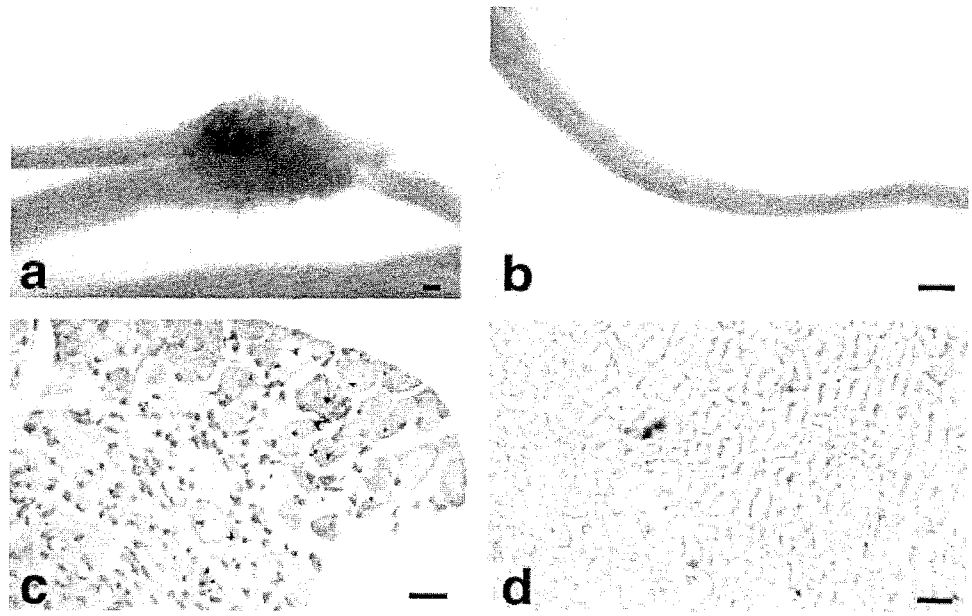

Fig. 3. Whole mount (**a, b**) and histologic (**c, d**) preparations for β-galactosidase activity stained with BluoGal (a–c) and X-gal (d). a: Punctate BluoGal staining in DRG of transgenic mice. Histologic sections of DRGs (c) reveal BluoGal reaction product in the cytoplasm of sensory neurons of DRGs (counterstained with nuclear fast red). Whole mounts of sciatic nerves stained with BluoGal (b) show barely detectable β-galactosidase activity even after staining for 24 hr. Cross sections of the sciatic nerve reveal scattered small deposits of X-gal reaction product within the cytoplasm of Schwann cells closely apposed to myelinated axons. Scale bars = 0.1 mm (a); 0.5 mm (b); 25 μm (c); 10 μm (d).

low level of expression of β-galactosidase driven by the −8.5-kb r*Pmp22* promoter in whole mounts and histologic sections of mouse sciatic nerves is in stark contrast to the robust β-galactosidase expression observed in myelin basic protein (MBP)-promoted *lacZ* transgenic mouse sciatic nerve sections stained in parallel (data not shown). Our control experiments indicated that the bicistronic reporter cassette was functional and did not bias toward expression of one or the other reporter genes. This finding, taken together with those of our previous adenovirus studies in rodent sciatic nerves in vivo that demonstrate that Schwann cells can efficiently use the poliovirus IRES to initiate internal translation of the β-galactosidase reporter gene in a bicistronic expression cassette (Colby et al., 2000), we conclude that 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 r*Pmp22*/CAT/*lacZ* 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.

Expression From the −8.5 kb r*Pmp22*/CAT/*lacZ* Reporter Construct Is Weakly Developmentally Regulated

In rodents, myelination of peripheral nerves occurs over the first 4 postnatal weeks. After birth, during active myelination, Schwann cells upregulate myelin genes including *Pmp22* at high levels. The approximately 200-fold increase in PMP22 mRNA peaks around 14–21 days postnatally (Snipes et al., 1992). To test whether the −8.5 kb r*Pmp22*/CAT/*lacZ* transgene was similarly regulated during postnatal development, three independent pools of sciatic nerves from homozygous line 16B animals were collected at 3, 7, 14, and 21 days of age and analyzed. CAT activity was assayed using ^{14}C-labeled chloramphenicol and normalized to the total protein content of the homogenate. As shown in Figure 4, the level of CAT detected in the sciatic nerve increased twofold 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 twofold upregulation does not compare to the 200-fold upregulation of the endogenous PMP22 mRNA (Snipes et al., 1992).

Because of the numerous animals required for the subsequent analyses, the remainder of the characterization of the −8.5 kb r*Pmp22*/CAT/*lacZ* transgene was carried out on line 16B. This decision was based on a number of considerations. First, line 16A did not breed well. Like transgenic mouse lines 16A and 18, line 16B demonstrated significant CAT expression levels in peripheral nerves, and shared similar increased CAT activity in peripheral nerves compared to that in other tissues. In addition, the relative expression of β-galactosidase in DRG and Schwann cells of peripheral nerves was similar between transgenic mouse lines 16A, 16B, and 18. Finally, up to this point, the expression pattern exhibited by the −8.5 kb r*Pmp22*/CAT/*lacZ* transgene closely resembles that of the −4 kb *Pmp22*/*lacZ* transgene described by Maier et al. (2002, 2003).

The −8.5 kb r*Pmp22*/CAT/*lacZ* Reporter Construct Is Not Reactivated During Nerve Regeneration

The expression of myelin genes including *Pmp22* is highly dependent on axonal contact. This is demonstrated clearly in three situations. First, the marked developmental upregulation of myelin genes in Schwann cells during postnatal development in vivo is not present to anywhere near the same extent in similarly aged Schwann cells grown in vitro (Suter et al., 1994). Second, marked downregulation

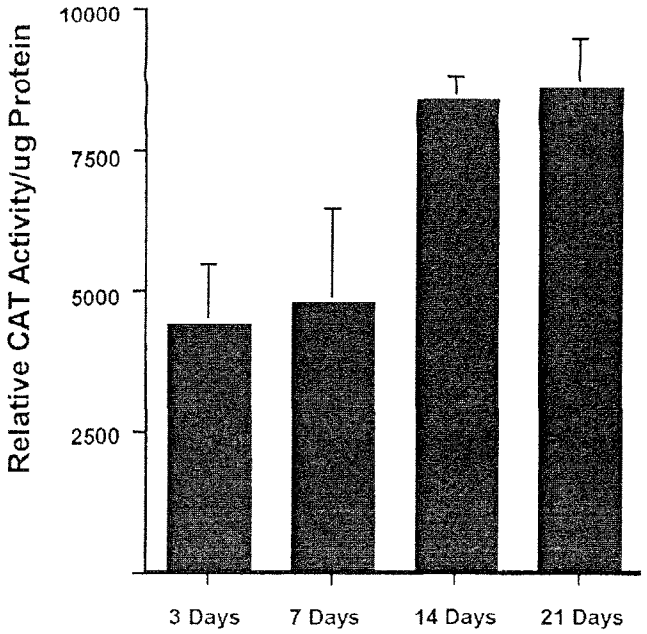

Fig. 4. The −8.5 kb r*Pmp22* promoter construct is weakly regulated during sciatic nerve development. Lysates from sciatic nerves from transgenic mouse line 16B were harvested at the indicated ages and assayed in triplicate for CAT activity expressed as counts/μg of total protein. The experimental value depicted in each column represents the average CAT activity of lysates as determined from three independent pools of sciatic nerves (ranging from 27 mice/pool for 3 days to 9 mice/pool at 21 days). Bars represent standard deviation.

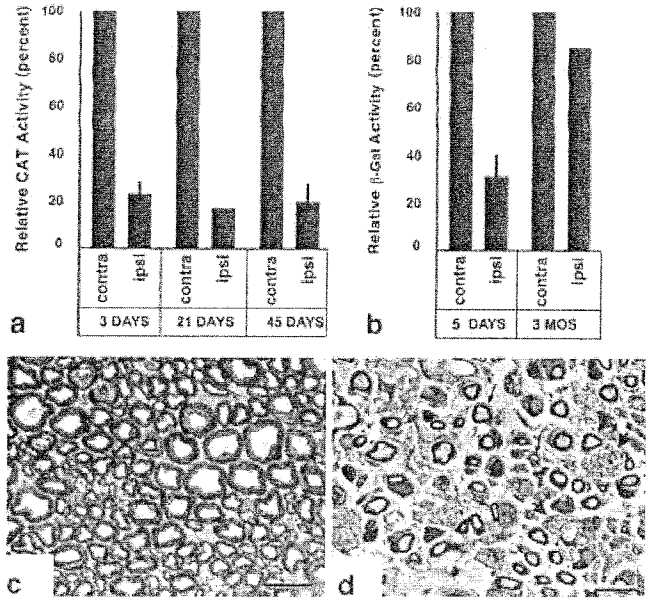

Fig. 5. Expression from the −8.5 kb r*Pmp22* promoter construct is downregulated 3 days after focal crush injury and is not upregulated in distal nerve segment by 45 days post-crush during nerve regeneration. Sciatic nerves from adult −8.5 kb r*Pmp22*/CAT/*lacZ* mice (line 16B) were focally crushed at the midthigh level. At the indicated times after lesion, the portion of the ipsilateral (ipsi) sciatic nerve distal to the crush was harvested and CAT activity was compared to that in the contralateral (contra) uninjured nerves (**a**). Each experimental value represents the average CAT activity of lysates as determined from three independent pools of sciatic nerves (eight nerves/pool). **b:** Data derived from a control −20, +60 kb *Pmp22*/*lacZ* mouse (line BMW 428), which does upregulate *Pmp22*-promoted β-galactosidase expression during nerve regeneration at 3 months after nerve injury as expected for the endogenous *Pmp22* gene. To verify completeness of crush injury, representative 0.5-μm-thick epon sections of the regenerating sciatic nerve from line 16B 21 days after crush is shown in **d**. For comparison, a normal contralateral nerve section is shown in **c**. These sections are stained with paraphenylenediamine and visualized under phase contrast optics. Note the presence of numerous phagocytic cells with foamy cytoplasm (representative phagocytes are indicated by the thick arrowhead) and the significant degree of regeneration evident by the number of thinly myelinated small caliber axons (representative thinly myelinated axons are indicated by the thin arrows). Scale bar = 10 μm.

of myelin genes occurs during Wallerian degeneration, when Schwann cells are deprived of contact with viable axons. Within 72 hr of lesion, both PMP22 mRNA and protein are downregulated sharply distal to a focal crush injury in sciatic nerves (Snipes et al., 1992). Third, if axons are allowed to regenerate, the PMP22 levels are upregulated over the ensuing 14–21 days as the regenerating axons are remyelinated (Snipes et al., 1992). We tested whether the −8.5 kb r*Pmp22*/CAT/*lacZ* transgene expression in Schwann cells exhibited a similar responsiveness to axonal denervation and reinnervation in the nerve crush injury model. We found that 3–4 days after crush injury, the expression of CAT was sharply downregulated in sciatic nerves of −8.5 kb r*Pmp22*/CAT/*lacZ* transgenic mice compared to that in the contralateral uninjured sciatic nerve (Fig. 5a). Unlike the endogenous *Pmp22* gene, however, significant expression from the transgene did not return during regeneration at 21 days post injury. Figure 5a shows that the level of CAT activity in the regenerated nerve up to 45 days after injury was not significantly different from CAT activity in distal sciatic nerves 4 days after injury. That the *Pmp22* gene contains *cis* regulatory elements that can be reactivated after Wallerian degeneration is shown in Figure 5b, which shows upregulation of a *Pmp22*-promoted *lacZ* reporter gene during nerve regeneration in a −20, +80 kb *Pmp22*/*lacZ* mouse (line BMW 428; Orfali et al., unpublished observations). The lack of increased CAT activity in injured sciatic nerves 45 days after crush injury did not reflect the absence of regeneration. As shown in 0.5-μm epon sections depicted in Figure 5c and 5d, there is significant regeneration taking place in the sciatic nerves of the transgenic mice 21 days after crush injury (Fig. 5d), as compared to that in the uninjured normal-appearing contralateral sciatic nerve (Fig. 5c). Histologic sections of the regenerating nerve (Fig. 5d) 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 r*Pmp22*/CAT/*lacZ* transgene is responsive to degeneration secondary to axonal loss, but not to remyelination that occurs during peripheral nerve regeneration. This result also suggests that regulation of myelin gene

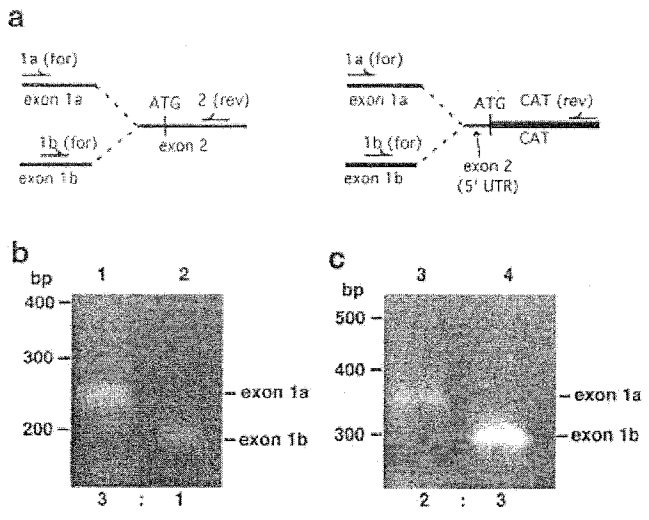

Fig. 6. Adult sciatic nerves from μ8.5 kb r*Pmp22*/CAT/*lacZ* promoter transgenic mice (line 16B) preferentially produce mRNA containing the exon 1b transcript (**c**) as compared to the endogenous PMP22 mRNA (**b**). **a:** RT-PCR design. PCR was carried out on reverse-transcribed RNA derived from the sciatic nerves of wild-type (**b**) or from the −8.5 kb r*Pmp22*/CAT/*lacZ* (line 16B; c) mice. Semiquantitative PCR was carried out using primers specific for the endogenous PMP22 transcript (primer 2 [rev] left, a; representative results in b) or for the CAT-containing transcript (primer CAT [rev] right, a; representative results in c). Note that the forward exon 1a- and exon 1b-specific primers are shared between the endogenous and transgene *Pmp22* sequence, whereas the exon 2 reverse and CAT reverse primers are specific to the endogenous *Pmp22* and transgene cDNAs, respectively. PCR products in b and c were generated with the following primers: lane 1, 1a (for) and 2 (rev); lane 2, 1b (for) and 2 (rev); lane 3, 1a (for) and CAT (rev); and lane 4, 1b (for) and CAT (rev). Numerical values under each lane represent the ratio of total SYBR Gold staining for each PCR product as an average of three separate RT-PCR experiments. SYBR Gold-stained DNA was imaged using a Storm Phosphorimager and quantitated with ImageQuant software.

expression during development differs from induction of myelin gene expression during regeneration and that these two methods of *Pmp22* upregulation (development and regeneration) are controlled through separate mechanisms.

The −8.5 kb r*Pmp22*/CAT/*lacZ* Reporter Construct Preferentially Activates Transcription From Exon 1b in Myelinating Schwann Cells

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 PNS, whereas expression of the exon 1b-containing PMP22 transcript is more widespread, although at much lower levels, in several different tissues (Suter et al., 1994). To test whether the expression pattern delivered by the −8.5 kb r*Pmp22*/CAT/*lacZ* construct recapitulates features of the endogenous PMP22 mRNA expression, we carried out semiquantitative RT-PCR on RNA isolated from sciatic nerves of transgenic animals (line 16B) to determine the relative amounts of the two major nerve transcripts, containing either exon 1a or 1b. As shown in Figure 6, when we used primers specific for endogenous PMP22 transcripts we obtained a 3:1 ratio of 1a:1b transcript, which is comparable to ratios obtained from previous RNase protection studies (Suter et al., 1994). The 1a:1b transcript ratio was 2:3, however, when we amplified the products of the transgene using identical PMP22 exon 1a and 1b transcript-specific forward primers but with a CAT-specific reverse primer (on exon 2). These experiments were carried out in triplicate with similar results. Although these experiments were not designed to quantitate absolute amounts of any specific transcript, it is clear that relative to transcription from the endogenous *Pmp22* gene, 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 r*Pmp22*/CAT/*lacZ* transgene.

DISCUSSION

We have analyzed 8.5 kb of the r*Pmp22* gene for its ability to promote PMP22-like expression of a compound CAT/*lacZ* reporter gene in transgenic mice. We have shown that this −8.5 kb r*Pmp22*/CAT/*lacZ* transgene targets expression to peripheral nerve in a weakly developmentally regulated manner. Furthermore, we have demonstrated that the −8.5 kb r*Pmp22*/CAT/*lacZ* transgene contains *cis* regulatory elements that when expressed in Schwann cells respond to loss of axonal signals, but not to axon regeneration.

Both the −8.5 kb r*Pmp22*/CAT/*lacZ* transgene reported here and the −4 kb *Pmp22*/*lacZ* transgene reported by Maier et al. (2002) target reporter gene expression to peripheral nerves, and both demonstrate a predilection for expression in DRG neurons over Schwann cells. In addition, both transgenes lack elements necessary to drive high levels of reporter gene expression that would be expected for the endogenous *Pmp22* promoter in adult peripheral nerves. As shown by the RT-PCR studies in Figure 6, the relatively low expression of the −8.5 kb r*Pmp22*/CAT/*lacZ* transgene in peripheral nerves correlates with its inability to transcribe mRNA significantly from the myelin-associated r*Pmp22* promoter P1, despite containing 4 kb of genomic DNA upstream of exon 1a. The lack of transcription from PMP22 promoter P1 is also true for the −4 kb *Pmp22*/*lacZ* construct, which by design does not include the PMP22 promoter P1. The preferential expression of the −8.5 kb r*Pmp22*/CAT/*lacZ* construct in peripheral nerve is also in agreement with the studies of Saberan-Djoneidi et al. (2000) demonstrating that *cis* regulatory elements within 300 bp upstream of the transcription start site of PMP22 exon 1a can regulate reporter gene expression when transiently transfected into Schwann cell lines, but not other cell types. The sum of the in vitro studies suggests that a reporter construct with *cis* regulatory elements from −4 to −5.5 kb of the *Pmp22* gene can target expression specifically, or at least preferentially, to Schwann cells. Hai et al. (2001) concluded that their construct containing up to −3.5 kb of the human *Pmp22* gene was not capable of

significantly activating the *Pmp22* promoter P1. In summary, to a first approximation, the −8.5 kb r*Pmp22*/CAT/*lacZ* construct in our mouse line 16B, which contains 4 kb upstream of the first exon of the r*Pmp22* gene (part of promoter P1), although it is intermediate in size between the −4 and the −10 kb *Pmp22*/*lacZ* constructs analyzed by Maier et al (2002), has many properties expected from the −4 kb *Pmp22*/*lacZ* (P2) transgene, which lacks regulatory input from the myelination-associated promoter P1. The −8.5 kb r*Pmp22*/CAT/*lacZ* construct in transgenic mice also behaves, up to a point, like *Pmp22* promoter/reporter constructs transfected into Schwann cells lacking axon-derived signals.

Further analysis of the −8.5 kb r*Pmp22*/CAT/*lacZ* transgenic mice, however, reveals that the r*Pmp22* promoter construct contains regulatory elements that do respond to axon-derived signals. Accordingly, these mice upregulate reporter gene (CAT) expression in peripheral nerve during development and downregulate reporter gene expression in peripheral nerve within the first 3–4 days of Wallerian degeneration distal to the site of focal nerve crush injury, similar to the endogenous *Pmp22* gene. Surprisingly, the same *cis* regulatory elements in the −8.5 kb r*Pmp22*/CAT/*lacZ* transgene that that respond to loss of axonal signals do not respond to reintroduction of the axonal signals by upregulating reporter gene expression during peripheral nerve regeneration. Maier et al. (2003) did not analyze the −4 kb *Pmp22*/*lacZ* transgene for its ability to confer developmental expression or to respond to nerve injury. In addition, transient transfection studies in the absence of neurons are unsuitable for identifying mechanisms requiring axon–Schwann cell interactions. Taken together, our findings indicate the existence of at least two axon–Schwann cell signaling mechanisms: one operative for myelin development or maintenance, and the other for remyelination after nerve regeneration. There is precedence for the concept that nerve development and regeneration may be two separate processes. Using zebrafish transgenesis, Udvadia et al. (2001) showed that 1 kb of the rat GAP-43 proximal promoter contained *cis* regulatory elements required to direct reporter gene expression during neuronal development in the optic nerve, but not during optic nerve regeneration after focal nerve crush injury. Indirect evidence that myelin genes may be regulated by separable mechanisms during development and maintenance is derived from studies of transgenic mice with myelin basic protein (*Mbp*)-promoted β-galactosidase expression. When the *Mbp*/*lacZ* mice were crossed with *Tr-J* mice, which carry a point mutation in the *Pmp22* gene, *Mbp*-promoted β-galactosidase expression was high in oligodendrocytes and Schwann cells during early postnatal development but was specifically extinguished in Schwann cells as animals matured (Farhadi et al., 1999). These studies reinforce the concept that discrete *cis*-acting modules, which may exist many kilobases from one another, are often required for gene expression. Such a modular organization and combinatorial network of *cis* regulatory elements has been particularly well demonstrated for the *Mbp* gene (Farhadi et al., 2003) and are likely to be operational for the *Pmp22* gene.

The fact that the dosage of the r*Pmp22*/CAT/*lacZ* transgene insertion does not correlate with the level of expression of reporter genes indicates that the −8.5 kb r*Pmp22*/CAT/*lacZ* construct lacks locus control activity. This is not surprising because autonomous locus control activity has only been demonstrated thus far for a relatively small number of transgenes (Li et al., 2002). Because activity of this 8.5 kb of proximal r*Pmp22* promoter has not been analyzed previously, and we only analyzed transgenic mouse line 16B in detail, it is possible that the effects that we ascribe to the transgene do not represent autonomous r*Pmp22* promoter activity but are due rather to proximity effects caused by random insertion of the transgene into the mouse genome. In either case, our findings indicate that regulation of the −8.5 kb r*Pmp22* gene fragment in response of axon signals during nerve development, Wallerian degeneration, and nerve regeneration can be uncoupled. Our initial characterization of the −8.5 kb r*Pmp22*/CAT/*lacZ* transgene including the relative tissue distribution of reporter gene activity and the predilection for transgene expression in DRG neurons that is promoted by the −8.5 kb r*Pmp22* fragment strongly supports our interpretation that the findings in line 16B are due to intrinsic activity of the −8.5 kb r*Pmp22* promoter fragment because all features of the initial line 16B characterization were shared by lines 16A and 18 using the identical construct. In addition, this staining pattern has been described independently for the −4 kb *Pmp22*/*lacZ* construct (Maier et al., 2002).

We have shown that the −8.5 kb r*Pmp22*/CAT/*lacZ* transgene is regulated by loss of axonal contact after nerve injury. In tissue culture, activation of cAMP-responsive signaling pathways has been hypothesized to mimic the effects of axonal contact on myelin gene expression and raise the possibility that cAMP-responsive elements in the −8.5 kb r*Pmp22*/CAT/*lacZ* transgene may mediate: (1) developmental regulation of the −8.5 kb r*Pmp22*/CAT/*lacZ* transgene during myelination; (2) the response of the −8.5 kb r*Pmp22*/CAT/*lacZ* transgene to loss of axonal contact; or (3) the lack of response of the −8.5 kb r*Pmp22*/CAT/*lacZ* transgene during nerve regeneration. 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, which may act as transcriptional silencers. Sequence comparisons of the human, rat, and mouse *Pmp22* genes aligned by PIPMAKER and scanned byTRANSFAC (Heinemeyer et al., 1998) or aligned by Vista (Frazer et al., 2004) and scanned by rVista for conserved putative CREB or CRE-BP1 transcription factor binding sites fail to demonstrate evolutionary conservation for the position of these putative CREB and CRE-BP1 binding sites in either the rat or mouse genomes. A highly conserved CREB binding site, however, was identified approximately 100 bp upstream of the CREB binding site identified by Saberan-Djoneidi et al. (2000). We identified only a single putative CRE-BP1 binding site between exon1a and 1b that was conserved among the proximal −8.5 kb of the human, rat, and mouse *Pmp22* genes. It will thus be difficult to test whether the −8.5 kb r*Pmp22*/CAT/*lacZ* construct utilizes these putative cAMP response

elements to transduce axon/glial signals using rat or mouse *Pmp22*-promoted reporter genes in transgenic mice.

The lack of conservation of the CREB and CREBP1 sites raises the overall question of whether the regulatory elements of the r*Pmp22* gene are evolutionarily conserved. By Vista analysis, the overall identity of rat and mouse *Pmp22* genes from −8.5 to 0 kb is 75% with approximately 95% identity between PMP22 ORFs. There are 20 regions of at least 90% identity over 100 bp between rat and mouse *Pmp22* in noncoding putative regulatory regions. In addition, one noncoding region immediately 5′ of exon 1a exhibits 100% identity between the rat and mouse *Pmp22* genes, and 95% identity between human, rat, and mouse *PMP22* genes. For reference, comparing human and mouse genomes overall, 5′ and 3′ untranslated regions of exons are approximately 76% identical, whereas intron sequence is approximately 69% identical between the two species (Waterston et al., 2002). That overexpression of the human *PMP22* gene results in a CMT-like phenotype in transgenic mice (Huxley et al., 1996) and overexpression of mouse *Pmp22* results in a CMT-like phenotype in transgenic rats (Sereda et al., 1996) is further evidence that the major regulatory elements for *PMP22* are conserved among humans and rodents.

In summary, −8.5 kb of the rat *Pmp22* gene, like the endogenous mouse *Pmp22* gene, is able to direct expression to DRG neurons and Schwann cells in adult peripheral nerves in vivo. In addition, like the endogenous *Pmp22* gene, the −8.5 kb r*Pmp22*/CAT/*lacZ* transgene is developmentally regulated and competent to respond to loss of axonal signals by downregulating gene expression during early Wallerian degeneration. Unlike the endogenous *Pmp22* gene, however, the −8.5 kb r*Pmp22*/CAT/*lacZ* transgene confers a low overall level of reporter gene expression, is not strongly developmentally regulated, and is not responsive to axonal signals for remyelination in adult animals. The RT-PCR studies indicate that the −8.5 kb r*Pmp22*/CAT/*lacZ* transgene lacks the necessary elements present in more distal portions (−6 to −10.5 kb) of the *Pmp22* gene that are required to activate significant expression from the myelin-associated P1 promoter in response to axonal contact, the major determinant of PMP22 expression in peripheral nerve. Nonetheless, our studies extend previous work on the regulation of the *Pmp22* gene by showing that there is a proximal region (0 to −8.5 kb) in the r*Pmp22* gene that responds to axonal signals, and that the axon-responsive molecular mechanisms that regulate myelin gene expression during development, Wallerian degeneration, and remyelination are not identical.

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