G. Jackson Snipes, M.D., Ph.D.

Progress Report for Moran Award 2003-2004.

Summary of Aims: Briefly, we proposed to investigate the regulation of the two major myelin structural genes in the peripheral nervous system, *Mpz* and *Pmp22*. A number of studies have investigated the regulation of the proximal *Pmp22* and *Mpz* promoter elements by transient transfection, but it is clear that additional regulatory elements are required for high levels of MPZ and PMP22 expression. Thus, our first two aims are:

Aim 1. Identify important regulatory elements within the *Pmp22* gene.

Aim 2. Identify important regulatory elements within the *Mpz*/P0 gene.

We proposed to map regulatory elements using a series of deletion mutants of the *Pmp22* and *Mpz* genes, respectively, driving the expression of the *lacZ* reporter gene in transgenic mice.

Mutations in the transcription factor gene, *SOX10,* causes CMT. *In vitro* evidence suggests that SOX10 directly regulates the expression of *Mpz*/P0 and that disease-causing mutations in SOX10 interfere with the ability of SOX10 to transactivate the *Mpz* gene.

Aim 3. Determine the significance of *SOX10* mutations on myelin gene expression *in vivo*.

We proposed to determine the contribution of *SOX10* to the transcription of *Mpz*/P0 by disrupting putative *SOX10* binding sites in the *Mpz/P0* proximal promoter by expressing wild-type and mutated *Mpz*/P0/*lacZ* constructs in transgenic mice. We will also replace the proximal *Mpz* promoter containing the putative SOX10 binding sites (from 0 to –200 bp) with a minimal heat shock promoter (HSP) to determine if these SOX10 binding sites are necessary for the function of the remainder of the *Mpz* locus control region.

Summary of Progress

-8 kb proximal to the *Pmp22* translation start site inefficiently directs reporter expression to peripheral nerves in transgenic mice. Initially, we cloned 8 kb of rat *PMP22* gene upstream of the translation start site in exon 2 and fused it to a bicistronic reporter cassette containing both chloramphenicol acetyl transferase (CAT) and the bacterial β-galactosidase (*lacZ*) genes. When expressed in multiple lines of transgenic mice, -8 kb of the rat *PMP22*/CAT/*lacZ* transgene targeted expression to peripheral nerves (by CAT assay), with expression in scattered Schwann cells (by β-galactosidase expression), but also in dorsal root ganglion (DRG) neurons. We have found that the expression of the –8 kb *PMP22*/CAT/*lacZ* transgene was weakly developmentally regulated and was down-regulated in Schwann cells by loss of axonal contact, but, unlike the endogenous *Pmp22* gene, it was not re-induced during nerve regeneration. Semi-quantitative RT-PCR revealed that expression from the –8 kb *PMP22*/CAT/*lacZ* construct resulted from activation of promoter P2, but not the myelination-associated promoter, P1. We conclude that the proximal 5' 8 kb of the *Pmp22* gene directs expression to peripheral nerves, but lacks *cis*-regulatory elements required to recapitulate myelin-like expression. In addition, these studies indicate that there are separable *cis*-regulatory elements in the *Pmp22* gene that respond independently to loss and gain of axon-derived signals. We are preparing a manuscript describing these findings.

Analysis of the *Pmp22* gene by interspecies sequence comparison.

Interspecies sequence comparisons identify regions of significant homology within noncoding regions of genes. We performed interspecies sequence comparisons for *PMP22* using PipMaker, (1) and Vista (2) and found 10 regions (designated A-J) of moderate homology (75% homology over 100 bp) of which 3 have relatively high homology (>86% homology over 100 bp) across all three species in noncoding, non-repetitive regions of the *Pmp22* gene (fig. C2). These regions of homology span over 45 kb, from 18 kb upstream of the first coding exon (exon 2) to 3 kb downstream of penultimate exon, exon 5. Previous studies by Maier et al (2002) revealed an upstream "late myelination specific element (LMSE)" from –10/-6.6k kb of the *Pmp22* gene (3). Conserved regions C and D that we identified by interspecies *Pmp22* sequence comparisons would be a candidate for the LMSE activity. Taken together with our finding of upstream *Pmp22* promoter elements responsive to axon loss, but not axon regeneration, these results suggested that the *Pmp22* regulatory region is composed of discrete autonomous cis-regulatory elements. To summarize, the overall expression pattern of the *Pmp22* cannot be wholly explained by the activity of the –10/0 region, indicating the existence of further upstream or downstream elements in the regulation of the *Pmp22* gene. Interspecies sequence comparisons suggest candidate regions containing *Pmp22* regulatory elements downstream of the first coding exon.

A 100 kb *Pmp22/lacZ* transgene, but not a –21 kb *Pmp22/lacZ* transgene, efficiently directs expression to myelinating Schwann cells. We subcloned the *lacZ* reporter gene in frame with the start codon of *Pmp22* by homologous recombination in E. coli to generate a 100 kb *Pmp22/lacZ* transgene containing –20 kb upstream of the start codon and all 3' introns and exons (fig. C3). We also prepared a transgene containing only –21 kb of *Pmp22* driving *lacZ* (-21 kb *Pmp22/lacZ*). The 100 kb *Pmp22/lacZ* transgene promoted high levels of reporter gene expression in myelinating Schwann cells in peripheral nerve (4/6 lines) derived from several rounds of pronuclear injections. The -21 kb *Pmp22/lacZ* transgene, on the other hand, exhibited elevated expression in myelinating Schwann cells in only 1/6 lines (line BM10). The other 5/6 lines exhibited β-galactosidase expression in peripheral nerves, but mainly in dorsal root ganglion neurons as we observed for the –8 kb rat *PMP22/lacZ* transgene. The 100 kb *Pmp22/lacZ* transgene in transgenic mice shares many features with the regulation of the endogenous *Pmp22* gene: it is strongly developmentally regulated; it is expressed strongly in myelinating Schwann cells; it is appropriately down-regulated by loss of axonal contact during Wallerian degeneration, and it is strongly re-expressed during the remyelination that accompanies nerve regeneration. In addition, transcription of *Pmp22* exon 1a is activated in the nerves of mice carrying the 100 kb *Pmp22/lacZ* transgene. Thus, the 100 kb *Pmp22/lacZ* transgene appears to contain most, if not all, of the *cis*-regulatory elements required for *Pmp22*-like expression.

Targeted single-site insertion of transgenes at a defined "docking site" (the *Hprt* locus) is a promising approach to *in vivo* promoter analysis. One of the major limitations of trangenesis by pronuclear injection is that the transgene integrates into different chromosomal sites and with different copy numbers in each transgenic line. In order to minimize insertional effects, we adopted a targeted transgene insertion approach pioneered by Bronson et al (4). In this system, transgenes are targeted by homologous recombination to a partially deleted hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) locus on the X chromosome in male ES cells. Thus, a single copy of the transgene is targeted to a single chromosomal site (the *Hprt* locus). Apparently, the regulatory region of the *Hprt* gene permits but does not direct heterologous gene

expression. Indeed, several investigators have adapted this system for the study of gene regulation (4-6). Thus, we targeted –21 kb *Pmp22/lacZ* and –11 kb *Pmp22/lacZ* to the *Hprt* locus in male ES cells and analyzed transgenic mice derived from them following blastocyst injection. We found that both the –21 kb *Pmp22/lacZ/Hprt* and the –11 kb *Pmp22/lacZ/Hprt* mice expressed β-galactosidase in a pattern indistinguishable from the other 5/6 lines of –21 kb *Pmp22/lacZ* mice, all of which failed to express high levels of β-galactosidase in peripheral nerves. Our interim conclusion was that –21 kb and –11 kb of the *Pmp22* gene does not effectively target transgene expression to myelinating Schwann cells whereas –21 kb plus downstream (3') elements does (e.g. the 100 kb *Pmp22/lacZ* construct).

Our results with the –21 kb and the –11 kb *Pmp22/lacZ* transgenes differ substantially from Dr. Suter's –10 kb *Pmp22/lacZ* transgene in that we got two different expression patterns with the –21 kb transgene: 1/6 resembled Dr. Suter's –10 kb *Pmp22/lacZ* and the other 5/6 plus the –21 kb *Pmp22/lacZ/Hprt* resembling the –8 kb *Pmp22*/CAT/*lacZ* mice. We are collaborating with Dr. Suter to determine whether these differences are significant, or whether they reflect a negative regulatory element between –10 and –11 kb of the *Pmp22* gene. In addition, by sequence comparison, Dr. Suter's construct is deleted for 500 bp between –10 and –9 kb relative to our –21 and -11 kb constructs and the murine genome database (NCBI) which may also explain the differences between our results, Dr. Suter has provided us with his –10 kb *Pmp22/lacZ* transgene for recombination into the *Hprt* locus. We have successfully recombined the –10 kb Pmp22/lacZ transgene into the Hprt locus in ES cells and have begun to create mice carrying the targeted transgene in collaboration with Dr. M. Matzuk (BCM, Pathology).

<u>Hypothesis: Downstream (3') elements are important for the regulation of the *Pmp22* gene</u>
We hypothesize that *Pmp22*, like many other genes, has a locus control region (LCR) that is required for activation. By definition, the presence of an LCR in a transgene confers dosage dependent expression of the (trans)gene independent of the site of integration (7). The 5' portion of the *Pmp22* gene spanning up to –21 kb from the translation start site does not contain an LCR since only 1/6 of our –21 kb *Pmp22/lacZ* transgenes and 3/23 of Suter's –10 kb *Pmp22/lacZ* transgenes showed significant levels of expression in myelinating Schwann cells. Based on our results with the 100 kb *Pmp22/lacZ* BAC, we suspect that our 100 kb *Pmp22/lacZ* transgene does contain the LCR, which would place it 3' to exon 2. Compared to our –21 kb *Pmp22/lacZ* mice, and Dr. Suter's –10 kb *Pmp22/lacZ* mice, the 100 kb *Pmp22/lacZ* mouse demonstrates relatively higher levels of expression in a larger percentage of transgenic mouse lines; and, the 100 kb *Pmp22/lacZ* mouse lines upregulate reporter gene expression at an earlier time point (3 days postnatal vs. 10 days postnatal) compared to the –10 kb *Pmp22/lacZ* mouse. Taken together these results suggest that there are *cis*-regulatory elements in the 3' portion of the *Pmp22* gene that are responsible for early postnatal expression (like the endogenous *Pmp22* gene) and may contribute to the overall expression levels of *Pmp22*. If confirmed, the functional organization of the *Pmp22* gene would resemble that of the *Mpz*/P0 gene (see below) in which both 5' and 3' *cis*-regulatory elements are required to recapitulate the expression pattern of the endogenous *Mpz*/P0 gene. We have created deletion constructs derived from the 100 kb *Pmp22/lacZ* transgene. We are currently performing pronuclear injections with a –14, +14 kb *Pmp22/lacZ* construct to test the effects of proximal 3' regulatory elements on the expression of the *Pmp22* gene.

<u>*Mpz*/P0 is an excellent model for the study of myelin gene regulation</u>.

We have demonstrated that the two major transmembrane components of peripheral nerve myelin, *PMP22* and *MPZ*/P0 are temporally and spatially co-expressed in myelinating Schwann cells suggesting that they may share common regulatory features. Thus, either, and optimally both, could potentially be used to study myelin gene regulation and interrogate axon-glial interactions. Our intergene (*Pmp22* vs. *Mpz*/P0) sequence analyses, however, so far have failed to identify regions of significant homology between these two genes. By comparison, the *Mpz*/P0 gene is relatively small (the coding region spans 6 kb); it is only expressed in Schwann cells; it encodes for a single mRNA species; and, appropriate *Mpz*/P0 /*lacZ* constructs, which require both 3' and 5' elements relative to the first coding exon, can efficiently model endogenous *Mpz*/P0 gene regulation (8). In order to circumvent some of the difficulties associated with the study of the *Pmp22*, we have initiated a parallel approach to the study of myelin gene expression exploiting the relative simplicity of the *Mpz*/P0 gene.

13 kb of the *Mpz*/P0 gene contains the regulatory elements necessary for *Mpz*-like expression.
Similar to our approach for *Pmp22*, we have isolated bacterial artificial chromosomes containing the murine *Mpz*/P0 gene and contiguous sequences known to be required for efficient *Mpz*/P0 expression. We then engineered an *Mpz*/P0/*lacZ* gene/reporter construct by cloning *lacZ* (with a transcription termination/polyadenylation signal) in frame with the start codon for *Mpz*/P0 thereby creating a +7, -6 kb *Mpz*/P0/*lacZ* transgene. We then generated transgenic mice from this transgene using standard pronuclear injection. Our preliminary results indicate that a –7, +6 kb *Mpz*/P0/*lacZ* construct (3/3 lines) strongly targets expression of β-galactosidase to myelinating Schwann cells in transgenic mice. The β-galactosidase expression promoted by the –7, +6 kb *Mpz*/P0/*lacZ* construct appears to be appropriately upregulated during development, down-regulated following focal nerve injury, and upregulated during the remyelination associated with nerve regeneration. We have extended this analysis and found the following results for the indicated constructs: a) -5, +3 kb *Mpz/lacZ*-- (2/3 lines) strongly targets expression of β-galactosidase to myelinating Schwann cells in transgenic mice; b) –5, +1 *Mpz/lacZ*—0/7 lines strongly target expression of β-galactosidase to myelinating Schwann cells in transgenic mice, however, on extended analysis, 3/7 of these lines expressed β-galactosidase very weakly in myelinating Schwann cells; c) –2, +3 kb Mpz/lacZ—1/6 lines weakly express b-galactosidase in peripheral nerves. From this analysis we conclude that the proximal Mpz/lacZ promoter (-2 kb) contains elements that can target expression to myelinating Schwann cells. In addition, we anticipate that 3' elements (+1, +3 kb) have major enhancer function.

Interspecies sequence comparisons for the *Mpz*/P0 gene.
We performed interspecies sequence comparisons using PipMaker, (1) and Vista (2) gene and found 5 regions (designated A-E) of intermediate homology (approximately 75% homology over 100 bp) of which 3 have relatively high homology (>75% identity over 100 bp) across all three species in noncoding, non-repetitive elements of the *Mpz/P0* gene spanning over 8 kb from 2 kb upstream of the first coding exon (exon 1) to 1 kb downstream of penultimate exon, exon 6 (see fig. C5). Interestingly, the noncoding region from +1 to +3 kb contains significant sequence homology (> 86% identity over 100 bases) that is shared among the human, rat, and mouse *Mpz*/P0 genes.

Putative SOX10 binding sites have been identified in conserved noncoding region B in the *Mpz*/P0 gene, immediately adjacent to the proximal promoter region upstream of exon

1(proximal 600 bp 5' to exon 1). Although mutations in SOX10 cause CMT, and two SOX10 binding sites have been identified in the *Mpz*/P0 promoter in vitro, the significance of SOX10 binding in the context of *Mpz*/P0 expression in myelinating Schwann cells is unknown. Though functionally active, the proximal 1-2 kb of the *Mpz*/P0 gene (containing both SOX10 binding sites) is inefficient as a promoter for driving transgene expression to myelinating Schwann cells in transgenic mice. We have replaced the proximal promoter of *Mpz/lacZ* with a minimal hsp68 promoter and 3 kb downstream (0, +3 kb). Though still under investigation, the 0, +3 kb hsp68 *Mpz/lacZ* transgene does not appear to direct expression to myelinating Schwann cells. Thus, the 0, +3 kb region does not function autonomously as an enhancer, but may interact with endogenous elements, such as the SOX10 binding sites, in the proximal promoter. To address the function of the SOX10 binding sites, we have mutated, separately and together, the two putative SOX10 in the proximal *Mpz* promoter and are preparing to analyze their activity as a single copy insertion in a defined site, the *Hprt* locus (see below).

Mpz/P0 in *Hprt*

We have successfully targeted the –7, +6 *Mpz*/P0/*lacZ* construct to the *Hprt* locus in ES cells and derived mouse lines designated –7, +6 *Mpz*/P0/*lacZ*/*Hprt* (in collaboration with Dr. Martin Matzuk, Baylor College of Medicine) that express high levels of β-galactosidase in the peripheral nerves). Thus, the *Hprt* locus is permissive for *Mpz*/P0/*lacZ* expression. This should prove to be an invaluable model for the analysis of single-copy *Mpz*/P0/*lacZ* transgenes at a defined locus. During the past year, we have been characterizing these mice along with the other *Mpz/lacZ* mice and we are preparing a manuscript describing our findings.

Statement of completion

The work described in this progress report is ongoing and provides the basis for an NIH grant application that has been submitted. We envision three manuscripts from the results described above.

References cited.

1. Schwartz, S., Z. Zhang, K.A. Frazer, A. Smit, C. Riemer, J. Bouck, R. Gibbs, R. Hardison, and W. Miller. 2000. PipMaker--a web server for aligning two genomic DNA sequences. *Genome Res* 10:577-586.
2. Mayor, C., M. Brudno, J.R. Schwartz, A. Poliakov, E.M. Rubin, K.A. Frazer, L.S. Pachter, and I. Dubchak. 2000. VISTA : visualizing global DNA sequence alignments of arbitrary length. *Bioinformatics* 16:1046-1047.
3. 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.
4. Bronson, S.K., E.G. Plaehn, K.D. Kluckman, J.R. Hagaman, N. Maeda, and O. Smithies. 1996. Single-copy transgenic mice with chosen-site integration. *Proc Natl Acad Sci U S A* 93:9067-9072.

5. Vivian, J.L., W.H. Klein, and P. Hasty. 1999. Temporal, spatial and tissue-specific expression of a myogenin-lacZ transgene targeted to the Hprt locus in mice. *Biotechniques* 27:154-162.
6. Evans, V., A. Hatzopoulos, W.C. Aird, H.B. Rayburn, R.D. Rosenberg, and J.A. Kuivenhoven. 2000. Targeting the Hprt locus in mice reveals differential regulation of Tie2 gene expression in the endothelium. *Physiol Genomics* 2:67-75.
7. Li, Q., K.R. Peterson, X. Fang, and G. Stamatoyannopoulos. 2002. Locus control regions. *Blood* 100:3077-3086.
8. 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* 11:1577-1586.