EVOLUTIONARILY CONSERVED NON-CODING SEQUENCES CONFER TRANSCRIPTIONAL REGULATION TO THE *MYELIN BASIC PROTEIN* GENE

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September 2005

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Your file Votre référence
ISBN: 978-0-494-25141-6
Our file Notre référence
ISBN: 978-0-494-25141-6

ACKNOWLEDGEMENTS

First, I would like to express my sincere gratitude to my thesis supervisor, Dr. Alan Peterson, for providing continued guidance and support throughout the course of my graduate studies. I have learned a great deal from him and, among other influences, my way of thinking about designing experiments and interpreting data has greatly developed as a result of his mentoring. Also, Dr. Peterson has shown great patience and flexibility in accommodating my accompanying clinical pursuits. For all of these together, I thank him very much.

There are several laboratory colleagues to whom I owe special thanks for sharing their expertise and providing assistance during my investigations. In particular, special thanks are due to Hana Friedman, Priscila Valera, Irene Tretjakoff, Heidi Furtenbacher, France Bourdeau, Tess Fernandez, Christine Dy, Melissa Beaudoin, and Margaret David. In particular, Mrs. Valera undertook the dissection of the whole mount specimens and Mrs. Tretjakoff has performed the bulk of pronuclear injections, embryonic stem cell injections, and blastocyst transplants.

On a personal level, I am indebted to my family members for their support and, in particular, to my father who has been the single greatest influence in my life. He has raised me to be curious about our surrounding world and has been a pillar of support throughout my life and studies. Also, Dr. Reza Forghani has been a longtime close friend and colleague throughout medical and graduate studies. We have been through a lot during our academic pursuits and I thank him for his friendship.

Finally, financial support for this work was provided by the Canadian Institutes of Health Research, the Multiple Sclerosis Society and the Multiple Sclerosis Foundation of Canada. Fellowship support was provided by a Canadian Institutes of Health Research Doctoral Award.

ABSTRACT

Myelin formation is an evolutionarily late acquisition that contributes to fast conduction in vertebrate axons. Continuous bidirectional signaling is required for the acquisition and maintenance of the mature nerve fiber phenotype. Myelin Basic Protein (MBP) is a representative structural component of compact myelin that is co-ordinately regulated with other myelin protein genes, primarily at the transcriptional level. In an effort to define the regulatory network controlling MBP expression, I located and functionally characterized conserved regulatory sequences in 5' non-coding sequence. First, I identified four widely-spaced segments of preferential conservation in orthologous human-mouse genomic sequences (termed modules 1-4) that range from 0.1 to 0.4 kb. In order to compare qualitative and quantitative regulatory programs mediated by modular and inter-modular sequences, a novel *in vivo* transgenesis system was adopted and validated. Targeted insertions of single-copy reporter constructs at a predetermined location within the *hprt* locus allowed for direct inter-construct regulatory program comparisons. The proximal modules M1 and M2 confer relatively low-level expression in oligodendrocytes, primarily during early postnatal development. The upstream M3 module confers high-level oligodendrocyte expression extending throughout maturity. Furthermore, constructs devoid of M3 fail to target expression to newly myelinating oligodendrocytes in the mature CNS. High-level and continuous expression is conferred to myelinating or remyelinating Schwann cells by M4. M3 also confers expression to Schwann cells but only transiently during active myelin elaboration and only when isolated from surrounding MBP sequences. These observations define the

regulatory roles played by a complex network of conserved non-coding MBP sequences and lead to a combinatorial model in which specific permutations of regulatory sequences are engaged differentially in various glial cell states. This experimental system also was shown to provide sufficient quantitative resolution to reveal the regulatory functions contributed by particular modular sub-domains and individual enhancer elements. M4 regulatory activity requires simultaneous contributions from elements located in both a core targeting as well as surrounding enhancing sub-domains with Sox10 and Krox-20 M4 binding appearing to mediate targeting and enhancer function, respectively. From this investigation, the complex network of elements and transcription factors that control expression of one important myelin gene has begun to emerge. This knowledge should lead to a deeper understanding of the regulatory mechanisms controlling the overall myelination program and, ultimately, to novel therapeutic strategies effective in ameliorating the consequences of inherited or acquired demyelinating disease.

RÉSUMÉ

La myéline est une acquisition récente dans l'évolution qui contribue à la conduction rapide de l'influx nerveux dans les axones des vertébrés. Une communication continue entre les neurones et les cellules gliales est nécessaire pour la formation et le maintien du phénotype de la fibre nerveuse mature. La Protéine Basique de la Myéline (MBP) est un composant structurel de la myéline compactée qui est principalement regulé, avec d'autre gènes de protéines de la myéline, au niveau transcriptionnel. Dans le but de définir le réseau de régulation controlant l'expression de la MBP, j'ai localisé et charactérisé la fonction de séquences régulatrices conservées durant l'évolution présentent dans la région 5' flanquante du gène. Dans un premier temps, j'ai identifié quatre segments (dénommés module 1-4) largement espacés d'une longueur variant entre 0,1 et 0,4 kb et présentant une conservation préférentielle dans les séquences orthologues humaine et murine. Afin de comparer de manière qualitative et quantitative les programmes de régulation médiés par les modules et les séquences inter-modulaires, une nouvelle technique de transgénèse a été utilisée et validée. L'insertion ciblée des constructions dans le locus *hprt* à permis la comparaison directe des programmes de régulation. Les modules proximaux M1 et M2 confèrent une expression relativement faible dans les oligodendrocytes durant le développement postnatal précoce. Le module M3 situé plus en amont confères un haut niveau d'expression dans les oligodendrocytes tout au long du développement. De plus, les constructions ne contenant pas M3 ne s'expriment pas dans les oligodendrocytes formant de la myèline dans le système nerveux mature. Une expression importante et continue dans les cellules de Schwann au cours de la myélination ou de la remyélination est donnée par M4. Une expression transitoire est

dirigée dans les cellules de Schwann durant la myélination par M3, dans ce cas uniquement lorsque ce module est isolé des séquences flanquantes du gène de la MBP. Ces observations définissent les rôles régulateurs joué par un ensemble de séquences non-codantes conservées du gène de la MBP menant à un modèle combinatoire dans lequel des combinaisons spécifiques de séquences régulatrices sont engagées dans les différents types cellulaires et aux différents stades de differentiation. Ce systême experimental a une résolution quantitative suffisante pour révéler les fonctions régulatrices apportées par des sous-domaines et des éléments individuels des enhancer. La régulation donnée par M4 requière les contributions simultanées d'éléments localisés au coeur de l'enhancer ainsi que d'éléments dans les séquences flanquantes. La liaison des facteurs de transcription Sox10 et Krox-20 à M4 médie, respectivement, le ciblage et l'amplification de l'expression. De cette étude, le réseau complexes des éléments et des facteurs de transcription controlant l'expression d'un gène important de la myéline apparaît. Cette connaissance devrait permettre une compréhension plus profonde des méchanismes contrôlant la myélination et la découverte de nouvelles stratégies thérapeutique visant à améliorer les conséquences des maladies de la myéline.

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3. A 0.3 kb HSP68 minimal promoter inserted at the *hprt* locus drives high level expression only in endothelial cells, with no detectable expression in either oligodendrocytes or Schwann cells
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14. When isolated from surrounding MBP sequences, module 3 confers comparatively low-level, but sustained, expression to myelinating primary Schwann cells; transient expression is only conferred to remyelinating Schwann cells co-incident with peak MBP re-expression following injury
15. A 1.0 kb human module 4 sequence (from -15.5 to -14.5 kb) targets high level and continuous transgene expression to myelinating Schwann cells

16. Following injury, module 4 targets high level expression to remyelinating adult Schwann cells coincident with the onset of MBP re-expression and remyelination
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3. Preparation of wild type and *Trembler-J* sciatic nerves for *in vivo* footprinting analysis
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Chapter 4

1. *Tr*^{J}/+ mice bearing the -8.9 kb-LacZ transgene gradually shut off reporter expression during post-natal development to undetectable levels

CONTRIBUTIONS OF AUTHORS

All the investigations described in this thesis were performed in the laboratory of Dr AC Peterson, my Ph.D. supervisor, and therefore he is senior author on all the manuscripts (chapters 1 to 4). The roles played by additional authors on each manuscript are discussed below. These discussions include a brief overview of my own role for maximal clarity.

Chapter 1

THE MYELIN BASIC PROTEIN GENE: A PROTOTYPE FOR COMBINATORIAL MAMMALIAN TRANSCRIPTIONAL REGULATION

Hooman F. Farhadi, Alan C. Peterson

I wrote this chapter to be included in an upcoming *Advances in Neurology* volume titled *Multiple Sclerosis and Demyelinating Diseases.*

Chapter 2

A COMBINATORIAL NETWORK OF EVOLUTIONARILY CONSERVED *MYELIN BASIC PROTEIN* REGULATORY SEQUENCES CONFERS DISTINCT GLIAL-SPECIFIC PHENOTYPES

Hooman F. Farhadi, Pierre Lepage, Reza Forghani, Hana C.H. Friedman, Wayel Orfali, Luc Jasmin, Webb Miller, Thomas J. Hudson, Alan C. Peterson

Journal of Neuroscience, 2003, 23(32):10214-23

HF Farhadi: I provided the mouse -5.8 to -3.1 kb MBP non-coding sequence to Dr P. Lepage to undertake the sequencing reactions (along with the rest of the mouse and human clones which were already available). I undertook comparative human-mouse sequencing using a number of software programs including MacVector; ultimately, I constructed a percentage identity plot using the web-based PIPMaker Program.

I designed and constructed all of the *hprt*-docked constructs detailed in Figure 1. I designed all of the experiments analyzing transgene expression in the various transgenic lines. I performed all of the demyelination and peripheral nerve injury experiments and undertook the electron microscopic analysis.

I designed and undertook the experiments quantitating *in vivo* transgene expression levels. Finally, I was responsible for the packaging of experiments and preparation (text and figures) of this manuscript leading to its publication.

P Lepage and TJ Hudson: Dr Lepage is a post-doctoral fellow in the Hudson laboratory. Under the supervision of Dr Hudson, he performed the isolation of the human clones and supervised the sequencing of the orthologous human and mouse MBP 5' flanking sequences.

R Forghani: Dr Forghani was a graduate student in the laboratory of Dr Peterson. He was involved in the initial derivation of some of the constructs.

H Friedman: Dr Friedman is a research associate in the laboratory of Dr Peterson. She was involved in the design of the control experiments comparing quantitative transgene expression levels (inset of Figure 7).

W Orfali: Dr Orfali was a graduate student in the laboratory of Dr. GJ Snipes. He was involved in the initial derivation of one construct (-3.1 kb/lacZ/SCE1 0.5 kb).

L Jasmin: Dr Jasmin is an investigator at UCSF. He initially described the CTB-saporin demyelination paradigm in rats and I learned the technique from him and adapted it to mice.

W Miller: Dr Miller is an investigator at Penn State University. He is an expert in bioinformatics and provided guidance on our comparative sequence analysis and on using the PIPMaker software.

Chapter 3

E Denarier: As primary contributor, conducted four-way interspecies Mod4 sequence comparison identifying multiple conserved motifs, designed bulk of *hprt* docked constructs bearing Mod4 subdomains and mutations, and undertook EMSAs.

R Forghani: Dr Forghani was a graduate student in the laboratory of Dr Peterson. He performed the initial work analyzing the *in vivo* function of various Mod4/Schwann cell enhancer 1 subdomains.

HF Farhadi: I analyzed the expression profile and quantitated the expression levels of the Mod4/Schwann cell enhancer 1 containing constructs promoted by MBP-related sequences. In addition to the -9.4, -8.98, and -8.9 kb constructs, other constructs that I designed ultimately did not target expression to Schwann cells but helped to tailor the focus of the experimental design (see above: contribution 13 under chapter 2).

R Drouin: The *in vivo* footprinting gels were carried out in Dr Drouin's laboratory using his protocol (with some modifications).

Chapter 4

MBP-LACZ TRANSGENE EXPRESSION IN JUVENILE AND ADULT *TREMBLER-J* MICE

Hooman F. Farhadi, Reza Forghani, Alan C. Peterson

Annals of the New York Academy of Sciences, 1999, 883:538-9

HF Farhadi: I analyzed transgene expression in developing and mature wild-type and $Tr^{J}/+$ mice. I was also responsible for the text and figure.

R Forghani: Dr Forghani was involved in the initial analysis of the developmental expression profile of Schwann Cell Enhancer 1 bearing constructs.

CHAPTER 1

GENERAL INTRODUCTION

PREFACE

Central and peripheral nervous system myelinogenesis represent fundamental adaptations of vertebrates and are hallmarks of oligodendrocyte and Schwann cell maturation, respectively. Multilayered lipoprotein myelin sheaths serve to insulate axons, facilitate saltatory conduction, and contribute to neuronal function. The mechanisms underlying myelinating glial cell differentiation and maturation from multipotent precursors have become increasingly well-characterized, with essential transcriptional regulators identified in recent years. In marked contrast, the signaling pathways governing the tightly controlled processes of myelin assembly and long-term stability remain poorly understood.

The primary objective of this thesis is to gain further insight into the transcriptional regulation of a representative myelin protein gene, *Myelin Basic Protein (MBP)*, during development, maturity, and following injury. This chapter provides a review of the background rationale and details the objectives for the investigations presented in subsequent chapters.

As this chapter is being published in a book on multiple sclerosis, it includes the general findings and conclusions of my thesis research (which are presented in greater detail in subsequent chapters), in addition to the objectives and rationale.

THE MYELIN BASIC PROTEIN GENE: A PROTOTYPE FOR COMBINATORIAL MAMMALIAN TRANSCRIPTIONAL REGULATION

Hooman F. Farhadi and Alan C. Peterson

In Press: Advances in Neurology: Multiple Sclerosis and Demyelinating Diseases

SUMMARY

In this chapter, we review recent investigations that expose the strategies evolution adopted to control the complex gene expression programs realized by the "myelin gene family". We focus on the mechanism controlling transcriptional regulation of one family member, the myelin basic protein (*MBP*) gene. Regulatory activity at the *MBP* locus arises from widely dispersed enhancer modules consisting of short non-coding sequences that are highly conserved throughout mammals and in most non-mammalian vertebrates. Each confers a discrete regulatory sub-program to the overall *MBP* expression phenotype with expression in oligodendrocytes conferred through

multiple proximal modules and the proximal promoter while Schwann cells rely upon a far upstream module. Beyond their well-characterized autonomous functions, such modules engage in combinatorial relationships to refine both cell-type specificity and quantitative expression phenotypes. Of relevance to myelin cell biology, this combinatorial organization underlies an unsuspected maturation program in which the transcription factor repertoire regulating *MBP* expression evolves with age. Thus, the combination and/or relative activity of transcription factors engaged during primary myelin elaboration is not present in mature myelin maintaining oligodendrocytes or, most remarkably, in remyelinating oligodendrocytes in mature animals. Should this circumstance also exist in humans, fundamental characteristics of the cells targeted in adult MS lesions, as well as those attempting myelin repair, are specific to the mature nervous system.

INTERDEPENDENCE OF AXONS AND THEIR MYELINATING GLIA

Myelin formation represents a relatively recent evolutionary adaptation of vertebrates. The electrical insulation afforded by compacted myelin membranes in the central and peripheral nervous systems allows for rapid and energy efficient conduction over long distances while respecting necessary size constraints. Within fast conducting myelinated nerve fibers, the morphological and functional phenotypes of axons and their supporting glia develop and are maintained through continuous bi-directional signaling throughout development and in the adult.

Significant differences exist in the way myelinating glia interact with axons in the central and peripheral nervous systems. Whereas a single oligodendrocyte may myelinate

scores of different axons, a myelinating Schwann cell associates with a single axon. The correlation between the caliber of the innervating axon and myelination is much tighter in the PNS than in the CNS (Aguayo et al., 1977; Peters et al., 1991). When Schwann cells are isolated from the innervating axon, either through denervation *in vivo* or isolation in culture, they revert to a non-myelinating phenotype and down-regulate the genes for the major myelin associated proteins to low basal levels (LeBlanc and Poduslo, 1990; Lemke and Chao, 1988; Trapp et al., 1988). Isolated oligodendrocytes, on the other hand, continue to extend their processes and induce and maintain ~30% of the normal level of expression of myelin genes (Abney et al., 1981; Bradel and Prince, 1983; Zeller et al., 1985).

Despite these differences, myelin formation and maintenance is the final step in the maturation of both oligodendrocytes and Schwann cells, and involves a complex and tightly regulated process that is supported by an intimate axon-glial physical inter-relationship. The synthesis and assembly of myelin specific components in all terminally differentiating glial cells must be precisely orchestrated to give rise to myelin. On the one hand, myelination leads to local changes in the cytoarchitecture of the axon as well as to more systemic changes in the biology of myelinated neurons including phosphorylation status and slow anterograde axonal transport (Aguayo et al., 1979; Brady et al., 1999; de Waegh et al., 1992; Kirkpatrick et al., 2001). Also, the clustering of sodium and potassium channels at the node and the juxtaparanode, respectively, is dependent on oligodendrocytes (Baba et al., 1999; Kaplan et al., 1997; Rasband et al., 1999) and Schwann cells (Arroyo et al., 1999; Joe and Angelides, 1992).

Conversely, axons also appear to control multiple features of their myelin sheaths (Michailov et al., 2004; Notterpek and Rome, 1994). Part of the control that axons exert over glial cells appears to be mediated through the coordinated regulation of specific developmentally regulated genes encoding myelin specific proteins. Early *in vitro* experiments showed that, in addition to neurons being mitogenic for oligodendrocytes and Schwann cells (DeVries et al., 1982; Wood and Bunge, 1986), isolation of glial cells from axons results in a downregulation of myelin specific genes with only partial restoration of expression in co-cultures (David et al., 1984; Macklin et al., 1986). *In vivo* differentiation of oligodendrocytes in the optic nerve is closely related to axonal integrity (Kidd et al., 1990; McPhilemy et al., 1990; Valat et al., 1988). Furthermore, modulation of myelin protein gene expression by axons is also observed under various experimental conditions, such as following nerve transection (LeBlanc and Poduslo, 1990; McPhilemy et al., 1990; Scherer et al., 1992).

It seems likely that both soluble and cell-mediated signals from adjacent axons are integrated into the developmental profile of oligodendrocyte precursors resulting in cell differentiation, up-regulation of myelin gene expression, and formation of mature myelin. For instance, oligodendrocyte maturation and survival is influenced by neuregulins expressed on axons (Fernandez et al., 2000; Vartanian et al., 1999). Neuregulin exposure induces morphological changes in cultured oligodendrocytes (Canoll et al., 1999). In the absence of the neuregulin receptor ErbB2, while many oligodendrocyte precursors develop, few of these cells mature and those that do fail to interact with axons and do not produce myelin (Kim et al., 2003; Park et al., 2001). In the PNS, axonally derived neuregulin promotes differentiation along a glial path (Britsch et al., 1998; Sandrock et

al., 1997) and has recently been shown to function as a key regulator of myelin sheath thickness in vivo (Michailov et al., 2004). Moreover, NGF has recently been shown to mediate potent, but inverse effects on myelination by Schwann cells and oligodendrocytes, likely through changes in the axonal signals that control myelination (Chan et al., 2004).

Other candidates for axonally derived soluble factors include fibroblast growth factors (Qian et al., 1997), thyroid hormone (Barres et al., 1994), and adenosine (Stevens et al., 2002). In particular, adenosine released in an activity-dependent manner by dorsal root ganglia axons promotes differentiation of oligodendrocyte precursors into MBP-expressing cells containing multiple processes and forming compact myelin sheaths. Myelination also appears to be regulated by electrical activity alone (Demerens et al., 1996; Stevens and Fields, 2000). Treatment of CNS explant cultures with tetrodotoxin results in significantly fewer myelin internodes transiently while treatment with α-scorpion toxin (a selective sodium channel activator) results in a significant increase. Finally, axonal cell surface molecules such as L1, MAG, NCAM, and N-cadherin may also regulate formation of the myelin sheath (Martini, 1994).

POTENTIAL *MBP* TRANSCRIPTIONAL REGULATORS

Glial cells and their progenitors have historically been characterized primarily by their morphologies and expression of a small number of lineage and myelin markers. However, exciting insight into the regulatory machinery and the signaling pathways that control their major expression features is rapidly emerging (Wegner, 2001). Despite the fact that only few such factors are well characterized, they are providing progressively

greater insight into the requirements for lineage specification, progression and terminal differentiation (Arnett et al., 2004; Stolt et al., 2002). Notably, as the number of factors implicated in myelin cell biology grows, insights into unanticipated spatial and temporal oligodendrocyte heterogeneity have emerged; where both the Nkx6 transcription factor and the Sonic hedgehog signaling system were thought to be essential for the origin of the oligodendrocyte lineage, recent investigations demonstrate that oligodendrocytes appearing later in the dorsal spinal cord share no such requirements (Cai et al., 2005; Vallstedt et al., 2005).

MYELIN PROTEIN GENE PROMOTER ANALYSIS

Developing a comprehensive catalog of the transcription factors controlling oligodendrocyte gene expression in all developmental states would provide a foundation from which novel interventions will likely emerge. While many difficulties are yet to be addressed before we develop such understanding, genome wide strategies capable of evaluating the transcription factor repertoire are currently under development and limited application. Importantly, transcriptional regulation in complex organisms is not a simple matter of turning a gene on at the right time in the right cell type. Rather, the basic mechanism emerging involves scores of factor/DNA element interactions that may be engaged to effect positive, negative, or neutral consequences on gene output. Further, not all relevant interactions are likely to occur in the cells positively regulating the locus; active silencing in inappropriate cell types or at specific stages of lineage progression also may be required. Thus, to complement the insights expected from genome-wide molecular approaches, it is essential that multiple model loci are sufficiently well

characterized to expose the actual structure/function relationships within their *cis*-linked regulatory sequences. It is our goal to introduce a myelin gene into this model locus category.

The promoters of myelin protein genes have been analyzed using *in vitro* preparations and for several, regulatory sequences which identify relevant transcription factors regulating myelin formation have emerged (Brown and Lemke, 1997; Clark et al., 2002; He et al., 1991; Miskimins et al., 2002; Monuki et al., 1993; Monuki et al., 1990; Monuki et al., 1989; Peirano and Wegner, 2000; Shy et al., 1996; Wei et al., 2003, 2005). However, since no *in vitro* model fully recapitulates the myelinating glial phenotype, and given the apparent importance of bi-directional signaling with intact axons, reliable dissection of control mechanisms with *in vitro* techniques has proved difficult.

Ideally, direct insight into the complex *in vivo* axon-glia relationships should be achievable through mouse transgenic technology. Following this strategy, several laboratories have investigated the regulatory capacity of sequences flanking representative myelin protein gene members. Transgenic mice have been derived bearing reporter constructs regulated by various lengths of 5' flanking sequence from the *proteolipid protein*, *MBP*, *protein zero*, *peripheral myelin protein 22*, and *2', 3' cyclic-nucleotide 3'-phosphodiesterase* genes. Targeting to oligodendrocytes and/or Schwann cells has been demonstrated with varying efficiency (Foran and Peterson, 1992; Gow et al., 1992; Gravel et al., 1998; Maier et al., 2002; Messing et al., 1992; Wight et al., 1993; Wrabetz et al., 1998).

Unfortunately, until recently, reliable and efficient identification of *cis*-regulatory sequences using classic transgenic methodologies has been seriously limited. Variability

in transcriptional efficiency attributable to unpredictable transgene copy numbers and local chromatin effects at the site of integration combined to confound the interpretation of regulatory phenotypes and have precluded fine structure analysis. To resolve these issues, and to locate and characterize the elements regulating *in vivo* transcription of the *MBP* locus, we employed a recently described controlled transgenesis strategy wherein single-copy construct insertion at the hypoxanthine-guanine phosphoribosyltransferase (*hprt*) locus allows for high resolution interconstruct qualitative and quantitative comparisons (Bronson et al., 1996; Cvetkovic et al., 2000; Vivian et al., 1999) (see Figure 1).

Several features of *MBP* and its regulatory phenotype make it an ideal candidate gene for elucidating mechanisms underlying axon-glia interactions. As a major component of the major dense line, MBP plays an essential and rate-limiting role in CNS myelin formation (Kirschner and Ganser, 1980; Privat et al., 1979). Also, it is a well-characterized experimental auto-antigen with potential roles at multiple levels in immune mediated mechanisms of myelin destruction (Lutton et al., 2004). It is present in both CNS and PNS myelin where it accounts for 5-15% and 30-40% of myelin protein content, respectively (Greenfield et al., 1973; Hahn et al., 1987; Milek et al., 1981). *MBP* appears to be differentially regulated throughout oligodendrocyte lineage progression, achieving highest levels in terminally differentiated myelinating cells with modestly reduced levels maintained throughout maturity into senescence. Expression also appears to be tightly regulated under numerous experimental conditions involving demyelination and remyelination (LeBlanc and Poduslo, 1990; Woodruff and Franklin, 1999). Both oligodendrocytes and Schwann cells regulate the appearance of MBP primarily at the

transcriptional level (Wiktorowicz and Roach, 1991) with such expression coordinated in time and place with other myelin-related genes in both cell types in response to axonal cues (Gordon et al., 1990; Notterpek et al., 1999). Finally, *MBP* is a potential susceptibility gene for MS in the context of a small Finnish population (Pihlaja et al., 2003), raising the intriguing possibility that dysregulated MBP expression may be a component in MS onset.

Investigations using classic transgenic preparations have shown that the proximal MBP promoter targets developmentally appropriate temporal expression to oligodendrocytes but not Schwann cells (Foran and Peterson, 1992; Goujet-Zalc et al., 1993; Gow et al., 1992) while 5' flanking sequences extending to approximately -6 kb or -9 kb yield more robust expression (Farhadi et al., 2003; Forghani et al., 2001). Additionally, the -9kb sequence also targets expression to developing, mature, and remyelinating Schwann cells.

COMBINATORIAL CONTROL OF TRANSCRIPTION

The regulation of gene expression in multi-cellular organisms is complex and for each locus, occurs through the coordinated action of multiple transcription factors acting on large numbers of regulatory elements. A common theme of such regulatory mechanisms is a combinatorial organization (Halfon et al., 2000; Yuh et al., 1998). A combinatorial strategy of transcriptional regulation allows the organism to dynamically control gene expression in response to a variety of environmental or developmental signals through different combinations of a limited number of transcriptional regulators.

The *cis*-regulatory system of the developmentally regulated *endo16* gene of the sea urchin has been investigated to perhaps greater depth than any other locus and thus serves as a useful model (Yuh et al., 1998, 2001). Its major regulatory sequence is contained within approximately 2300 bp and consists of several clusters of target sites that operate as separable modular regulatory units that execute distinct qualitative or quantitative functions. Through a detailed spatiotemporal analysis of endoderm reporter expression in numerous lines bearing different permutations of normal or mutated enhancer elements, Davidson and colleagues successfully developed a quantitative computational model that appropriately reflects the integrated output of defined regulatory sequences. Of significance, most *endo16* elements confer only small quantitative effects that, in aggregate, determine the integrated expression programming.

Although the analysis of *endo16* greatly enhanced our ability to contemplate the fine structure of the regulatory networks functioning in vertebrate and mammalian systems, elucidating the transcriptional regulatory network in such higher order organisms represents a much more daunting task; each experimental approach, either computational or functional, provides only partial answers (Fickett and Hatzigeorgiou, 1997; Fickett and Wasserman, 2000; Werner, 1999). Currently, a synthesis of multiple approaches is employed that includes genomic sequence annotation, cross-species sequence comparisons, functional analysis of reporter constructs and abstract model building.

Patterns of gene regulation and the corresponding regulatory controls are often conserved across species and cross-species sequence comparison has emerged as a powerful means to reveal functionally relevant regulatory sequences. Relying on the

principle that selective pressure over functional regulatory sequences causes them to evolve slower than surrounding unselected sequences, these so called phylogenetic footprints are expected to confer various regulatory functions (Cawley et al., 2004; Saluja and Kohane, 2003). In this regard, the mouse genome has received considerable attention given that its genomic sequences are now easily accessible and that there is a wealth of similarity to many human-relevant biological and disease processes (including myelination and inherited and acquired myelin diseases). Using recently developed computational methods and evolving genomic databases from an increasing number of species, interspecies sequence comparisons are performed in an efficient and comprehensive manner (Dermitzakis et al., 2002; Frazer et al., 2003; Schwartz et al., 2000; Wasserman et al., 2000; Waterston et al., 2002; Wingender et al., 2001). While the general usefulness of human-mouse sequence comparisons as a systematic guide to functionally relevant regulatory sequences remains untested, evolutionarily conserved sequences (in the range of hundreds of base pairs) are associated with many loci and to date, many such conserved non-coding sequences have been assigned regulatory functions (Farhadi et al., 2003; Gottgens et al., 2000; Loots et al., 2000; Oeltjen et al., 1997; Pennacchio et al., 2001; Tumpel et al., 2002).

EVIDENCE FOR COMBINATORIAL CONTROL OF *MBP* TRANSCRIPTION

The *MBP* transcriptional unit maps to chromosome 18 in both mouse and human where it is distributed over a length of approximately 32 kb and 45 kb, respectively. Interestingly, it is contained within a much larger transcriptional unit (spanning 105 kb in mouse and 179 kb in human) that includes the gene *Golli* (gene expressed in the

oligodendrocyte lineage) at its 5' end. The first three *MBP* exons and the proximal MBP promoter contribute to translated *Golli* exons (Campagnoni et al., 1993; Pribyl et al., 1993). While non-coding domains have an average level of human-mouse conservation approximating 30-40%, with an upper limit near 80% (Jareborg et al., 1999), the MBP genomic domain fortuitously appears to have undergone evolutionary change at a comparatively greater tempo revealing an overall level of conservation at the lower end of this scale.

Given this circumstance, sequences conserved functionally should be clearly highlighted as conserved regulatory regions between highly substituted non-functional adjacent sequence. Therefore, in our initial attempts to highlight *MBP* regulatory sequences through phylogenetic footprints, we used thresholds of identity of 75% over a pre-specified minimum length (100 bp) to define sequence conservation. We scanned 25 kb of human and 12 kb of mouse 5' flanking orthologous sequences and identified four islands of marked sequence conservation (termed modules 1-4). All modules were ligated into reporter genes that were introduced, in single copy, 5' of the *hprt* locus and investigated for *cis*-regulatory activity in subsequent lines of transgenic mice (Farhadi et al., 2003) (also see Figure 1).

A diverse range of *in vivo* expression phenotypes, including different cell specificities, developmental programming and expression levels was observed (Figures 2 and 3). From these observations, it became evident that the MBP regulatory mechanism controls transcription through a combination of seemingly autonomous modular contributions that are overlain with functions derived from integrated arrangements (Figure 4).

Thus, multiple sub-programs were discerned and amongst these, perhaps the most striking is the requirement of module 3 for high-level expression in mature mice. This observation requires that the MBP relevant transcription factor repertoire of mature oligodendrocytes differs from that of the less mature oligodendrocytes in pre-weaning mice, either as a response to an intrinsic developmental program or as a change secondary to extrinsic differences in the environment provided in the juvenile versus the mature brain. In either case, this observation defines a novel level of temporal oligodendrocyte heterogeneity.

To explore the potential origin of such age-associated heterogeneity, we investigated the regulatory phenotype of oligodendrocytes remyelinating in the mature nervous system. Transgene expression was evaluated in mice bearing constructs containing only modules 1 and 2 (Farhadi et al., 2003). In response to intrathecal injection of saporin conjugated to the beta subunit of cholera toxin, oligodendrocytes mount a significant remyelination response (Jasmin et al., 2000) but, surprisingly, we obtained no evidence indicative of transgene expression in newly myelinating oligodendrocytes bearing these constructs devoid of module 3. In contrast, observations from randomly targeted MBP-LacZ transgenic lines regulated by 5' flanking sequences containing module 3, reveal that adult newly myelinating oligodendrocytes do express their transgene reporter coincident with new myelin deposition (Finsen and Peterson, 2001).

From these observations, it is apparent that at least part of the regulatory mechanism used to control *MBP* transcription during primary development is not in service during myelin repair in the mature CNS. In the context of MS lesions, while some

controversy exists regarding the density of the progenitor population in regions of demyelination (Lassmann et al., 1997; Wolswijk, 1998b; Zhang et al., 1999), it is nevertheless clear that such cells possess atypical myelination capabilities. In this regard, oligodendrocyte precursors have clearly been identified lying directly within chronic plaques of MS patients (Wolswijk, 1998a). Why these cells do not aggressively repair the demyelinated lesions, and why those oligodendrocytes that eventually do enter an active myelination program slowly elaborate only thin myelin, remain largely unanswered questions. As the differences observed in the MBP transcriptional mechanism operating in primary development and in oligodendrocytes remyelinating in the mature brain may change expression of an essential myelin constituent, they may contribute to the protracted and incomplete process of remyelination often observed in the diseased or injured adult CNS.

To identify the regulatory sequences and factors that confer the module 3 expression phenotype, and in an effort to characterize the regulatory cascade functioning during adult myelin maintenance and regeneration, an intense analysis of module 3 elements and their relationship to expression in mature and remyelinating oligodendrocytes is currently underway (Dionne et al., 2005). In the initial round of investigations, we focused on Nkx6.2/Gtx, an oligodendrocyte-specific homeodomain protein known to avidly bind TAAT-containing consensus sites (Awatramani et al., 2000; Awatramani et al., 1997) and that is coordinately expressed with several myelin-specific mRNAs in multiple cell states (Sim et al., 2000). While a module 3 construct bearing mutations in two of three Nkx6.2/Gtx consensus sites retains continuous oligodendrocyte targeting activity, quantitative analysis revealed a significant decrease in transcriptional

efficiency (Farhadi et al., 2003). Thus, this transcription factor, or related homeodomain proteins, appears to function within a complex framework finely modifying module 3 output.

Taken together, observations summarized in this chapter demonstrate that different combinations of regulatory sequences control *MBP* expression during development, myelin maintenance and remyelination. Though the picture is far from complete and notable exceptions are emerging, vast numbers of positive *cis*-regulatory elements account for the major spatiotemporal features of *MBP* expression. The specific temporal, spatial, and quantitative output from the *MBP* gene is determined by the particular combination of evolutionarily conserved *cis*-regulatory binding sites that are engaging transcription factors. To arrive at a more complete picture of this complex *cis*-regulatory circuitry, the fine structure of individual enhancers as well as more in-depth analysis of modular interactions and negative regulatory activities will be required. Fortunately, the HPRT based controlled strategy of transgenesis is sufficiently robust to reveal the role played by individual elements and, consequently, is ideally suited to support such investigations (Denarier et al.). Additionally, initial *in vitro* experiments, including transfection analysis, DNase I footprinting, and electrophoretic mobility shift assays, are underway by our group and others (Taveggia et al., 2004) using the conserved modular sequences.

CONCLUSION

Although nothing of therapeutic relevance for patients with MS has emerged so far from past and current investments in basic gene regulation mechanisms, this endeavor

appears poised to yield insights with practical consequences. The convergence of recent discoveries in genome organization, along with the unexpected developments of strategies to modulate gene output (small interfering RNAs, chimeroplasty, exon skipping, etc.) suggest that opportunities to control the stability and/or responsiveness of myelin forming cells, in clinically meaningful ways, is within reach. Beyond this emerging potential, greater understanding of the mechanisms regulating gene expression is, of itself, offering a disease relevant window into the myelination program in effect at different stages of oligodendrocyte maturation. By achieving insight into the molecular mechanisms regulating myelin formation and repair, we anticipate that novel opportunities to stabilize myelin in the diseased CNS and to promote repair following demyelinating episodes will become evident.

ACKNOWLEDGEMENTS

The investigations discussed in this chapter were supported by the Canadian Multiple Sclerosis Society and Foundation, the Canadian Institutes for Health Research (CIHR) and Genome Canada/Genome Quebec. HFF was supported through a CIHR Doctoral Fellowship. We thank our many laboratory colleagues who made the investigations described in this chapter possible. In particular, we thank Drs. Eric Denarier, Hana Friedman and Melissa Beaudouin for help with figures and helpful comments on the manuscript.

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Figure 1: Diagram depicting deletion in the *hprt* locus where constructs are docked and the genotype of the transgene locus following homologous recombination of the construct bearing targeting vector. HAT selection following transfection with appropriate targeting vectors allows survival only of clones in which homologous recombination has simultaneously restored deleted HPRT expression and inserted a single copy of the experimental construct, at a known site and orientation 5' of the *hprt* locus (Bronson et al., 1996). *PHF6* indicates the next 5' locus.

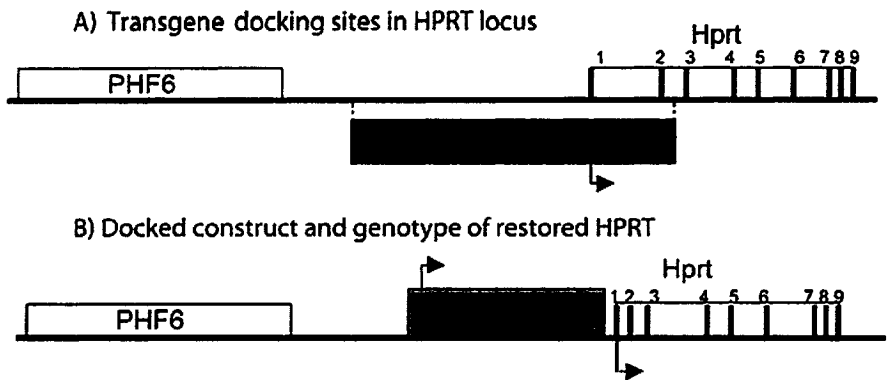
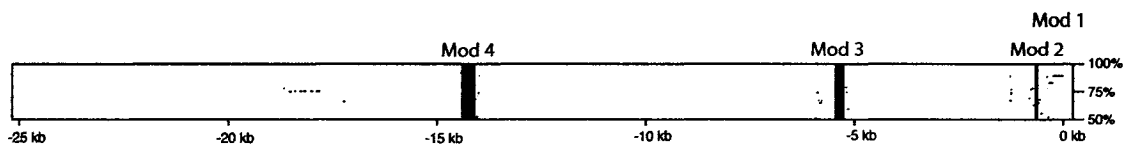
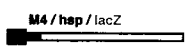

A) Transgene docking sites in HPRT locus
Hprt
1 2 3 4 5 6 7 8 9
PHF6
B) Docked construct and genotype of restored HPRT
Hprt
1 2 3 4 5 6 7 8 9
PHF6

Figure 2: Phylogenetic footprints revealed by comparison of 5' *MBP* sequence from mouse and human. The human sequence is displayed on the X-axis while the % identity, measured in a 100 bp window, is indicated on the y axis. In the proximal promoter sequence, extensive and high levels of conservation are encountered that in this analysis, define two separable modules. More upstream is module 3 and further upstream yet is module 4. Whole mount histochemical preparations displayed below show that module 4 containing constructs express in Schwann cells, and not oligodendrocytes, while constructs bearing the more 3' modules are expressed in oligodendrocytes only. In both constructs, *lacZ* is the reporter gene. In the Mod3/2/1 construct, MBP 5' flanking sequence was ligated directly to *lacZ* while in the module 4 construct, a minimal 300 bp heat shock protein promoter was introduced between module 4 and the lacZ reporter.



Mod 4 (-Mod3/2/1)

Mod3/2/1 (-Mod4)



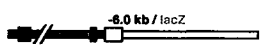


Figure 3: Sciatic nerve and cervical spinal cord samples were obtained from transgenic mice containing constructs bearing different combinations of *MBP* regulatory modules. Samples were recovered at postnatal day 18 when myelin elaboration, *MBP* and construct expression are at, or near, maximal levels. As constructs were docked in an X chromosome site, for one potentially active construct copy to be available in all cells, analyzed mice were either transgene bearing males or homozygous females. Mice analyzed in this study were derived from intercrosses between C57Bl/6, 129PAS and 129OLA inbred strains and therefore, had variable genetic backgrounds. Truncated modules are indicated by *. Bars represent means and error bars SD.

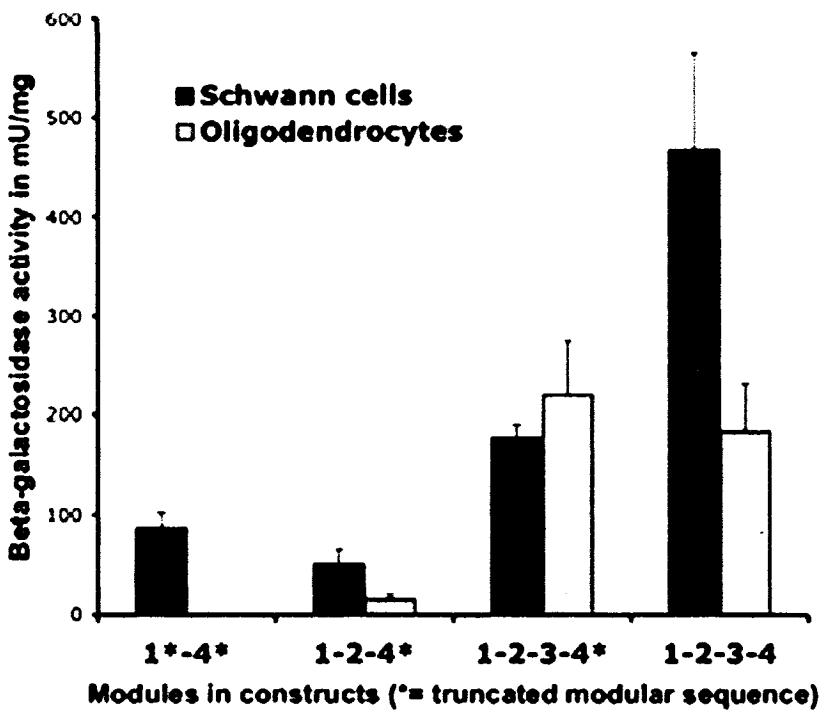
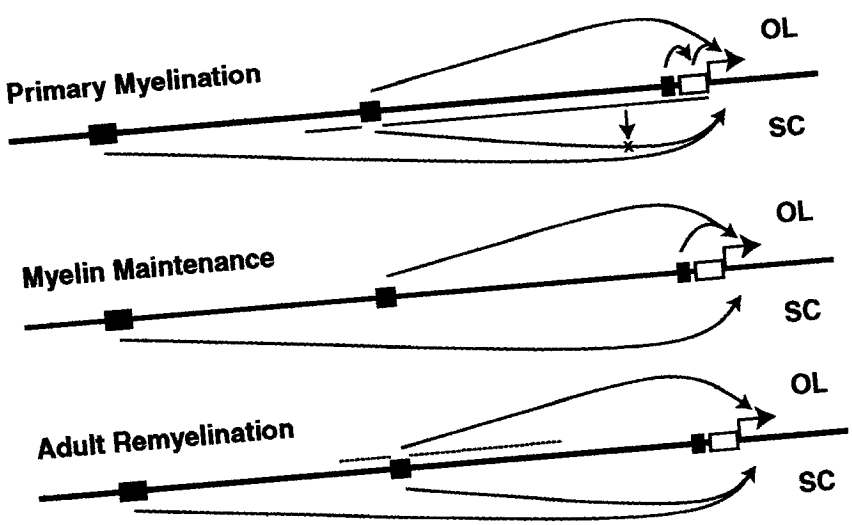

Schwann cells
Oligodendrocytes
Beta-galactosidase activity in mU/mg
600
500
400
300
200
100
0
1*-4*
1-2-4*
1-2-3-4*
1-2-3-4
Modules in constructs (*= truncated modular sequence)

Figure 4. The 5' flanking region of the mouse MBP locus along with its 4 regions of high inter-species conservation (colored rectangles) is shown. The positive relationships defined so far that can drive expression in developing, mature and remyelinating cells are represented as solid arrows. Those above the flanking sequence positively regulate expression in oligodendrocytes while solid arrows below positively regulate expression in Schwann cells. Dotted lines represent the general location of various negative regulatory activities.


Primary Myelination
OL
SC
Myelin Maintenance
OL
SC
Adult Remyelination
OL
SC

CHAPTER 2

PREFACE

This chapter presents the main bulk of primary data contributed by this thesis. It also provides the scientific basis for the ongoing use of high-resolution *in vivo* controlled transgenesis strategies to elucidate the transcriptional mechanisms and machinery regulating *MBP* expression in development and maturity. A complex network of evolutionarily conserved regulatory motifs is observed to differentially regulate *MBP* expression in myelinating glia during various differentiation states as seen during development, maturity, or following injury. As such, an investigative tool is validated for the identification of the relevant transcription factors and cellular signaling engaged in such diverse glial states.

A COMBINATORIAL NETWORK OF EVOLUTIONARILY CONSERVED *MYELIN BASIC PROTEIN* REGULATORY SEQUENCES CONFERS DISTINCT GLIAL-SPECIFIC PHENOTYPES

Hooman F. Farhadi, Pierre Lepage, Reza Forghani, Hana C.H. Friedman, Wayel Orfali, Luc Jasmin, Webb Miller, Thomas J. Hudson, Alan C. Peterson

ABSTRACT

Myelin basic protein (MBP) is required for normal myelin compaction and is implicated in both experimental and human demyelinating diseases. In this study, as an initial step in defining the regulatory network controlling MBP transcription, we located and characterized the function of evolutionarily conserved regulatory sequences. Long-range human-mouse sequence comparison revealed over 1 kb of conserved non-coding MBP 5' flanking sequence distributed into four widely spaced modules ranging from 0.1 to 0.4 kb. We demonstrate first that a controlled strategy of transgenesis provides an effective means to assign and compare qualitative and quantitative *in vivo* regulatory programs. Using this strategy, single-copy reporter constructs, designed to evaluate the regulatory significance of modular and inter-modular sequences, were introduced by homologous recombination into the mouse *hprt* locus. The proximal modules M1 and M2

confer comparatively low-level oligodendrocyte expression primarily limited to early postnatal development while the upstream M3 confers high-level oligodendrocyte expression extending throughout maturity. Further, constructs devoid of M3 fail to target expression to newly-myelinating oligodendrocytes in the mature CNS. Mutation of putative Nkx6.2/Gtx sites within M3, while not eliminating oligodendrocyte targeting, significantly decreases transgene expression levels. High-level and continuous expression is conferred to myelinating or remyelinating Schwann cells by M4. In addition, when isolated from surrounding MBP sequences, M3 confers transient expression to Schwann cells elaborating myelin. These observations define the *in vivo* regulatory roles played by conserved non-coding MBP sequences and lead to a combinatorial model in which different regulatory modules are engaged during primary myelination, myelin maintenance, and remyelination.

INTRODUCTION

The myelin sheath is a specialized glial membranous organelle essential for rapid and energy efficient action potential conduction. Perturbations of the sheath, as in multiple sclerosis (MS), may also lead to axonal degeneration (Bjartmar et al., 2003). The process of remyelination is invariably prolonged as compared to primary myelin deposition, and often results in thin sheaths with only partial recovery of conductive properties. Thus, an understanding of the mechanisms that control myelination and remyelination are key to the development of therapeutic strategies to treat demyelinating diseases.

The search for potential regulators of glial cell development and axon-glia signaling has led to the identification of numerous candidate transcription factors including the basic-helix-loop-helix proteins Olig1 and Olig2, the homeodomain proteins Tst-1/SCIP/Oct-6 and Nkx6.2/Gtx, the zinc finger protein Krox-20/Egr2, and the HMG-domain protein Sox-10 (Awatramani et al., 1997; Bermingham et al., 1996; Britsch et al., 2001; Topilko et al., 1994; Zhou and Anderson, 2002). However, the upstream glial signaling pathways and the full complement of interacting transcription factors remain to be elucidated.

Mouse transgenesis provides an ideal system to examine regulatory phenotypes under normal and experimental conditions. Using this approach, various lengths of 5' flanking sequence from the *proteolipid protein, MBP, protein zero, peripheral myelin protein 22, and 2',3' cyclic-nucleotide 3'-phosphodiesterase* genes have been demonstrated to target reporter expression to oligodendrocytes or Schwann cells (Foran and Peterson, 1992; Gow et al., 1992; Gravel et al., 1998; Maier et al., 2002; Messing et al., 1992; Wight et al., 1993; Wrabetz et al., 1998). However, identifying *cis*-regulatory sequences by this method has been limited by variability associated with copy number and position effects at random transgene insertion loci. The recent description of a controlled transgenesis strategy permitting single-copy construct insertion by homologous recombination at the *hprt* locus resolves these issues allowing for higher resolution inter-construct comparisons (Bronson et al., 1996; Cvetkovic et al., 2000; Vivian et al., 1999).

Regulatory sequences are thought to constitute a small fraction of the non-coding portion of the mammalian genome (Waterston et al., 2002). Computational sequence

analysis and inter-species comparisons have been used to identify conserved non-coding sequences with potential gene regulatory properties. To date, however, evolutionarily conserved sequences associated with only a few loci have been assigned regulatory functions (Gottgens et al., 2000; Loots et al., 2000; Oeltjen et al., 1997; Pennacchio et al., 2001).

In this study, we combined human-mouse non-coding sequence comparison with targeted transgenesis at the *hprt* locus to identify the *in vivo cis*-regulatory network controlling MBP, an essential constituent of central and peripheral myelin (Peterson and Bray, 1984; Readhead et al., 1987). We find that the regulatory programming of MBP is accounted for by four widely spaced conserved sequence modules. Each module confers a distinct quantitative and spatiotemporal expression pattern defining specific regulatory programs for primary myelination, myelin maintenance, and remyelination following injury. Taken together, our observations highlight this combined approach as a valuable tool for assigning qualitative and quantitative *in vivo* regulatory phenotypes and provide a basis for modeling the *cis*-regulatory system controlling myelin protein gene expression.

MATERIALS AND METHODS

Isolation of human and mouse MBP clones

Human MBP PAC clone 248_D_12 was isolated by screening the RPCI.1 Human PAC library with chromosome 18 STS marker WI-9286, and validated with Golli/MBP exon-specific primers. PAC DNA was prepared from 2L cell cultures in LB-Broth containing 30 μg/ml of kanamycin. DNA was isolated using a midi-prep kit following manufacturer's instructions (Qiagen). PAC DNA was sheared using a sonicator to an

average of 2 Kb fragments. The ends of the fragments were repaired with Mung Bean Nuclease (New England Biolabs). The fragments were gel purified twice to select for 2 kb fragments and ligated into an M13mp18 vector digested with *SmaI*. The reactions were then transformed into XL-2 competent cells (Stratagene). Individual plaques of M13 subclones were grown for 16 hours at 37°C in 0.5 ml of 2X YT with 10 µl of log-phase TG-1 bacterial cells. Single strand M13 DNA for sequencing was obtained from 100 µl of the culture supernatant using magnetic beads (PerSeptive Diagnostics) following manufacturer's instructions. Isolation of a mouse MBP genomic lambda DashII clone (approximately 15 kb) was described previously (Foran and Peterson, 1992).

Sequencing

Sequencing of the M13 clones was done with the Dye Primer Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Sequencing reactions included approximately 400 ng of M13 DNA in 1.5 µl and 3 µl of reaction mixture for each of the four labeled primers. The thermal cycling parameters were: 15 cycles at 96°C for 10 s, 55°C for 5 s and 70°C for one min, followed by 15 cycles at 96°C for 10 s and 70°C for 1 min. The four reactions were mixed and purified with an ethanol precipitation. Samples were loaded and run on an ABI 377 Sequencer according to the manufacturer's instructions (Applied Biosystems). A portion of the mouse sequence (-12 Kb to -3 Kb) was obtained by walking using specific oligonucleotide primers and analyzed on a LICOR sequencer. Ambiguous mouse and human sequences were verified using the DyeTerminator system (Applied Biosystems) with specific primers. The sequences were supplemented with

available sequences present in GenBank, followed by assembly into contigs and analysis with the STADEN (version 1997.1) and MacVector 7.0 software packages.

Generation of reporter constructs

A -3.1 kb mouse MBP/lacZ construct (Foran and Peterson, 1992), was used to derive constructs containing -165 bp (*EheI*), -300 bp (*StuI*), -377 bp (*ApaI*), or -794 bp (*BseRI*) of proximal promoter sequence driving lacZ expression. A -9.4 kb construct was derived by the addition of a 0.44 kb *PvuII-SacII* fragment to a previously described -8.9 kb MBP/lacZ construct (clone 8) (Forghani et al., 2001). Clone 8 was also used to isolate a -5.8 to -3.1 kb *KpnI-XbaI* fragment encompassing M3. Deletion of a 0.6 kb fragment containing M3 was performed by digestion with *AvrII-BglII* (spanning -5.0 to -4.4 kb). Both -5.8 to -3.1 kb fragments (either containing M3 or not) were in turn ligated upstream of the -300 bp proximal promoter sequence coupled to the coding region of lacZ. All of the above MBP-promoted constructs also include either the complete previously described Schwann cell enhancer 1 (SCE1) sequence (*SacII-SacI* fragment from -8.9 to -8.3 kb; (Forghani et al., 2001), or truncated versions of 0.2 kb SCE1 (*BstEII-BglII* from -8.8 to -8.6 kb), 0.5 kb SCE1 (*SacII-NaeI* from -8.9 to -8.4 kb) or 0.44 kb of upstream SCE2 sequence (*PvuII-SacII* from -9.4 to -8.9 kb; (Forghani et al., 2001), as shown in Fig. 1C.

Constructs containing M3 alone were generated by ligation of a 0.38 kb *BtgI-AvaII* fragment upstream of either -300 bp of proximal promoter sequence (as above) or a 0.3 kb HSP68 minimal promoter ligated to lacZ (clone p610ZA; a gift from R. Kothary, University of Ottawa). To test whether the two putative Nkx6.2/Gtx sites on the (+)

strand of M3 are involved in targeting function, complementary M3 sequence encoding oligonucleotides bearing mutations that abolish Nkx6.2/Gtx binding (GT*TAAT*GC → GT*TGGC*GC and TT*TAAT*TC → TT*TGGC*CC; custom made by Sheldon Biotechnology Centre, McGill University) (Awatramani et al., 1997) were annealed and cloned into M3. The mutated sequence (M3 δnkx6.2) was subsequently ligated to the 5' end of -300 bp/SCE1. The resultant mutant (M3 δnkx6.2/-300 bp/SCE1) was confirmed by sequencing of both strands.

M4-containing sequences were similarly ligated upstream of the heterologous HSP68 minimal promoter; namely, a mouse 1.1 kb *PvuII-PvuII* fragment (spanning -9.4 to -8.3 kb), a mouse 0.4 kb *BstXI-AvrII* fragment (spanning -9.1 to -8.7 kb), and a human 1.0 kb *BssSI-EcoRI* fragment (spanning -15.5 to -14.5 kb). A construct containing modular sequences with minimal surrounding sequence was generated by sequential ligation of M4 1.1 kb and M3 (0.38 kb fragment) upstream of -794 bp of proximal promoter sequence ligated to lacZ.

All of the above constructs were directionally inserted into a slightly modified version of the *hprt* targeting vector pMP8SKB (a gift from Sarah Bronson, Pennsylvania State University).

Cell culture and electroporation

BK4-ES cells bearing the *hprt* docking site (also a generous gift from Sarah Bronson) or a derivative on a mixed genetic background (C57Bl/6 and 129) generated in our laboratory were grown on gamma-irradiated murine embryonic fibroblasts in high-glucose DMEM (Life Technologies) supplemented with 15% heat-inactivated FBS, 1%

L-glutamine, 1% MEM amino acids, 1% sodium pyruvate, 1% penicillin-streptomycin, 0.1 mM 2-mercaptoethanol, and 50 μl of LIF (Life Technologies). A quantity of 5–7 x 10^6 BK4-ES cells were electroporated with 40 μg of linearized DNA (250 V and 500 μF, Gene Pulser II; Bio-Rad Laboratories). Homologous recombinants were selected on HAT-supplemented medium, containing 0.1 mM hypoxanthine, 0.0004 mM aminopterin, and 0.016 mM thymidine (Sigma). HAT-resistant colonies were picked 14-21 days later for propagation.

Generation of transgenic mice

All experiments involving animals were conducted in accordance with McGill University animal care guidelines. Targeted ES cells were injected into C57BL/6-derived blastocysts that were then transplanted into the uteri of recipient females. Resulting chimeric males were bred with C57BL/6 females, and the F1 agouti female offspring were backcrossed with C57BL/6 males. Genotyping was performed by PCR analysis of genomic DNA with lacZ coding sequence-specific primers.

Demyelination/nerve injury models

To induce spinal cord demyelination, adult female mice (2-3 months of age) were injected intrathecally at the L4-5 level with 0.3 μg of CTB-Saporin (Advanced Targeting Systems) dissolved in sterile 0.9% saline. Tissues were analyzed following a 6 wk recovery period. To induce peripheral nerve regeneration, adult male or female mice (2-3 months of age) were anesthetized and unilateral sciatic nerve crushes were performed

(2X 10 seconds) at mid-thigh level with a No. 5 forceps. Sciatic nerves were analyzed at 14 or 21 days post crush (dpc) injury.

Histochemical detection of β-galactosidase activity

Histochemical staining was performed as described previously (Forghani et al., 2001). Briefly, mice were anesthetized and perfused transcardially with 0.5% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Following post-fixation for an additional hour, whole mounts or tissue sections (brain, spinal cord, or dorsal root ganglia) were incubated at 37°C in staining solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, 0.02% Nonidet P-40, and 0.4 mg/ml Bluo-Gal (Sigma).

Microscopy

Stained whole mount specimens and spinal cord vibratome sections were viewed on a Wild M5A stereomicroscope and photographed with a Zeiss AxioCam HRc. Adjacent vibratome sections were embedded in epon. Following trimming, semi-thin (1 μm) and thin (10 nm) sections were prepared using an ultramicrotome (Reichert Jung), and processed for light microscopy and electron microscopy (Phillips CM-10), respectively.

Quantitation of β-galactosidase expression levels

Cervical spinal cords were dissected from C57Bl/6 backcrossed P18 mice and homogenized with two 10 second bursts of a Polytron homogenizer in 10 volumes (0.7

ml) of lysis solution (100 mM potassium phosphate, 0.2% Triton-X-100). Total protein concentrations were then measured for all extracts in triplicate by the Bradford microassay procedure (Bio-Rad). Two separate methodologies were employed for quantitation of activity levels. Absolute activity levels differed in the two techniques but relative levels were conserved. The Galacto-Star chemiluminescent assay system (Applied Biosystems) with readings performed on a Revelation MLX luminometer (Dynex Technologies) was used following manufacturer's instructions for comparison of expression levels from independently derived transgenic lines bearing identical constructs (Fig. 4A, inset). The fluorescent substrate DDAO galactoside (Molecular probes) with quantitation using the Typhoon phosphorimager and ImageQuant software (Molecular Dynamics) was used for comparison of activity levels between different constructs (Fig. 4A). Standard curves were generated by assaying serial dilutions of β-galactosidase (Roche Diagnostics) in triplicate.

RESULTS

Human-mouse sequence comparison reveals four conserved modules

To search for evolutionarily conserved MBP regulatory elements, human (25 Kb) and mouse (12 kb) 5' flanking sequences were sequenced and compared. Figure 1A shows a percentage identity plot (PIP) generated by the PIPMaker program (Schwartz et al., 2000), with the human sequence represented on the x-axis. This comparison revealed a relatively high level of interspecies polymorphism equivalent to that observed at the ß-globin locus (Hardison et al., 1997). Using the threshold criteria of 75% identity over 100 bp, four conserved modules were identified. These range in length from 106 bp to 369 bp

(Fig. 1B) and demonstrate percent identities of 87%, 78%, 79%, and 82% (M1 to M4, respectively). All inter-modular sequences are longer in human than in mouse with further human-mouse conservation present only in short sequences (<30 bp), typically adjacent to the modules (as defined by the above criteria).

We further evaluated the modular sequences for the presence of known regulatory elements linked to MBP regulation. We searched for such elements using both MacVector 7.0 software and the Transcription Factor Database (TRANSFAC; http://transfac.gbf.de/TRANSFAC/) (Wingender et al., 2001). Over 250 experimentally characterized murine *cis*-regulatory sites were recognized in M1 to M4 including putative high-affinity recognition sites for the glial-associated factors Krox-20/Egr2, Sox-10, and Nkx6.2/Gtx.

The *hprt* locus does not deregulate MBP-promoted reporter gene expression in myelinating glia

To determine if regulatory function is conferred by the conserved non-coding MBP sequences, we applied a controlled transgenesis strategy permitting direct inter-construct comparisons of both qualitative and quantitative regulatory phenotypes. Reporter constructs containing different configurations of modular and inter-modular sequence were inserted, in single-copy and in a known orientation, at a predetermined site at the *hprt* locus (Bronson et al., 1996; Cvetkovic et al., 2000; Vivian et al., 1999) (Fig. 1C). We show first that the chosen *hprt* docking site provides a transcriptionally favorable environment with representative constructs displaying spatiotemporal expression patterns paralleling that observed with multiple independently-derived random

insertion transgenic lines. A relatively weak MBP promoter (-3.1 kb) (Foran and Peterson, 1992) drives readily detectable reporter gene expression (Figures 2A,B,E,F) while stronger promoters (-9.4 kb, Fig. 2C,G; and -8.9 kb, see below) drive markedly higher expression levels. These *hprt*-docked constructs also contain the previously described SCE1 sequence which, as again expected from numerous random insertion lines (Forghani et al., 2001), targets continuous high-level Schwann cell expression.

Despite ubiquitous expression of the endogenous *hprt* gene, its 5' flanking sequence provides a relatively neutral environment. A sensitive *hprt*-docked enhancer trap construct, regulated by 0.3 kb of the HSP68 promoter, is expressed only in cardiomyocytes (data not shown) and blood vessels (including in the CNS; Fig. 2D,H) and, at trace levels, in spinal cord dorsal gray matter (data not shown). Thus *hprt*-associated enhancers do not drive expression in myelinated glia or neurons. Although further features of the *hprt*-docking site chromatin and regulatory environment remain to be elucidated, this circumstance supports the use of direct inter-construct regulatory program comparisons made throughout this investigation.

Multiple pathways mediate oligodendrocyte targeting during development

The design of reporter constructs analyzed here was based upon the modular structure of conserved MBP 5' flanking sequence. Subsequent analysis of reporter gene expression was performed in either tissue sections or whole mount preparations of spinal cords and brain (oligodendrocytes), or spinal roots and sciatic nerves (Schwann cells). Expression was analyzed mainly at P18, corresponding to peak MBP mRNA accumulation in the CNS (Mathisen et al., 1993), at multiple mature ages when stable and

lower MBP mRNA levels are observed, and in the remyelinating CNS and PNS of mature mice.

To begin, we evaluated the putative regulatory function of M1 and M2 sequences using a series of proximal promoter sequences extending to -794 bp. No oligodendrocyte expression was detected from constructs regulated by either -165 bp (data not shown) or -300 bp (Fig. 3F, see below) of proximal promoter sequence. In contrast, extending the promoter to –377 bp, and thus including the complete M1 sequence, leads to expression in oligodendrocytes during primary myelination (Fig. 3A) which shuts off by about P30 (data not shown). The sequence extending to -794 bp, and thus including M2, results in higher levels of reporter gene expression at P18 (Fig. 3B, see below) and extends the period of expression (Fig. 3C) (construct also differs in content of truncated Schwann cell enhancer sequences ligated 3'of lacZ). Like the M1-regulated construct, expression in oligodendrocytes is transient and, at three months of age, only a small oligodendrocyte sub-population expresses the -794 bp regulated reporter (data not shown).

To determine whether M3 has regulatory function, a series of five constructs was evaluated. In contrast to M1- or M2-containing constructs, a construct bearing the 0.38 kb M3-containing sequence, ligated to the heterologous HSP68 promoter, was expressed at high-levels not only in juvenile mice but throughout maturity and senescence (Fig. 3D, E). Further, an identical CNS expression pattern is observed when either the 0.38 kb or a 2.7 kb (from -5.8 to -3.1 kb) M3-containing sequence is ligated to the non-targeting (minimal) -300 bp MBP promoter (Fig. 3F,G,H). Finally, a construct otherwise identical to that bearing the -5.8 to -3.1 kb sequence but deleted of a 0.6 kb M3-containing fragment, fails to express in oligodendrocytes at any age (Fig. 3I).

Taken together, these results demonstrate that major oligodendrocyte targeting functions reside within the first three MBP modules, corresponding to a total of 0.7 kb of conserved sequence. Depending upon the stage of CNS maturation, M1, M2, and M3 all contribute to oligodendrocyte expression. While M1- and M2-containing constructs are expressed robustly in oligodendrocytes of juvenile mice during primary myelination, M3 alone drives continuous expression in the mature CNS.

M1 and M2 fail to target expression during adult CNS remyelination

A demyelinating insult in the mature CNS results in local maturation of oligodendrocyte progenitors that subsequently initiate expression of myelin genes (including MBP) and partially remyelinate denuded axon segments. Amongst MBP-regulated constructs that express in oligodendrocytes during primary development, the –3.1 kb regulated construct fails to be re-activated in such newly-myelinating adult brain oligodendrocytes while a -8.9 kb regulated construct is expressed robustly (Finsen and Peterson, unpublished observations). Using a novel spinal cord demyelination strategy (Jasmin et al., 2000), we show here that neither the –3.1 kb (Fig. 4A,C) nor the –794 bp sequence (Fig. 4B,D) drive expression in newly myelinating oligodendrocytes analyzed at six weeks following the demyelinating insult. β-galactosidase histochemical labeling and electron microscopic analysis of adjacent spinal cord cross-sections reveals no detectable activity in oligodendrocytes elaborating thin compact myelin sheaths typical of adult remyelination. Taken together, these results suggest that M3, essential for expression throughout myelin maintenance, may also be required for MBP expression during myelin repair in the adult CNS.

M3 and M4 targeting to myelinating Schwann cells depends on combinatorial interactions

MBP also is expressed by Schwann cells myelinating PNS axons and the previously designated 0.6 kb SCE1 sequence (Forghani et al., 2001) located at -8.9 to -8.3 kb partially overlaps M4 (-9.1 to -8.7 kb). All *hprt*-docked constructs containing M4, including minimally promoted constructs, express in myelinating Schwann cells during both primary development and regeneration following sciatic nerve crush (Fig. 5A,B,F). Further, constructs regulated by the human M4 sequence also express robustly, demonstrating multiple levels of human-mouse conservation in the mechanism regulating Schwann cell MBP expression (Fig. 5C,D).

Our combined data provide strong evidence that major MBP regulatory sub-programs are conferred through each of the modules identified by sequence conservation. In addition, in the course of these functional studies, evidence of combinatorial regulatory control extending beyond individual modules was encountered. Previous investigations showed that MBP 5' flanking sequences initiating at the proximal promoter must extend into M4 to confer Schwann cell expression (Foran and Peterson, 1992; Forghani et al., 2001; Gow et al., 1992). Thus, it was unexpected that a construct bearing only M3 sequences would drive expression in Schwann cells. Nonetheless, when isolated from adjacent inter-modular sequences, M3 drives robust, albeit transient, Schwann cell expression during both development and sciatic nerve remyelination (Fig. 5E,G,H). As the control HSP68 construct is not expressed in either developing or remyelinating sciatic nerve Schwann cells (Fig. 5I), this activity is attributable to M3 alone.

Further evidence for combinatorial regulatory control was revealed for M4-containing constructs. When divorced from further MBP sequence, M4-containing constructs express ectopically in satellite glia of dorsal root ganglia (Fig. 5A-D and data not shown), whereas the -9.4 kb construct shows no such expression (Fig. 2C). The M4 1.1 kb/M3/M2/M1 construct (which contains all the modules with only minimal surrounding sequence) reveals a regulation program similar to that conferred by contiguous MBP sequences extending to –9.4 kb and, as observed in the chimeric preparation shown in Figure 6, appears not to express in satellite glia. Thus, elements serving to constrain the spectrum of glial cell types expressing MBP also likely reside within modular sequences.

The un-masked M3 Schwann cell enhancer activity and the M4-mediated satellite cell targeting suggest that either inter-modular or modular sequences can serve to restrict the cell-specific enhancer function contained in modular sequences. As combinatorial regulatory activity underlies the above two targeting phenotypes, it raised the possibility that such interactions might also regulate quantitative phenotypes.

Quantitative comparison of *hprt*-docked reporter gene expression levels

For the well-characterized *endo-16* locus of sea urchin (Bolouri and Davidson, 2002; Yuh et al., 1998, 2001), quantitative functions have been shown to play a major role in the overall *cis*-regulatory program. To determine if MBP regulatory sequences similarly display a variety of complex quantitative phenotypes, we first set out to validate quantitative comparisons between *hprt*-docked constructs by determining whether independently derived transgenic lines bearing identical constructs accumulate similar

levels of ß-galactosidase. Figure 7 (inset) shows that pairs of transgenic lines derived from independent embryonic stem cell clones bearing either -3.1 kb/SCE1, -8.9 kb, or -9.4 kb constructs display similar ß-galactosidase activity levels (across the broad range of quantitative phenotypes predicted from the histochemical preparations). Thus, we conclude that differences in inter-construct ß-galactosidase activity levels can be reliably ascribed to the complement of regulatory elements contained within each construct.

Our investigations reveal extensive quantitative modulation since mice bearing the various constructs demonstrated a wide range of oligodendrocyte ß-galactosidase activity levels (Fig. 7). There is an approximately 25-fold difference between the expression levels of the -377 bp/SCE1 (0.2 kb) and -9.4 kb transgenic lines. In particular, significant enhancement appears to be conferred through modular activity mediated by M3. For example, the M3/hsp and M3/-300 bp/SCE1 lines have ß-galactosidase activity levels approximately 8-fold that of -794 bp/SCE2 (0.44 kb) and 36% that of the -9.4 kb line. In addition, certain inter-modular sequences also appear to be associated with significant quantitative activity; the -(5.8-3.1) kb/-300 bp/SCE1 transgenic line displays approximately 2.1-fold ß-galactosidase activity as compared to the M3/-300 bp/SCE1 line, possibly related to short conserved sequences lying adjacent to M3.

To identify functional regulatory elements *in vivo*, we extended our quantitative analysis to examine the potential contribution of putative binding sites for transcription factors already implicated in oligodendrocyte maturation. We initially focused on putative sites within M3, as we show that it is the dominant regulator of transgene expression in mature oligodendrocytes. Nkx6.2/Gtx is an oligodendrocyte-specific homeodomain protein known to avidly bind TAAT containing consensus sites

(Awatramani et al., 2000; Awatramani et al., 1997). Two evolutionarily conserved putative Nkx6.2/Gtx binding sites (both containing the essential TAAT core) on the (+) strand of M3 were mutated in a manner to abolish binding (Awatramani et al., 2000; Awatramani et al., 1997). The resultant M3 δnkx6.2/-300 bp/SCE1 construct (bearing the M3 sequence mutations GT*TAAT*GC → GT*TGGC*GC and TT*TAAT*TC → TT*TGGC*CC) retains continuous oligodendrocyte targeting activity (data not shown). However, quantitative analysis at P18 reveals a statistically significant decrease (~35%) in oligodendrocyte ß-galactosidase activity levels (Fig. 7). Thus, Nkx6.2/Gtx (or related Nkx homeodomain protein) mediated regulation through these elements is not essential for M3 oligodendrocyte targeting function but transcriptional efficiency *in vivo* is finely modulated through transcription factor binding at one or both of these two homeodomain sites.

DISCUSSION

Multiple modules differentially regulate MBP expression in oligodendrocytes

In this investigation, we have validated and applied an efficient strategy for the *in vivo* identification and functional characterization of MBP regulatory sequences. Initially, comparison of large regions of human and mouse MBP non-coding sequences identified four widely-spaced conserved non-coding modules, totaling ~1 kb in length. Using a novel targeted transgenesis paradigm that allows for single-copy insertion at the *hprt* locus, each module was shown to confer a unique cell-targeting and quantitative gene programming function, seemingly irrespective of absolute spacing from the transcriptional start site. A model summarizing the basic combinatorial targeting and

quantitative programs of the MBP regulatory system is presented in Figure 8. Taken together, our results demonstrate that three MBP modules differentially control major cell-state dependent targeting activities and expression levels during CNS primary myelination and myelin maintenance while modular and inter-modular interactions contribute to maintain glial specificity.

Several *in vivo* studies have focused on the MBP proximal promoter region and together have demonstrated that short segments are sufficient to activate oligodendrocyte-specific and developmentally-regulated reporter gene expression (Goujet-Zalc et al., 1993; Gow et al., 1992; Miskimins et al., 1992; Turnley et al., 1991; Wrabetz et al., 1998). Other studies, using similar promoters, have revealed ectopic expression in a number of non-neural tissues (Asipu et al., 2001; Yoshioka et al., 1991). In this study of *hprt*-docked constructs, the shortest segment mediating oligodendrocyte-specific targeting was the M1-containing -377 bp sequence, and it conferred activity only during the postnatal period encompassing primary CNS myelin deposition. Truncation of M1 (-300 bp, Figure 3F; and -165 bp, data not shown) led to complete loss of oligodendrocyte targeting. It is notable that the first 138 bp of the MBP promoter also is a widely expressed exon included in an overlapping transcriptional unit (Campagnoni et al., 1993). Thus, conservation in this portion of M1 likely includes constraints imposed by this coding function. In contrast to the present findings, a previous study (Goujet-Zalc et al., 1993) using random insertion transgenesis, reported oligodendrocyte targeting from a construct regulated by the same -300 bp promoter sequence. However, construct multimerization and enhancer trapping are commonly observed at random insertion

transgene loci, potentially leading to novel regulatory element interactions (Heard et al., 1999).

Extension of proximal promoter sequence to include M2 as well as M1 (with the -794 bp construct) increases oligodendrocyte reporter gene expression (~37%) at P18 and modestly extends the period of expression, at least in a subpopulation of oligodendrocytes. Only M3-containing constructs were uniformly expressed at high levels in oligodendrocytes throughout primary development, adulthood, and senescence. At P18, the M3/hsp construct (which contains no other MBP sequence except the 0.38 kb fragment encompassing M3) was expressed at ~8-fold the level of the -794 bp construct and at ~36% that of the -9.4 kb construct (which contains the entire complement of modules). Thus, M3 appears to be the primary regulator of MBP expression both in terms of enhancing expression levels during primary myelin deposition and independently regulating expression during myelin maintenance.

These findings are consistent with previous reports demonstrating that proximal promoter sequences, up to -1.3 and -3 kb in length, targeted low level expression to myelinating oligodendrocytes that was attenuated with maturation (Foran and Peterson, 1992; Miskimins et al., 1992; Wrabetz et al., 1998). In contrast, a randomly integrated -1.9 kb proximal promoter sequence did confer high-level oligodendrocyte reporter gene expression in adult mice (Gow et al., 1992). Either the unpredictable effects of copy number or integration site account for this or the -1.9 kb promoter contains a unique combination of enhancer and repressor elements not revealed by the current analysis. We consider the second scenario less likely as the intervening region between -1.9 kb and M2

contains no modular sequence and there is minimal difference between the quantitative phenotypes of the -3.1 kb-and -794 bp-promoted constructs.

Throughout this investigation, targeted *hprt* targeting made it possible to use inter-construct comparisons to assign precise quantitative phenotypes. Analysis of these quantitative results suggests that most of the differences may derive from a simple additive relationship amongst the regulatory sequences. Notably, addition of the activities derived from constructs regulated by individual modular sequences results in a value approximating half that observed for the contiguous -9.4 kb sequence. This difference may indicate either the influence of combinatorial relationships or the exclusion of relevant sequence from the defined modules. The relatively large quantitative difference observed for M3 alone versus M3 in the context of a further 2.3 kb of surrounding sequence is consistent with the latter circumstance and suggests that not all functional regulatory sequences are grouped into distinct modules.

Among the few factors directly implicated in the coordinated activation of myelin gene expression, the homeodomain protein Nkx6.2/Gtx in particular has been shown to be expressed exclusively in differentiated oligodendrocytes (Awatramani et al., 1997). Notably, its temporal expression profile parallels that of myelin specific mRNAs in multiple cell states (Awatramani et al., 1997; Sim et al., 2000). In addition, *in vitro* assays have demonstrated that Nkx6.2/Gtx binds to a number of recognition sites within both MBP and PLP proximal promoters, although none of the MBP putative sites previously examined encode a high-affinity TAAT core (Awatramani et al., 2000; Awatramani et al., 1997). In contrast, M3 contains three such TAAT core domains and a construct bearing mutations in the two sites contained within the (+) strand shows decreased expression

levels at P18 (Fig. 7). Thus, Nkx6.2/Gtx (or related homeodomain proteins) appears to function within a complex network of factors impinging on M3 in oligodendrocytes. Investigations are ongoing to unmask the contributions to transcriptional efficiency by the remaining TAAT as well as other putative binding sites within M3.

Newly-myelinating adult oligodendrocytes do not recapitulate the developmental recruitment of MBP modules

CNS lesions in experimental demyelination and in diseases such as MS show delayed and frequently incomplete remyelination (Franklin, 2002). While no susceptibility locus has so far been unequivocally identified for MS, strong evidence points to the involvement of genetic factors (Pihlaja et al., 2003). Abortive adult remyelination could result from deficient recruitment of oligodendrocyte precursors (which involves both migration and proliferation), a relative decrease in the rate of differentiation of recruited precursors into myelination competent adult oligodendrocytes, altered myelin protein gene regulation, or a combination thereof (Franklin et al., 1997; Gensert and Goldman, 1997).

Our results suggest that the M3 elements that independently mediate expression throughout myelin maintenance in the uninjured adult may also be recruited for expression in newly-myelinating oligodendrocytes in the adult following a demyelinating insult. While M1 and M2 drive expression during development in the CNS, they neither maintain expression in the adult nor re-initiate expression following a demyelinating injury (Fig. 4). The differential MBP modular recruitment exhibited by newly-myelinating adult oligodendrocytes suggests that the limited remyelination capacity seen

throughout the CNS, across species and in numerous disease states, may thus involve critical differences in the programming of essential myelin genes as compared to primary development, possibly reflecting different transcription factor repertoires.

Schwann cells demonstrate a distinct pattern of modular recruitment

We showed previously that the M4-overlapping SCE1 sequence was both necessary and sufficient for developmentally regulated targeting to myelinating Schwann cells (Forghani et al., 2001). The experimental paradigm used here revealed additional Schwann cell enhancer activity within M3. This activity transiently targets expression to primary myelinating and adult remyelinating Schwann cells, coincident in both cases with the rapid phase of myelin deposition. In contrast to M4, which can target robustly during development and remyelination independent of any other MBP sequences, M3 activity is revealed only when it is isolated from other MBP sequences. While its Schwann cell enhancer role in the context of the endogenous locus remains to be established, M3 interacts specifically with Schwann cell transcription factors during myelin elaboration and therefore can be used effectively as a probe or marker for factors expressed at this maturation stage.

Conclusion

Our observations identify a complex of sequence motifs through which MBP expression is differentially controlled in the CNS and PNS during primary myelination, myelin maintenance, and *de novo* myelin formation in the adult. In addition to providing a tool to target precisely pre-determined levels of exogenous gene products to

myelinating glia, we believe it likely that such motifs will serve as effective probes leading to the identification of the relevant transcription factors engaged in such different glial states as seen during development, maturity, or following myelin perturbations secondary to genetic alterations or injury. Through such associations, linking myelin gene regulation to cellular signaling may then be possible (Stolt et al., 2002).

Finally, although it is likely that many strategies will be used in the functional analysis of regulatory sequences, this investigation demonstrates the effectiveness of the controlled *hprt* transgenesis technique in assigning both qualitative and quantitative *in vivo* regulatory phenotypes. With the emergence of the complete genome sequences for human, mouse, and other organisms (Lander et al., 2001; Venter et al., 2001; Waterston et al., 2002), comparative analysis can be applied on a genome-wide scale (Deloukas et al., 2001; Mural et al., 2002). As similar levels of functional understanding are achieved with further model loci, new insights into genome organization and the structure of regulatory networks appear likely.

ACKNOWLEDGEMENTS

This work was performed in collaboration with the RVH Transgenic Facility. We dedicate this paper to its Director, Dr. N. Bachnou, deceased, 2002. We thank P. Valera, I. Tretjakoff, H. Furtenbacher, T. Fernandez, C. Dy, and J. Fortin for their excellent technical support. This work was supported by grants from the CIHR and MS Societies of Canada and program grants from the MS Foundation of Canada and Genome Quebec. HFF and RF were supported through CIHR Doctoral Fellowships. PL was supported by a CIHR Post Doctoral Fellowship. TJH is a recipient of an Investigator Award from the CIHR and a Clinical Scientist Award in Translational Research from the BWF.

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Figure 1. Human-mouse sequence comparison identifies putative regulatory modules evaluated in reporter constructs. A, Alignment of human (25 kb) and mouse (12 kb) MBP 5' flanking sequences using the PIPMaker software. The human genomic sequence is represented on the x-axis and its percentage identity to the mouse sequence (from 50% to 100%) on the y-axis. This analysis reveals similarly ordered and oriented conserved sequence modules. Modular sequences, represented in distinct colors, are designated as module 1 through 4 (M1 to M4) proceeding 5' from the proximal promoter. In addition, an interspersed repeat lies at -19 kb of the human sequence. B, ClustalW alignment (MacVector 7.0) reveals that the individual modules vary in length from 106 to 369 bp in the mouse (lower limit of 75% sequence identity over at least 100 bp). Numbering is from the initiator ATG. C, Schematic representation, drawn to scale, of reporter constructs inserted in single copy at the mouse *hprt* locus. Enlarged boxes, colored as in panel (A), correspond to the conserved modules in their entirety. Inter-modular MBP sequences and HSP68 promoter sequences are represented in gray and black, respectively. SCE1 and SCE2 sequences (Forghani et al., 2001) are both represented in blue and gray. Human M4 is denoted by the symbol h. All constructs are ligated to a lacZ reporter gene.

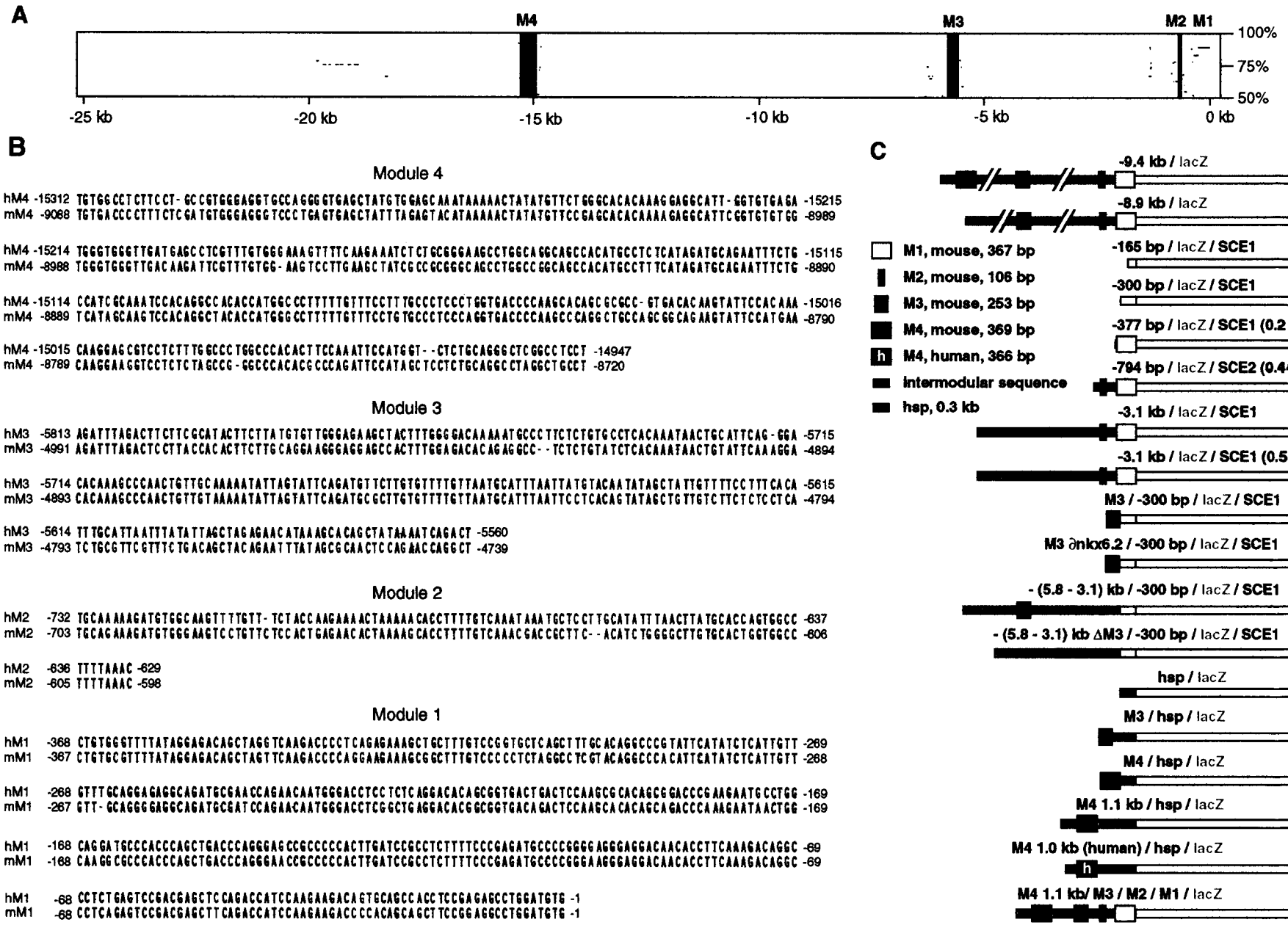

A
M4
M3
M2 M1
100%
75%
50%
-25 kb
-20 kb
-15 kb
-10 kb
-5 kb
0 kb
B
Module 4
hM4 -15312 TGTGGCCTCTTCCT-GCCGTGGGAGGTGCCAGGGGTGAGCTATGTGGAGCAAATAAAAACTATATGTTCTGGGCACACAAAGGAGGCATT-GGTGTGAGA -15215
mM4 -9088 TGTGACCCCTTTCTCGATGTGGGAGGGTCCCTGAGTGAGCTATTTAGAGTACATAAAAACTATATGTTCCGAGCACACAAAAGAGGCATTCGGTGTGTGG -8989
hM4 -15214 TGGGTGGGTTGATGAGCCTCGTTTGTGGGAAAGTTTTCAAGAAATCTCTGCGGGAAGCCTGGCAGGCAGCCACATGCCTCTCATAGATGCAGAATTTCTG -15115
mM4 -8988 TGGGTGGGTTGACAAGATTCGTTTGTGG-AAGTCCTTGAAGCTATCGCCGCGGGCAGCCTGGCCGGCAGCCACATGCCTTTCATAGATGCAGAATTTCTG -8890
hM4 -15114 CCATCGCAAATCCACAGGCCACACCATGGCCCTTTTTTGTTTCCTTTGCCCTCCCTGGTGACCCCAAGCACAGCGCGCC-GTGACACAAGTATTCCACAAA -15016
mM4 -8889 TCATAGCAAGTCCACAGGCTACACCATGGGCCTTTTTTGTTTCCTGTGCCCTCCCAGGTGACCCCAAGCCCAGGCTGCCAGCGGCAGAAGTATTCCATGAA -8790
hM4 -15015 CAAGGAGCGTCCTCTTTGGCCCTGGCCCACACTTCCAAATTCCATGGT--CTCTGCAGGGCTCGGCCTCCT -14947
mM4 -8789 CAAGGAAGGTCCTCTCTAGCCG-GGCCCACACGCCCAGATTCCATAGCTCCTCTGCAGGCCTAGGCTGCCT -8720
Module 3
hM3 -5813 AGATTTAGACTTCTTCGCATACTTCTTATGTGTTGGGAGAAGCTACTTTGGGGACAAAAATGCCCTTCTCTGTGCCTCACAAATAACTGCATTCAG-GGA -5715
mM3 -4991 AGATTTAGACTCCTTACCACACTTCTTGCAGGAAGGGAGGAGCCACTTTGGAGACACAGAGGCC--TCTCTGTATCTCACAAATAACTGTATTCAAAGGA -4894
hM3 -5714 CACAAAGCCCAACTGTTGCAAAAATATTAGTATTCAGATGTTCTTGTGTTTTGTTAATGCATTTAATTATGTACAATATAGCTATTGTTTTCCTTTCACA -5615
mM3 -4893 CACAAAGCCCAACTGTTGTAAAAATATTAGTATTCAGATGCGCTTGTGTTTTGTTAATGCATTTAATTCCTCACAGTATAGCTGTTGTCTTCTCTCCTCA -4794
hM3 -5614 TTTGCATTAATTTATATTAGCTAGAGAACATAAAGCACAGCTATAAAATCAGACT -5560
mM3 -4793 TCTGCGTTCGTTTCTGACAGCTACAGAATTTATAGCGCAACTCCAGAACCAGGCT -4739
Module 2
hM2 -732 TGCAAAAAGATGTGGCAAGTTTTGTT-TCTACCAAGAAAACTAAAAACACCTTTTGTCAAATAAATGCTCCTTGCATATTTAACTTATGCACCAGTGGCC -637
mM2 -703 TGCAGAAAGATGTGGGAAGTCCTGTTCTCCACTGAGAACACTAAAAGCACCTTTTGTCAAACGACCGCTTC--ACATCTGGGGCTTGTGCACTGGTGGCC -606
hM2 -636 TTTTAAAC -629
mM2 -605 TTTTAAAC -598
Module 1
hM1 -368 CTGTGGGTTTTATAGGAGACAGCTAGGTCAAGACCCCTCAGAGAAAGCTGCTTTGTCCGGTGCTCAGCTTTGCACAGGCCCGTATTCATATCTCATTGTT -269
mM1 -367 CTGTGCGTTTTATAGGAGACAGCTAGTTCAAGACCCCAGGAAGAAAGCGGCTTTGTCCCCCTCTAGGCCTCGTACAGGCCCACATTCATATCTCATTGTT -268
hM1 -268 GTTTGCAGGAGAGGCAGATGCGAACCAGAACAATGGGACCTCCTCTCAGGACACAGCGGTGACTGACTCCAAGCGCACAGCGGACCCGAAGAATGCCTGG -169
mM1 -267 GTT-GCAGGGGAGGCAGATGCGATCCAGAACAATGGGACCTCGGCTGAGGACACGGCGGTGACAGACTCCAAGCACACAGCAGACCCAAGAATAACTGG -169
hM1 -168 CAGGATGCCCACCCAGCTGACCCAGGGAGCCGCCCCCACTTGATCCGCCTCTTTTCCCGAGATGCCCCGGGGAGGGAGGACAACACCTTCAAAGACAGGC -69
mM1 -168 CAAGGCGCCCACCCAGCTGACCCAGGGAACCGCCCCCACTTGATCCGCCTCTTTTCCCGAGATGCCCCGGGAAGGGAGGACAACACCTTCAAAGACAGGC -69
hM1 -68 CCTCTGAGTCCGACGAGCTCCAGACCATCCAAGAAGACAGTGCAGCCACCTCCGAGAGCCTGGATGTG -1
mM1 -68 CCTCAGAGTCCGACGAGCTTCAGACCATCCAAGAAGACCCCACAGCAGCTTCCGGAGGCCTGGATGTG -1
C
-9.4 kb / lacZ
-8.9 kb / lacZ
M1, mouse, 367 bp
M2, mouse, 106 bp
M3, mouse, 253 bp
M4, mouse, 369 bp
h
M4, human, 366 bp
intermodular sequence
hsp, 0.3 kb
-165 bp / lacZ / SCE1
-300 bp / lacZ / SCE1
-377 bp / lacZ / SCE1 (0.2 kb)
-794 bp / lacZ / SCE2 (0.44 kb)
-3.1 kb / lacZ / SCE1
-3.1 kb / lacZ / SCE1 (0.5 kb)
M3 / -300 bp / lacZ / SCE1
M3 ∂nkx6.2 / -300 bp / lacZ / SCE1
- (5.8 - 3.1) kb / -300 bp / lacZ / SCE1
- (5.8 - 3.1) kb ΔM3 / -300 bp / lacZ / SCE1
hsp / lacZ
M3 / hsp / lacZ
M4 / hsp / lacZ
M4 1.1 kb / hsp / lacZ
M4 1.0 kb (human) / hsp / lacZ
h
M4 1.1 kb/ M3 / M2 / M1 / lacZ

Figure 2. The *hprt* docking site is permissive and does not de-regulate MBP targeting in myelinating glia. A, E, As predicted from pronuclear lines (Foran and Peterson, 1992; Forghani et al., 2001), a LacZ reporter construct driven by -3.1 kb of proximal promoter sequence and SCE1 is expressed in spinal cord (SpC) and brain oligodendrocytes and spinal root (SpR) Schwann cells of P18 mice (arrows). B, F, As observed with randomly integrated constructs, -3.1 kb-mediated oligodendrocyte expression gradually shuts off in adults while high level SCE1-mediated Schwann cell expression is maintained (shown at 6 wks). C, G, A reporter construct driven by -9.4 kb, and thus containing the full complement of recognized evolutionarily conserved sequence, is expressed at high levels in myelinating cells throughout development and maturity (shown at 12 wks). D, H, LacZ driven by the 0.3 kb HSP68 minimal promoter alone is expressed at high-levels only in CNS endothelial cells, with no detectable expression in either oligodendrocytes or Schwann cells (shown at 6 wks).

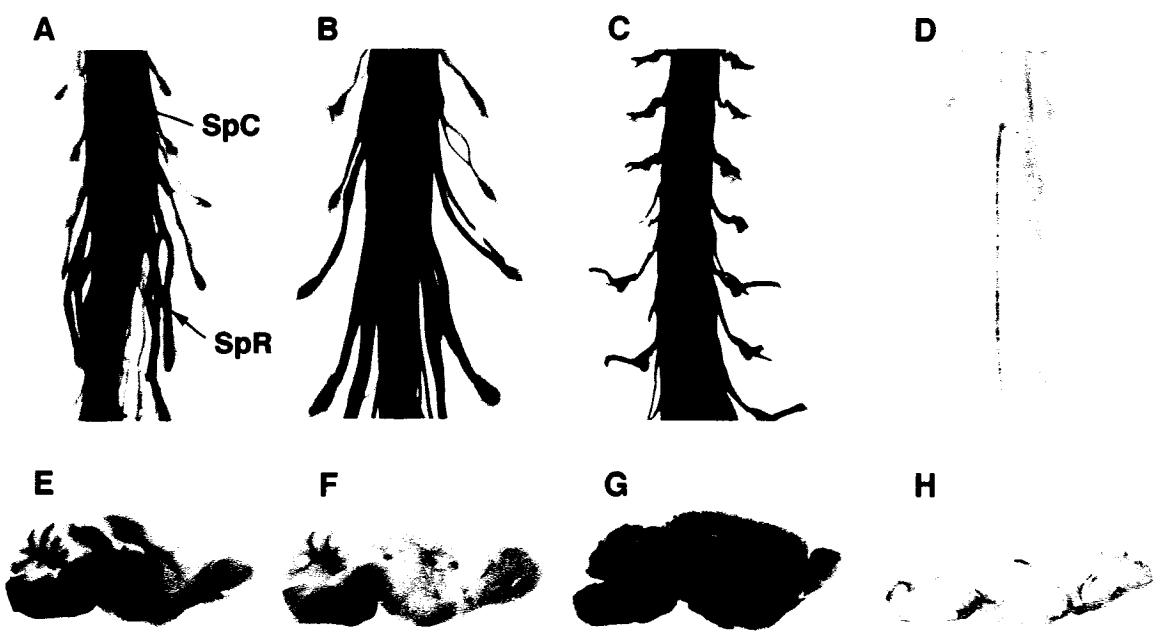
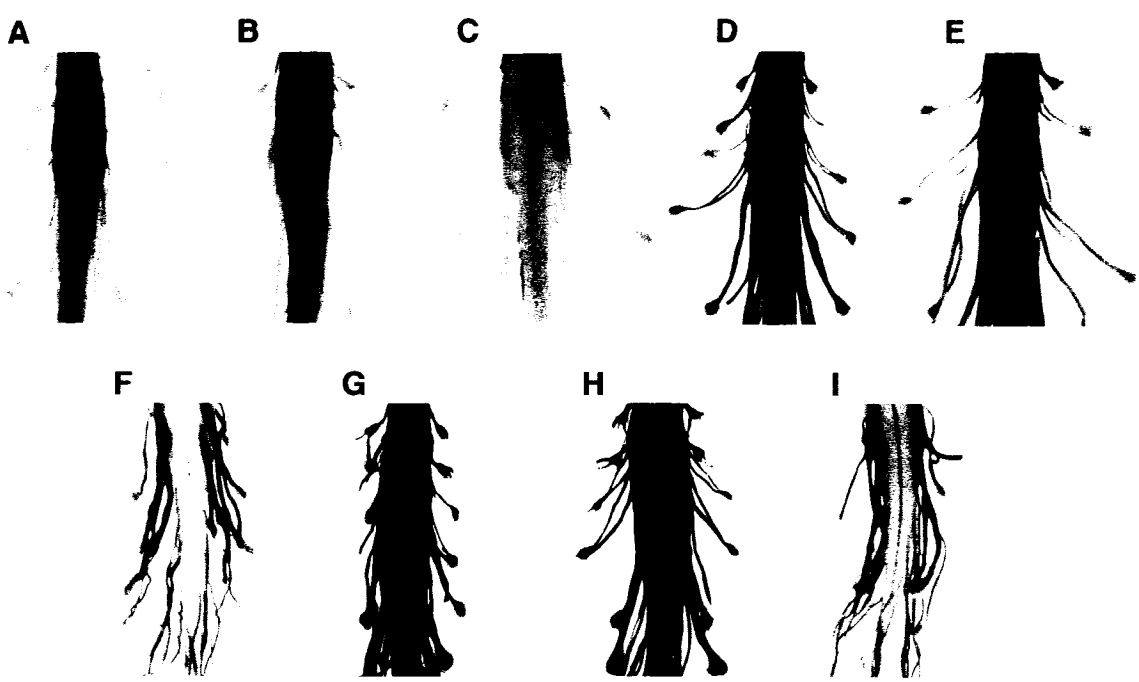

A
B
C
D
SpC
SpR
E
F
G
H

Figure 3. Multiple pathways mediate oligodendrocyte targeting during primary myelination. A, B, LacZ regulated by -377 bp/SCE1 (0.2 kb), which includes M1, or -794 bp/SCE2 (0.44 kb), which includes both M1 and M2, is expressed in spinal cord oligodendrocytes at P18. C, -794 bp/SCE2 (0.44 kb) mediated oligodendrocyte expression gradually shuts off in the adult (shown at 6 wks), in a pattern similar to that seen for the -3.1 kb/SCE1 construct. D, E, M3 has autonomous oligodendrocyte targeting ability. A 0.38 kb M3 fragment ligated to the HSP68 minimal promoter (0.3 kb) drives high level expression in spinal cord oligodendrocytes (shown at P18 and 24 wks, respectively). F, A truncated version of M1 (-300 bp) does not drive oligodendrocyte expression (shown at P18). G, H, Addition of either 0.38 kb or 2.7 kb (spanning from -5.8 to -3.1 kb) module-3-containing sequences to -300 bp MBP results in high level and continuous oligodendrocyte expression throughout development and adulthood (shown at P18). I, An otherwise identical construct, deleted of a 0.6 kb sequence spanning M3 (from -5.3 to -4.7 kb) fails to express in oligodendrocytes throughout development and adulthood (shown at P18). Constructs represented in (F-I) contain the SCE1 sequence ligated 3' of the lacZ gene, accounting for expression in Schwann cells (Forghani et al., 2001).


A
B
C
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E
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I

Figure 4. M1 and M2 do not drive expression in newly myelinating oligodendrocytes in adults. A, B, LacZ reporter constructs bearing -3.1 kb/SCE1 (0.5 kb) or -794 bp/SCE2 (0.44 kb), respectively, fail to express in newly myelinating adult oligodendrocytes six wks following spinal cord demyelination induced by intrathecal injection of CTb-saporin (Jasmin et al., 2000). C, D, Sections adjacent to histochemical preparations were processed for electron microscopy with boxed areas in (A-B) sampled in (C-D), respectively. Variable ß-galactosidase labeling is observed across the section but areas devoid of labeling contain profiles typical of newly-myelinating oligodendrocytes (boxed areas at 900X shown at 6300X on right).

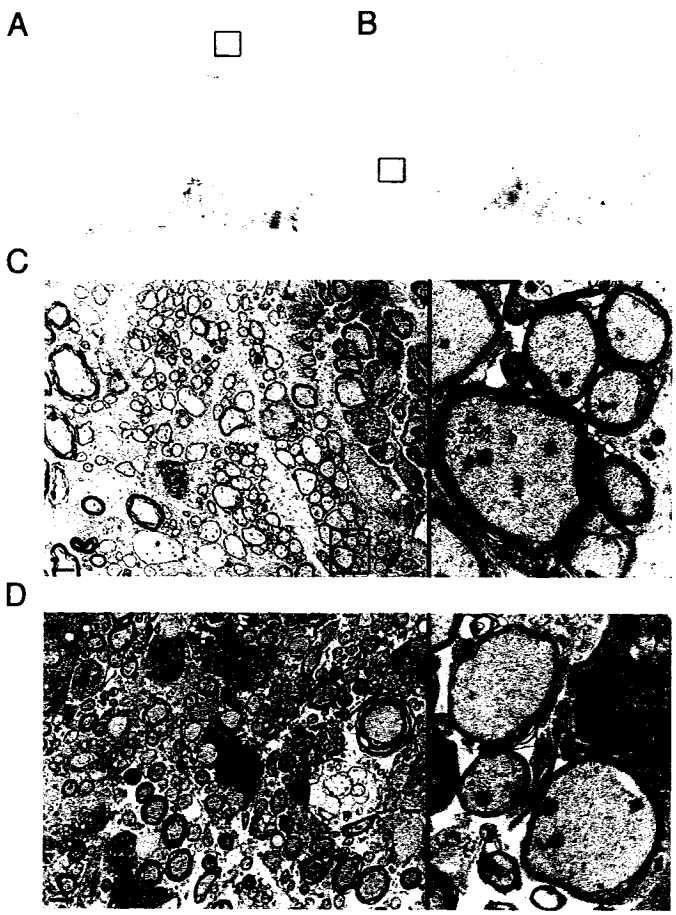
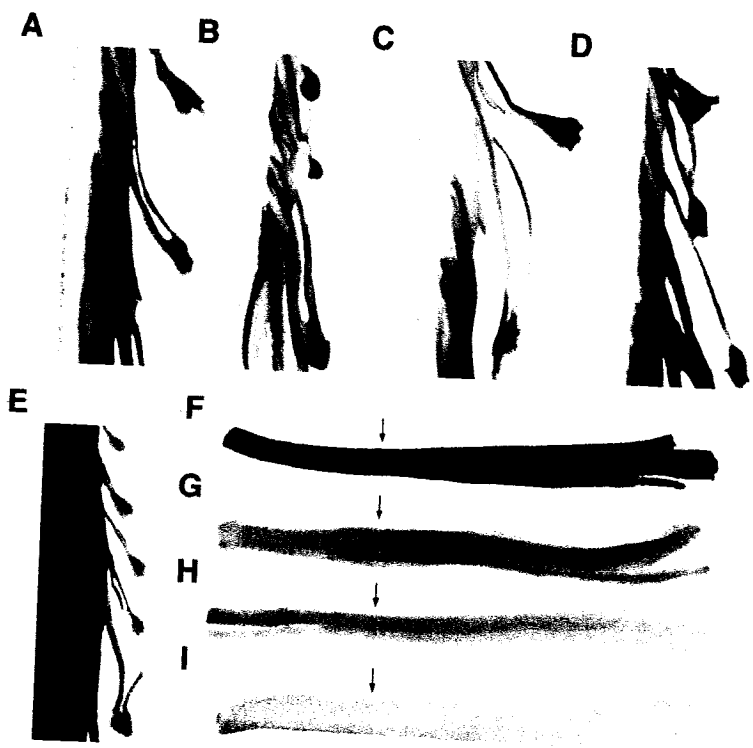

A
B
C
D

Figure 5. M3 and M4 individually target expression to myelinating Schwann cells. A, C, Reporter constructs M4 1.1 kb/hsp, M4/hsp, M4 1.0 kb (human)/hsp all demonstrate high level expression in spinal root Schwann cells throughout primary development and in the adult (all shown at 12 wks). D, A randomly inserted M4 1.0 kb (human)/hsp reporter construct similarly results in high-level Schwann cell expression (shown at 12 wks). E, The M3/hsp reporter construct drives high level expression in spinal root Schwann cells but only during active myelin formation in primary development (shown at P10). F, The M4 1.1 kb/hsp construct is expressed at high levels in both mature (left of arrow) and remyelinating (right of arrow) Schwann cells (shown at 21dpc). G, H, The M3/hsp construct also drives expression in Schwann cells during the initial phase of remyelination following sciatic nerve crush injury but gradually shuts off (portion of nerve to right of arrow), shown at 14 and 21 dpc, respectively. I, The control HSP68-promoted construct fails to express in either mature or remyelinating Schwann cells (shown at 14 dpc).


A
B
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I

Figure 6. Combined M1 to M4 targeting profile parallels that of -9.4 kb construct. The M4 1.1kb/M3/M2/M1 construct is expressed at high levels throughout development and maturity in both oligodendrocytes and Schwann cells but not in dorsal root ganglia (shown here in a chimera at 6 wks).

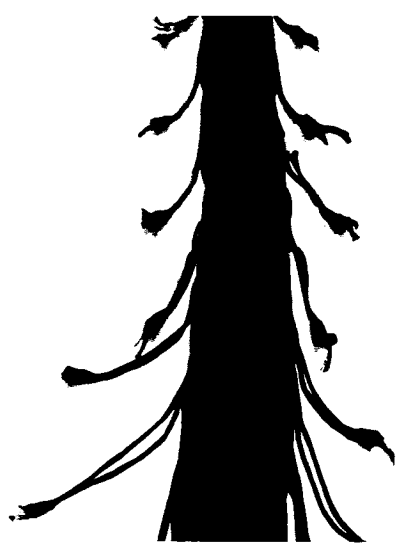


Figure 7. Multiple comparisons of β-galactosidase activity levels between transgenic lines validates quantitative analysis of *hprt*-docked reporter genes. Inset demonstrates that independently derived transgenic lines bearing the same construct at the *hprt* locus accumulate similar levels of β-galactosidase (units/μg total protein) in oligodendrocytes (comparisons between pairs of transgenic lines derived from independent ES clones bearing –3.1 kb/SCE1, -8.9 kb, and –9.4 kb constructs are shown). Comparisons of the expression levels achieved by the –9.4 kb reporter construct and constructs regulated by variously truncated MBP sequences demonstrate that significant enhancer activity is associated with all four modules.

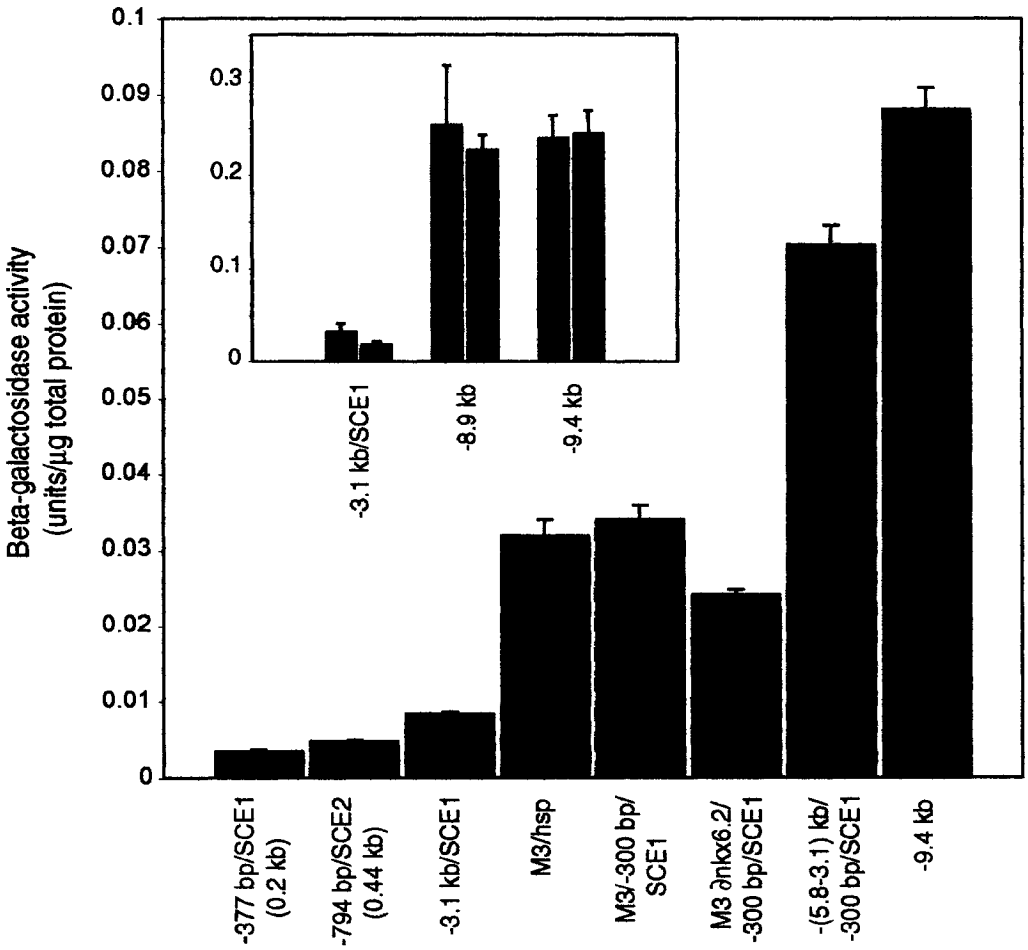
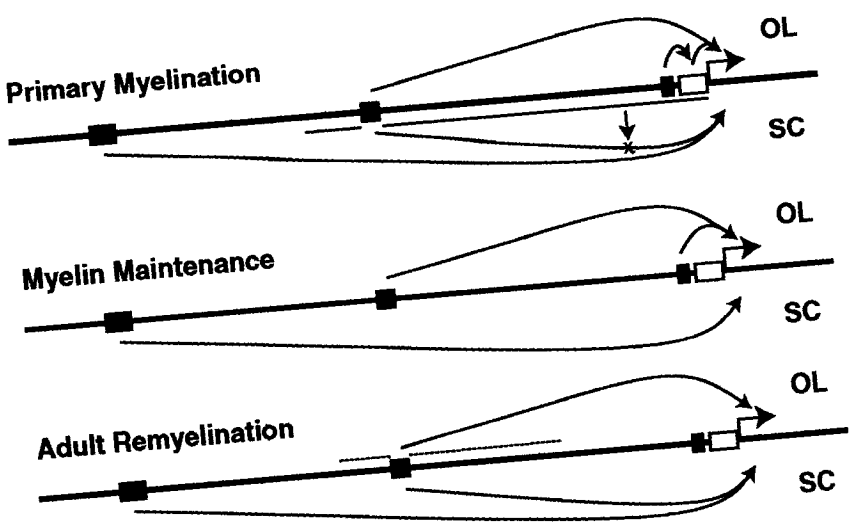

Beta-galactosidase activity
(units/µg total protein)
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0.3
0.2
0.1
0
-3.1 kb/SCE1
-8.9 kb
-9.4 kb
-377 bp/SCE1
(0.2 kb)
-794 bp/SCE2
(0.44 kb)
-3.1 kb/SCE1
M3/hsp
M3/-300 bp/
SCE1
M3 ∂nkx6.2/
-300 bp/SCE1
-(5.8-3.1) kb/
-300 bp/SCE1
-9.4 kb

Figure 8. Modular organization of the MBP *cis*-regulatory system. Schematic representation of recognized regulatory programs conferred by MBP modular sequences. Targeting activity is represented by solid arrows in oligodendrocytes (OL) and Schwann cells (SC). Potential inter-modular regulatory activity is represented by dotted lines.


Primary Myelination
OL
SC
Myelin Maintenance
OL
SC
Adult Remyelination
OL
SC

CHAPTER 3

PREFACE

This chapter presents data characterizing the functional organization of one of the MBP 5' flanking modules, module 4 located approximately 9 kb upstream of the transcriptional start site. As such, it represents one of several ongoing second generation studies evaluating the function of evolutionarily conserved regulatory motifs within a module. As seen for the sea urchin *endo-16* locus, enhancer activity requires contributions from a highly conserved targeting core as well as multiple flanking elements, with the latter modulating activity in a developmentally contextual manner.

FUNCTIONAL ORGANIZATION OF A SCHWANN CELL ENHANCER

Eric Denarier, Reza Forghani, Hooman F. Farhadi, Samar Dib, Nancy Dionne, Hana Friedman, Pierre Lepage, Thomas J. Hudson, Régen Drouin and Alan C. Peterson

Submitted to the Journal of Neuroscience

ABSTRACT

Myelin genes coordinately up regulate expression at the initiation of myelin formation, reach peak levels pre-weaning and maintain lower levels throughout maturity. For the *Myelin Basic Protein (MBP)* gene, this expression program is conferred in oligodendrocytes and Schwann cells through different upstream enhancers. In Schwann cells, expression is controlled by a 422 bp enhancer shown here to contain 22 mammalian conserved motifs $\geq$ 6 bps. To investigate their functional significance, different combinations of wild type or mutated motifs were introduced into reporter constructs that were inserted in single copy at a common HPRT docking site in ES cells. Lines of transgenic mice were derived and the subsequent qualitative and quantitative expression phenotypes were compared at different stages of maturation. In the enhancer core, seven contiguous motifs cooperate to confer Schwann cell specificity while different combinations of flanking motifs engage, at different stages of Schwann cell maturation, to modulate expression level. Mutation of a Krox-20 binding site reduces the level of reporter expression while mutation of a potential Sox element silences expression. The potential Sox motif also was found conserved in other Schwann cell enhancers suggesting

that it contributes widely to regulatory function. Our results demonstrate a close relationship between phylogenetic footprints and regulatory function and suggest a general model of enhancer organization. Finally, this investigation demonstrates that *in vivo* functional analysis supported by controlled transgenesis can be a robust complement to both molecular and bioinformatics approaches to regulatory mechanisms.

INTRODUCTION

Rapid conduction of action potentials is conferred to large vertebrate axons by their myelin sheaths. In the peripheral nervous system (PNS), myelin is elaborated and maintained by Schwann cells in response to axonal signals (LeBlanc and Poduslo, 1990). Coincident with initiation of myelin formation, genes such as *myelin basic protein (MBP), myelin protein zero (P0), and peripheral myelin protein 22 (PMP22)* are coordinately up-regulated (Stahl et al., 1990). Demonstrating the essential role played by myelin in PNS function, acquired or inherited disruptions in the formation or maintenance of myelin leads to well characterized and often severe neuropathies (Keller and Chance, 1999).

Several transcription factors are known components of the mechanism engaged to regulate Schwann cell differentiation. In the absence of the zinc finger transcription factor Krox-20, or the POU-domain transcription factor Oct-6, Schwann cells arrest at the promyelinating stage (Bermingham et al., 1996; Topilko et al., 1994). In Oct-6 mutants myelination resumes following a two-week pause while in Krox-20 mutants this block is insurmountable. Sox-10, known to cooperate with Oct-6 (Kuhlbrodt et al., 1998) and Krox-20 (Bondurand et al., 2001), also is required for myelin formation (Britsch et al., 2001). Thus, differentiation of the Schwann cell lineage is controlled through a regulatory

network of transcription factors acting at multiple stages of Schwann cell maturation. Also, evidence from *in vitro* studies suggests that these same factors may regulate individual myelin genes at later stages of maturation (Musso et al., 2001; Peirano et al., 2000).

Previous investigations designed to identify the regulatory sequences controlling myelin gene expression in Schwann cells exploited reporter constructs containing specific promoter or enhancer sequences. Regulatory sequences from MBP (Farhadi et al., 2003; Forghani et al., 2001), P0 (Feltri et al., 1999), PMP22 (Maier et al., 2003) and the transcription factors Oct-6 (Mandemakers et al., 2000) and Krox-20 (Ghislain et al., 2002) have been evaluated in transgenic mice. Such functional assays revealed diverse expression programs suggesting that myelin gene expression is controlled by a large and developmentally contextual transcription factor repertoire. However, neither the precise location nor functional relevance of individual elements has been defined clearly for any such locus in transgenic animals.

We show here, using a controlled strategy of transgenesis (Bronson et al., 1996), that phylogenetic footprints in the MBP Schwann cell enhancer are functional regulatory elements. Our findings further suggest a model of enhancer structure in which targeting elements are clustered in the enhancer core. Activity from multiple flanking motifs combines with the targeting core to confer fine control over cell specificity and expression levels. Notable differences in the enhancing elements engaged during myelin elaboration and maintenance demonstrate that the MBP expression program is controlled by different transcription factor repertoires at different stages of development. By specific mutation, we show that a binding site for Krox-20 and a motif with potential Sox-10

binding activity play major roles in enhancer function. Further, the Sox motif is conserved in other active sequences suggesting that it contributes widely to Schwann cell expression.

MATERIALS AND METHODS

Generation of constructs for HPRT docked transgenesis

The MBP –9.4 kb and –8.9 kb constructs were described previously (Farhadi et al., 2003). The construct –8.98kb was generated by cloning an annealed 80 bp oligonucleotide product (custom made by Sheldon Biotechnology Centre, McGill University, Montreal, Quebec, Canada) to the SacII site 5' of clone –8.9 kb. To clone Mod4 sub-fragments, we first generated an hsp-LacZ Entry vector in which hsp-LacZ is cloned into an Eco RV site of the pENTRIA vector (Invitrogen). Mod4 sub-domains were ligated upstream of the hsp promoter in reverse orientation relative to endogenous MBP. Entry vectors were used for *in vitro* recombination into the HPRT Destination vector that includes homology arms for the HPRT locus using the LR clonase reaction kit (Invitrogen). The final Destination vector was amplified, sequenced across the insert and linearized by Age I. 40 μg of targeting construct was used to transfect ES cells. Individual motif mutations were generated by a two step PCR introducing a TTGTT →CGAGC substitution in 135-M14mut, TGA→ATT in 135-M16mut, ACAA→CCGC in SA-M18mut, and ACA→GTT in SA-M20mut.

Derivation of transgenic mice

Transgenic animals bearing constructs in the HPRT docking site were generated by transfection of Destination constructs bearing the HPRT targeting cassette into the BK4 (for SA) or BPES5 cells, a laboratory derived heterozygous cell line. Homologous recombination simultaneously restores the deleted HPRT locus and inserts a single copy of the reporter construct into the HPRT 5' flanking region. Restoration of HPRT expression confers resistance to HAT selection thus permitting positive selection for clones derived from the desired homologous recombination event. Following selection and PCR screening, ES clones were injected into blastocysts. DNA prepared from subsequent chimeras or germline females was analyzed by Southern blot to check for single integration.

Remyelination experiments

To induce peripheral nerve regeneration, 2-month old male mice were anesthetized and unilateral sciatic nerve crushes were performed as described (Farhadi et al., 2003). The injury site was marked with India ink. Following 1, 2, or 4 weeks of recovery, injured and contralateral control nerves were harvested and separated into similar proximal and distal segments. β-galactosidase activity was measured in distal segments and compared to expression levels in uninjured contralateral samples.

Histochemical detection of β-galactosidase activity

Histochemical staining was performed as described (Forghani et al., 2001).

Quantitation of β-galactosidase activity

Sciatic nerves were dissected from backcross 2 to 3 C57BL/6 male mice and snap frozen in liquid nitrogen. 48 Samples were homogenized using a mixer mill (Qiagen) with 2 minute bursts in 250 µl of lysis buffer. Total protein concentrations were measured for all extracts in triplicate by the Bradford procedure (Bio-Rad) using a BSA standard curve. β-galactosidase activity was detected using the Galacto-Star chemiluminescent assay system (Applied Biosystems). Standard curves were generated with serial dilutions of β-galactosidase (Roche) in duplicate.

***In vivo* footprinting**

Peripheral nerves from wild type or *Trembler-J* (*TrJ*/+) mice, both on a C57Bl/6 background, were analyzed. *TrJ*/+ mice were evaluated here because the chronic demyelination/remyelination they experience leads to an enrichment of myelinating Schwann cells in their peripheral nerves. First, P4–7 wild type or adult *TrJ*/+ sciatic nerves were harvested, treated with DMS, and their DNA was then processed for Maxam & Gilbert sequencing. For *in vivo* samples, mice were sacrificed and their sciatic nerves exposed. Each nerve was then immersed in situ in Ringer's solution containing 0.5% DMS for 6 minutes, washed in cold Ca^{2+} and Mg^{2+} free PBS for 1-5 min, and finally transferred to buffer B+C (approximately 20 nerves per ml). DNA was prepared and treated as described (Drouin et al., 2001). A total of 800 bp of Mod4 containing sequence was analyzed on both strands using different primer sets. As a sequence marker, genomic DNA from liver was also prepared for sequencing. Methylated guanines from DMS exposures and chemically damaged DNA were converted into strand breaks by hot piperidine treatment. Strand break frequencies were estimated on an alkaline agarose gel

(Drouin et al., 1996). Consistent band intensity differences between *in vivo* and *in vitro* samples in two different experiments were scored as *in vivo* footprints.

Sequence analysis

The rat Mod4 sequence was obtained through the rat genome sequence available at http://genome.ucsc.edu/. An MBP containing BAC from the genomic Chicken BAC library (BACPAC resource, Oakland Research Institute) was identified using a PCR probe for chicken MBP exon 1. Positive clones were identified and amplified. DNA was sheared and ligated to pBluescript vectors. Sequences were aligned by ClustalW using MacVector 7.0 software. A VISTA plot of the Mod4 sequence was created using the VISTA server (http://gsd.lbl.gov/vista/).

Electrophoretic mobility shift assays

Oligonucleotide probes were produced by annealing the complementary strands and filling the two end overhangs with αP^{32} dCTP and Klenow. M11 CTAGCCGGCAGCCACATGCCTTTC, M11mut CTAGCCGGCAGAATTCT GCCTTTC, M12-5' CTAGTGCCTTTCATAGATGCAGAA, M12-5'mut CTAGTGCCTTTGAATTCTGCAGAA, M14 CTAGGGGCCTTTTTGTTTCCTGTG, M14mut CTAGGGGCCTTTCGAGCTCCTGTG, M16 CTAGTCCCAGGTGACCC CAAGCCC, M16mut CTAGTCCCAGGATTCCCCAAGCCC, M20 CTAGTA GCCGGGCCCACACGCCCA, Krox CTAGGTTGTACGCGGGGGCGGTTAGT. Sciatic nerve extracts were prepared as follows: 150 sciatic nerves from P10 mice were dissected, extracts were prepared and EMSA reactions (30µl) were performed as

described (Forghani et al., 1999). Bacterially expressed Krox was produced from a pET-Krox plasmid as described (Nardelli et al., 1992).

RESULTS

Inter-species sequence comparisons reveal conserved motifs within Mod4

The MBP 5' flanking sequence contains four non-coding sequences (Mod1 to Mod4) each with human/mouse conservation extending over 100 bps at 75% or greater identity (Farhadi et al., 2003). Mod4 lies approximately 9 kb upstream of the MBP start site and corresponds to a 422 bp region of conservation with Schwann cell enhancer activity. To reveal motifs that may correspond to functional transcription factor binding sites we extended the Mod4 sequence comparison to rat and chicken. Mouse/rat, mouse/human, and mouse/chicken comparisons yield respective sequence identities of 91%, 76% and 56%. Twenty-two motifs of at least 6 bps (M1 to M22) are conserved in many mammalian species and of these, 7 are invariant in the more distantly related chicken species (Fig. 1).

In vivo footprinting reveals protein/DNA interactions in the core Mod4 sequence

To locate the regions of DNA-transcription factor interaction within Mod4, we screened an 800 bp Mod4 containing sequence using the LMPCR based *in vivo* footprinting approach. DNA was derived from sciatic nerve samples from normal mice and, to potentially enrich for the population of Schwann cells actively elaborating myelin, additional samples were prepared from *Trembler-J* mice. Sciatic nerves were pre-treated with dimethylsulfate and DNA was purified and subsequently analyzed for footprints. Within the 800 bp region screened in this analysis, four footprints were detected, all

located in the core of the conserved Mod4 sequence. One is a protected guanine residue in motif 9, and three are hypersensitive sites, one between motif 10 and 11 and two in motif 15 (Fig. 1 and 2). These observations suggest that a protein complex binds to the most conserved Mod4 region while the sequences surrounding Mod4 lack similar interactions with transcription factors. Subsequent DNA-protein interaction analysis and functional characterization focused on Mod4, guided by the location of both phylogenetic and *in vivo* footprints.

MBP developmental programming is exposed by species-specific Mod4 expression

To determine if human and chicken Mod4 sequences are capable of productive interaction with mouse transcription factors, each was ligated to an hsp68-promoted LacZ reporter gene and investigated for expression in transgenic mice. To eliminate the variation associated with random integration at different insertion sites, constructs were docked, in single copy and predetermined orientation, in the 5' flanking sequence of the *hprt* locus (Bronson et al., 1996). Mod4 sequences from both species drove reporter gene expression in Schwann cells in mature mice (Fig. 3A and B). The human sequence conferred a typical post-natal expression phenotype initiating high-level expression at the commencement of myelin formation. In contrast, the chicken Mod4 reporter remained silent during pre-weaning development, when both the endogenous MBP locus and the human Mod4 reporter are highly expressed. Rather, expression of the chicken regulated reporter initiated in the peri-weaning period when myelin formation in the PNS is approaching a mature state. Thus, Mod4 elements essential for expression during mammalian myelin formation are either non-existent or too divergent in the chicken sequence to be recognized by mouse factors. Further, the location of such essential

elements must be limited to the polymorphisms existing between the chicken and human sequence (Fig. 1). The subsequent expression of the chicken Mod4 reporter in mature mice demonstrates that the MBP relevant transcription factor repertoire undergoes significant changes as Schwann cells progress from myelin elaboration to myelin maintenance.

Mapping of the targeting sequence within Mod4

To map the location of targeting elements within mouse Mod4, multiple Mod4 sub-domains were analyzed individually (Fig. 3, A and C). The 5' 210 bps lying between Bst XI and Sac II (BS), containing conserved motifs M1 through M9, failed to drive Schwann cell expression. In contrast, robust expression was conferred by the 3' 213 bp lying between Sac II to Avr II (SA) that contains motifs 10 to 22. Of note, two non-overlapping fragments of SA, 85 bps containing M10-M13 and 107bps containing M14-M20, individually showed no activity. Thus, one or more elements within each fragment are needed to confer targeting activity. As a 135 bp sequence containing M10-M16 drives Schwann cell expression, at least one of the essential elements is located within the 50bp containing M14-M16.

Subfragments of Mod4 confer responsiveness to axonal signaling

In rodents, the expression phenotypes of many myelin-related genes follow the program of myelin acquisition, with peak expression realized in the pre-weaning period (Stahl et al., 1990). To determine if the core enhancer sequences contained in the SA and 135 bp fragments are sufficient to confer normal developmental expression programming, the quantitative phenotypes realized from the respective reporter genes

were followed throughout development. Both yield post-natal expression phenotypes that closely follow the endogenous MBP program; e.g., for SA, β-galactosidase activity increases 6 fold from P2 through P10 followed by a decrease to one-fourth the peak level by 3 months of age (Fig. 4A). In a notable exception to normal programming, both sequences also drive low-level expression in fetal nerves commencing at E13.5 dpc (data not shown), indicating that they lack elements essential for normal silencing of MBP transcription in Schwann cell precursors.

High-level expression of genes encoding the major myelin proteins requires that Schwann cells achieve and maintain axonal contact. Following axon transection, denervated Schwann cells in the distal nerve segment abruptly down-regulate numerous myelin related genes such as MBP (LeBlanc and Poduslo, 1990). These are re-expressed, only when contact with in-growing axons is reestablished. To determine if the elements within the SA construct are sufficient to confer this injury/remyelination response, ß-galactosidase activity was evaluated in sciatic nerves regenerating in response to crush injury (Fig. 4B). Consistent with maintained axonal responsiveness, one week post-crush, ß-galactosidase activity in the distal segment was reduced to one-third that expressed by uninjured contralateral nerves. When in-growing axons reestablish contact with Schwann cells in the distal segment (approximately two weeks post-crush), ß-galactosidase levels increased, and by 4 weeks following injury, when axon regeneration and remyelination are largely complete, expression levels were indistinguishable from control nerves. The same axon responsive expression program was revealed for the 135bp construct (histochemical analysis data not shown). Thus, both the 213 bp SA and the internal 135bp

sequence are recognized by transcription factors that regulate MBP expression in response to axonal signals.

Mod4 contains distinct targeting and enhancing sub-domains

As observed in both developing and remyelinating preparations, Schwann cell targeting and responsiveness to axon signals are conferred by elements located within the core 135 bp sequence. To evaluate the potential role played by motifs located outside this domain, constructs differing in only small sets of additional motifs were analyzed for both qualitative and quantitative expression phenotypes. When compared to the 135 bp sequence, SA extends for 78 bps to include M17 to M22 and it drives expression at 3-fold greater levels (Fig. 5). In the 5' half of Mod4, where M1 to M9 are located, three constructs bearing contiguous MBP 5' flanking sequence, but terminating at different sites within or 5' of conserved Mod4 sequence, were analyzed. The sequence terminating at Sac II includes motifs 10 to 22. The sequence terminating at –8.98 kb adds M5 through M9 and demonstrates a marked increase in expression levels in both young and mature mice. Similarly, the further addition of the sequence containing M1 through M4 (-9.4 kb) leads to an additional increase in expression levels but, only during the pre-weaning period of myelin formation. Thus, one or all of the M1–M4 motifs confer regulatory information and recognize factor/s that distinguish pre-weaning myelin elaboration from myelin maintenance in mature mice.

Motifs in Mod4 sub domains are functional

In an attempt to assign function to specific elements we performed both band shift assays on motif specific oligonucleotides and expression analysis with mutated

constructs. Electrophoretic mobility shift assays (EMSAs) were performed with probes representing conserved motifs in the SA sequence (Fig. 1). While such putative elements could act in diverse cell types, we searched for those that engage transcription factors expressed in Schwann cells by incubating the oligonucleotides with nuclear extracts prepared from P10 sciatic nerves. Consistent shifts were detected with 4 of the 8 probes (M11, M12-5', M14 and M16) (Fig. 6A) and as one indication of specificity, none were competed with oligonucleotides bearing 3-5bp substitutions in the motif cores. Thus, some motifs appear to engage factors expressed directly in Schwann cells while those that were not shifted may require cooperative binding not achievable on short oligonucleotides or engage factors expressed in different cell types.

Motifs M14 and M16 are located in the 50 bp shown to be essential for Schwann cell targeting and M18 and M20 are the most conserved sites present in the enhancing region of SA. We therefore searched for transcription factor binding sites located in these conserved motifs. Using rVISTA software (Loots et al., 2002) several candidates with known relevance to Schwann cell biology and myelination were revealed. M14 and M18 contain a Sox family binding site with potential to engage Sox10. M16 contains sites for AP1 and nuclear receptors RORA1 and ER (Desarnaud et al., 2000; Miskimins and Miskimins, 2001) while M20 is a consensus binding site for Krox-20.

To determine directly if M20 can bind Krox-20, we performed a competition experiment using a Krox control oligonucleotide shown to bind Krox-20 in our extracts (Chavrier et al., 1988; Forghani et al., 1999). Using sciatic nerve extracts, the M20 oligonucleotide yields a complex of 3 retarded bands and, as predicted, competition was observed with the Krox oligonucleotide as diminished signal for the 2 lower bands (Fig.

6B). As a further test of specificity, we expressed Krox-20 in bacteria and using total bacterial extract for M20 band shift, a single complex is formed that again was specifically competed with the Krox control oligonucleotide. We conclude that motif M20 is able to bind Krox-20 and therefore, that Krox-20 is a likely factor contributing to the activity of the enhancer within Schwann cells. In addition, M8, in the 5' half of Mod4, has a sequence compatible with Krox-20 binding activity and consistent with this function, it was also found to bind bacterially expressed Krox-20 (data not shown). Thus, one or more Mod4 motifs appear to function through interactions with Krox-20.

To test the *in vivo* function conferred through individual motifs, constructs were derived in which mutations were introduced into the core of M14, M16, M18 and M20 motifs (Fig. 1) and the consequences of such mutations on expression phenotypes were analyzed in mice. M14 and M16 mutations were each introduced into the 135 bp sequence and both affected expression; the M14 mutation silenced expression throughout post-natal development, while the M16 mutation reduced the expression level to 20% of control values in young animals and to 40% in adults (Fig. 6C). The reduction to zero or near zero expression observed with both mutant constructs reveals the essential cooperativity between factors bound to these motifs in conferring functionality to the 135bp subdomain.

M18 and M20 were mutated in the context of the longer SA sequence. Both mutations caused expression levels to decline with activity decreased to 50% of control values at most time points. An exception is the M18 mutation at P4 where further reduction of activity approaching only 30% of the control value was observed.

As M14 and M18 share a common AACAA sequence (close to the Sox protein consensus AACAA(T/A)RG for Sox9 in Transfac), and both motifs play an essential role in enhancer function, they may bind a particularly critical factor. To determine if this factor could play a similar role in the regulation of other Schwann cell expressed genes, we searched for this motif in regulatory sequences from the Oct-6 SCE, Krox-20 MSE, PMP22 CR1, and a conserved region lying within 6 kb of sequence upstream of the P0 gene (Table 1). All sequences are included in constructs shown previously to confer Schwann cell targeting. As shown in Table 1, the motif was present in multiple copies and conserved in all of these functional sequences. Sox10, the candidate factor to bind M14, may therefore be a widely used component of the mechanism conferring expression in Schwann cells.

Functional organization of Mod4

The Mod4 organization emerging from this investigation shares numerous features with the regulatory mechanism controlling the *endo-16* locus of sea urchin (Yuh et al., 1998). Specifically, enhancer function is conferred through a small number of targeting elements operating in concert with large complexes of diverse factors that engage elements dedicated to modulating activity in response to physiological and developmental changes. When the different constructs investigated in the present study are aligned with the Mod 4 sequence conservation plot (Fig.7), the elements necessary for Schwann cell specificity are included in two peaks of high conservation corresponding to the 5' and 3' ends of the core 135 bp targeting sequence. In combination, they are essential for the basic Schwann cell targeting function while the two peaks of

conservation flanking this core confer various enhancing activities, some of which are restricted to specific developmental stages.

DISCUSSION

In this investigation, we show that M4 is composed of multiple conserved motifs, all of which contribute to the enhancer activity. We provide direct evidence that M14 is critical to enhancer function while three other motifs make important quantitative contributions. M14 and M18 present a consensus binding site for Sox10 known to be present in Schwann cells (Kuhlbrodt et al., 1998). This transcription factor contains a high mobility group domain that bends DNA to facilitate the cooperative binding of transcriptional activators (Bustin, 1999; Ellwood et al., 2000) and is known to modulate expression of myelin genes such as P0 in the PNS (Peirano et al., 2000) and MBP in the CNS (Wei et al., 2004). As suggested by our observations, it is also a critical factor in the formation of the MBP Schwann cell enhancer complex. While other M4 motifs were not found in the known sequences capable of driving Schwann cell expression in transgenic mice, the putative Sox10 binding site was found in multiple copies in all sequences and conserved in multiple species. These findings suggest an important, and perhaps central, role for Sox proteins in the regulation of Schwann cell expressed genes.

In addition to the two putative Sox10 binding sites, M4 also contains 2 motifs that are able to bind Krox20. Such potential binding site redundancy has been observed in multiple enhancers leading to a general model in which enhancers can activate through factor binding at multiple sites (Berman et al., 2002). In the present investigation, such redundancy was anticipated and we focused our functional analysis on short, but still

functional sub-sequences to illuminate the critical elements conferring Schwann cell targeting. In the 135bp construct no obvious binding site for Krox20 was found suggesting that Krox-20 is not crucial but rather, as suggested for the periaxin gene, is used to amplify the activity of the enhancer (Parkinson et al., 2003).

Using in vivo functional analysis, we demonstrate that M4 enhancer activity requires simultaneous contributions from elements located in both targeting and enhancing sub-domains. A more proximal MBP oligodendrocyte enhancer (Mod3) demonstrates a similar structure (Dionne et al., 2005) suggesting a general model of enhancer structure/function in which targeting, once established, achieves fine-tuning of expression phenotypes through the lateral recruitment of enhancing elements. Evolutionary diversification of such lateral elements could accommodate species-specific regulatory requirements and consistent with this hypothesis, divergence between mammalian and chicken M4 is more pronounced outside the targeting core. The extent to which this model is generally applicable will become evident only as the identity and location of functional elements in additional tissue specific enhancers become known.

A recent study by Taveggia et al. (2004) used reporter constructs to characterize the MBP sequences important for expression in transfected Schwann cells and oligodendrocytes. In such in vitro preparations, the Mod4 sequence was found to confer robust enhancer activity in both Schwann cells and oligodendrocytes. Amongst the transgenic lines we have evaluated to date, no construct regulated by Mod4, or its derivatives, expresses in oligodendrocytes but we cannot exclude the possibility that Mod4 modulates the quantitative oligodendrocyte expression phenotypes controlled by other MBP modules. Alternatively, as Taveggia et al. point out, glial cells cultured in the

absence of neurons may provide an unusual regulatory environment. Consistent with this potential in vitro limitation, we show here that Mod4 enhancer sub-domains are highly responsive to Schwann cell-axon interactions.

The experimental mutations introduced in this study led to dramatic effects on reporter gene expression supporting the view that natural mutations occurring in human regulatory sequences could cause, or significantly contribute to, gene deregulation and disease (Knight, 2005). If the organization of elements within MBP enhancers is typical, mutations in conserved motifs could variously silence, or significantly down regulate, transcription at one, or all, developmental stages. Further, maturation changes in the Schwann cell transcription factor repertoire, seen here for MBP regulation in juvenile and mature mice, could be a contributing factor in adult onset neuropathies.

Finally, this investigation highlights the role in vivo functional analysis can play in the investigation of mammalian regulatory mechanisms. Few techniques are capable of accurately revealing the expression phenotypes conferred by specific regulatory elements and these are most widely applied to non-vertebrate models or in vitro preparations. The results of this investigation further demonstrate that the controlled construct docking strategy introduced by Bronson et al. (1996) can be applied as an effective strategy to reveal high-resolution qualitative and quantitative in vivo expression phenotypes. By supporting comprehensive access to temporal, spatial and quantitative regulatory phenotypes, this robust in vivo approach emerges as an effective complement to both bioinformatics and molecular investigations on the structure and function of mammalian regulatory sequence.

ACKNOWLEDGEMENTS

This work was supported by grants from the Canadian Institutes of Health Research, The Canadian MS Foundation, the Canadian Genetic Diseases Network, Genome Canada and Genome Quebec. E.D. was supported by Commissariat à l'Energie Atomique, France. Doctoral fellowships were provided by CIHR (R.F. and H.F.F.), McGill University Health Centre (S.D.) and MS Society (N.D.). T.J.H. is a recipient of an investigator Award from CIHR and a Clinician-scientist Award in Translational Research from the Burroughs Wellcome Fund. R.D. holds the "Canada Research Chair in Genetics, Mutagenesis and Cancer". We thank P. Charnay for providing the pET-Krox plasmid. The authors are grateful to M. Beaudoin, F. Bourdeau, N. Dallaire, C. Dy, T. Fernandez, H. Furtenbacher, I. Paradis, I. Tretjakoff and P. Valera for excellent technical support.

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Figure 1. Sequence alignment of Mod4 from four different species. 22 motifs of at least 6 bp are conserved in mammals (highlighted and designated M1 to M22). Circles over the sequence indicate guanines footprinted by *in vivo* DNA analysis (open: protected; filled: hypersensitive). The Mod4 constructs are delineated by arrowheads along with their designations over the sequence. Oligonucleotides used in EMSA (M11 to M21) are represented by lines under the sequence and mutations are indicated as white rectangles.

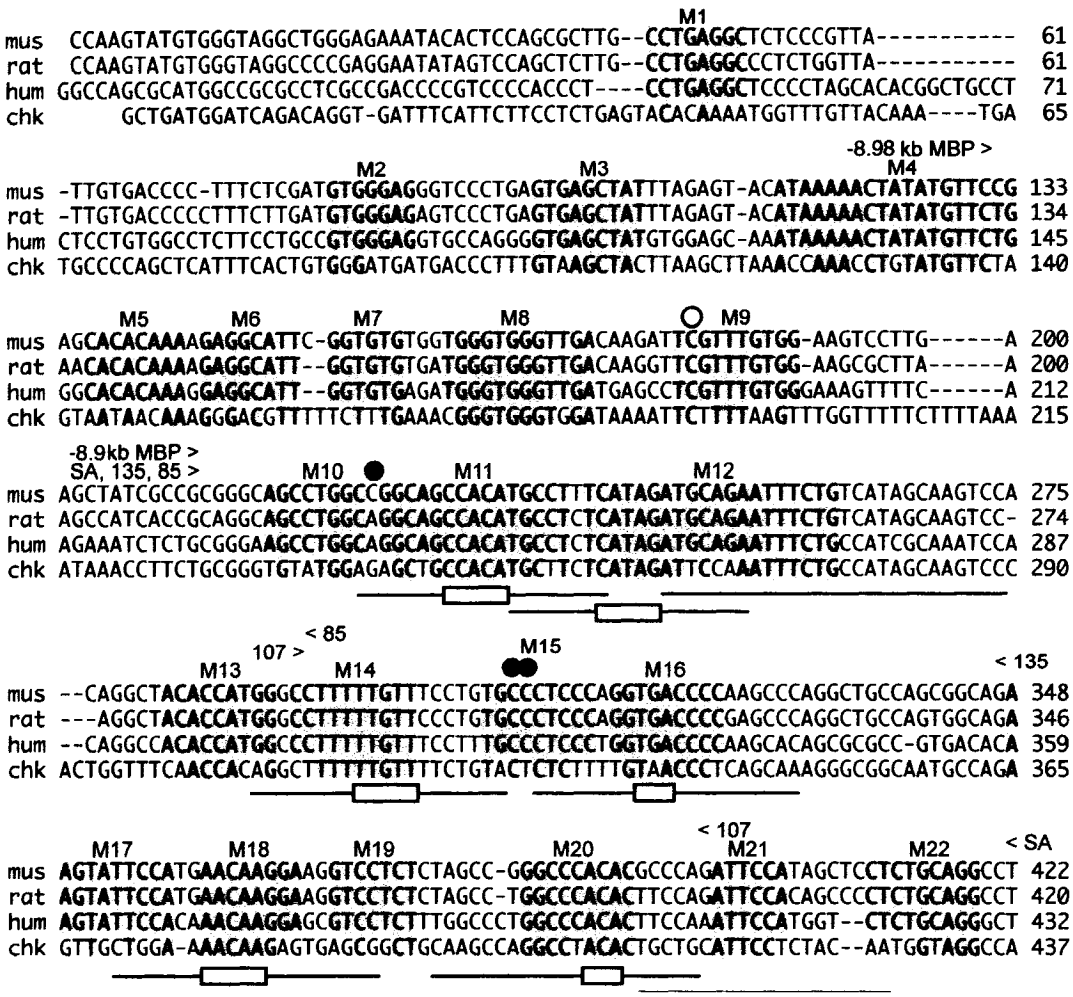

M1
mus CCAAGTATGTGGGTAGGCTGGGAGAAATACACTCCAGCGCTTG--CCTGAGGCTCTCCCGTTA---------- 61
rat CCAAGTATGTGGGTAGGCCCCGAGGAATATAGTCCAGCTCTTG--CCTGAGGCCCTCTGGTTA---------- 61
hum GGCCAGCGCATGGCCGCGCCTCGCCGACCCCGTCCCCACCCT----CCTGAGGCTCCCCTAGCACACGGCTGCCT 71
chk GCTGATGGATCAGACAGGT-GATTTCATTCTTCCTCTGAGTACACAAAATGGTTTGTTACAAA----TGA 65
-8.98 kb MBP >
M2 M3 M4
mus -TTGTGACCCC-TTTCTCGATGTGGGAGGGTCCCTGAGTGAGCTATTTAGAGT-ACATAAAAACTATATGTTCCG 133
rat -TTGTGACCCCCTTTCTTGATGTGGGAGAGTCCCTGAGTGAGCTATTTAGAGT-ACATAAAAACTATATGTTCTG 134
hum CTCCTGTGGCCTCTTCCTGCCGTGGGAGGTGCCAGGGGTGAGCTATGTGGAGC-AAATAAAAACTATATGTTCTG 145
chk TGCCCCAGCTCATTTCACTGTGGGATGATGACCCTTTGTAAGCTACTTAAGCTTAAACCAAACCTGTATGTTCTA 140
M5 M6 M7 M8 M9
mus AGCACACAAAAGAGGCATTC-GGTGTGTGGTGGGTGGGTTGACAAGATTCGTTTGTGG-AAGTCCTTG------A 200
rat AACACACAAAAGAGGCATT--GGTGTGTGATGGGTGGGTTGACAAGGTTCGTTTGTGG-AAGCGCTTA------A 200
hum GGCACACAAAGGAGGCATT--GGTGTGAGATGGGTGGGTTGATGAGCCTCGTTTGTGGGAAAGTTTTC------A 212
chk GTAATAACAAAGGGACGTTTTTCTTTGAAACGGGTGGGTGGATAAAATTCTTTTAAGTTTGGTTTTTCTTTTAAA 215
-8.9kb MBP >
SA, 135, 85 >
M10 M11 M12
mus AGCTATCGCCGCGGGCAGCCTGGCCGGCAGCCACATGCCTTTCATAGATGCAGAATTTCTGTCATAGCAAGTCCA 275
rat AGCCATCACCGCAGGCAGCCTGGCAGGCAGCCACATGCCTCTCATAGATGCAGAATTTCTGTCATAGCAAGTCC- 274
hum AGAAATCTCTGCGGGAAGCCTGGCAGGCAGCCACATGCCTCTCATAGATGCAGAATTTCTGCCATCGCAAATCCA 287
chk ATAAACCTTCTGCGGGTGTATGGAGAGCTGCCACATGCTTCTCATAGATTCCAAATTTCTGCCATAGCAAGTCCC 290
< 85
107 >
M15
M13 M14 M16 < 135
mus --CAGGCTACACCATGGGCCTTTTTGTTTCCTGTGCCCTCCCAGGTGACCCCAAGCCCAGGCTGCCAGCGGCAGA 348
rat ---AGGCTACACCATGGGCCTTTTTGTTCCCTGTGCCCTCCCAGGTGACCCCGAGCCCAGGCTGCCAGTGGCAGA 346
hum --CAGGCCACACCATGGCCCTTTTTGTTTCCTTTGCCCTCCCTGGTGACCCCAAGCACAGCGCGCC-GTGACACA 359
chk ACTGGTTTCAACCACAGGCTTTTTTGTTTTCTGTACTCTCTTTTGTAACCCTCAGCAAAGGGCGGCAATGCCAGA 365
< 107
M17 M18 M19 M20 M21 M22 < SA
mus AGTATTCCATGAACAAGGAAGGTCCTCTCTAGCC-GGGCCCACACGCCCAGATTCCATAGCTCCTCTGCAGGCCT 422
rat AGTATTCCATGAACAAGGAAGGTCCTCTCTAGCC-TGGCCCACACTTCCAGATTCCACAGCCCCTCTGCAGGCCT 420
hum AGTATTCCACAAACAAGGAGCGTCCTCTTTGGCCCTGGCCCACACTTCCAAATTCCATGGT--CTCTGCAGGGCT 432
chk GTTGCTGGA-AAACAAGAGTGAGCGGCTGCAAGCCAGGCCTACACTGCTGCATTCCTCTAC--AATGGTAGGCCA 437

Figure 2. *In vivo* footprinting experiments. DNA from pretreated sciatic nerves from P10 normal mice (WT) or adult *Trembler-J* mice (TR) were compared to purified DNA treated with dimethylsulfate (sequencing reaction lane C, T+C, A and G). Base 182 is protected while bases 225, 309 and 310 are hypersensitive. Footprinted bases are represented in Figure 1.

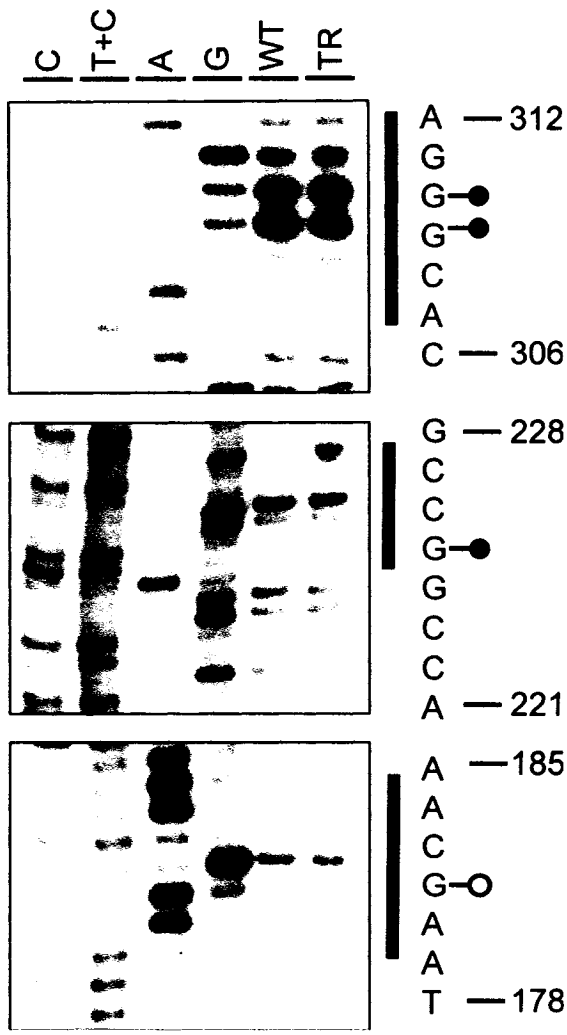
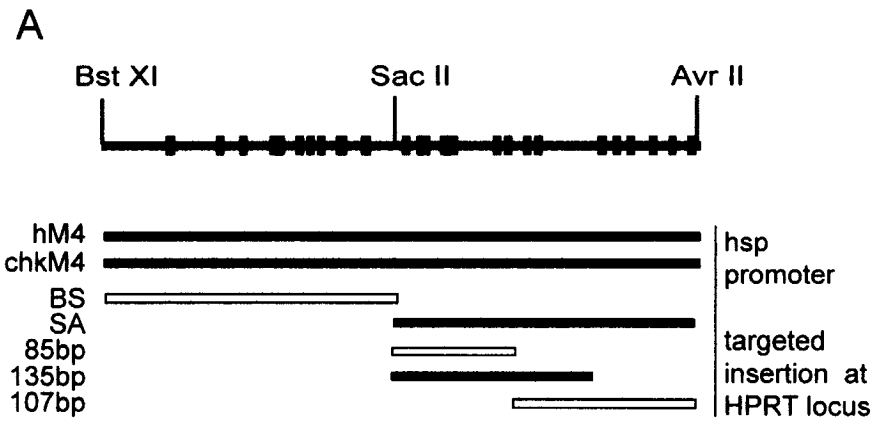
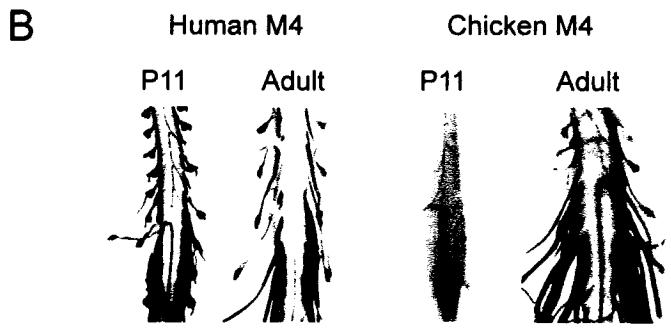

C
T+C
A
G
WT
TR
A — 312
G
G
G
C
A
C — 306
G — 228
C
C
G
G
C
C
A — 221
A — 185
A
C
G
A
A
T — 178

Figure 3. A, Distribution of motifs conserved in mammals and sequences analyzed as hspLacZ reporter constructs. Mod4 sequences that drive expression in peripheral nerves are indicated in blue. B, ß-galactosidase histochemistry of spinal cord and spinal root whole mount preparations. Human versus chicken Mod4 constructs at P11 and maturity. C, Whole mount preparations from mature mice bearing different Mod4 sub-domains. Note: central artery expression is observed with diverse reporter constructs docked at HPRT and is not related to Mod4 enhancer activity.


A
Bst XI
Sac II
Avr II
hM4
chkM4
BS
SA
85bp
135bp
107bp
hsp
promoter
targeted
insertion at
HPRT locus


B
Human M4
Chicken M4
P11
Adult
P11
Adult

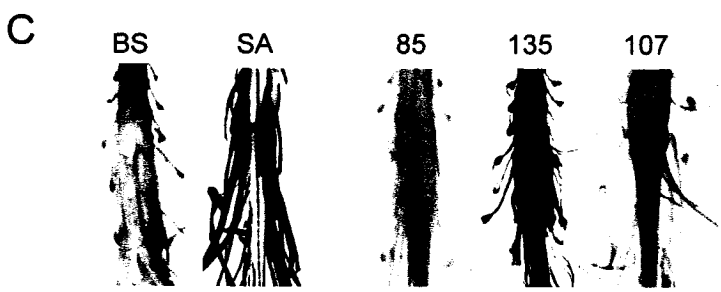
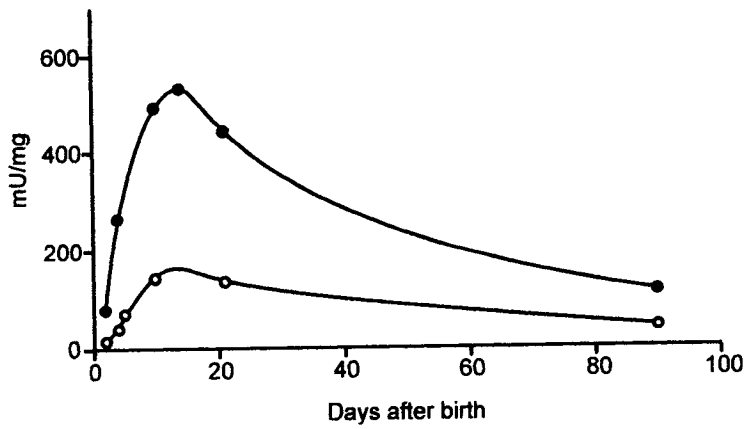

C
BS
SA
85
135
107

Figure 4. Reporter constructs are responsive to axonal signals. A, β-galactosidase activity in sciatic nerves peaks during early post-natal development. SA construct (black circles) and 135 construct (white circles). B, Expression of the SA construct following sciatic nerve injury and during remyelination of regenerating axons. β-galactosidase activity in distal nerve segments (white) 1, 2 and 4 weeks following nerve crush was compared to the uninjured contralateral nerve (black). Means +/- SD, $N \geq 5$. *** equals *t*-test result of $P<0.001$.

A


600
400
200
0
mU/mg
0
20
40
60
80
100
Days after birth

B

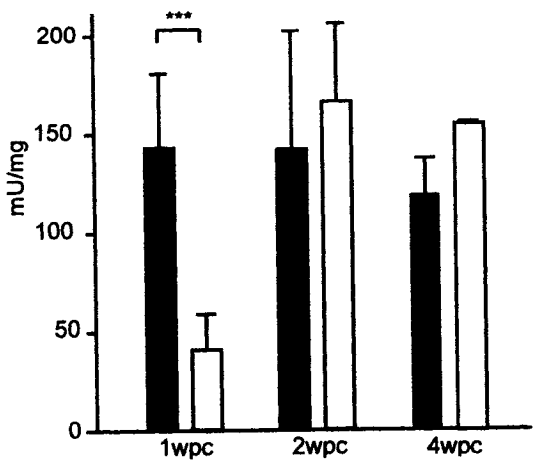

***
200
150
100
50
0
mU/mg
1wpc
2wpc
4wpc

Figure 5. Developmental expression of constructs containing progressive deletions of mod4. A, SA and 135 bp hsp promoter constructs. B, Constructs regulated by -8.9, -8.98, or -9.4 kb of MBP 5' flanking sequences containing different complements of Mod4 sequence. -8.9 kb terminates at the SacII site defining the 5' end of SA. -8.98 kb adds motifs M9 to M5 and -9.4 kb contains all 22 Mod4 motifs. Means +/- SD. *** equals *t*-test result of $P<0.001$, NS : not significant.

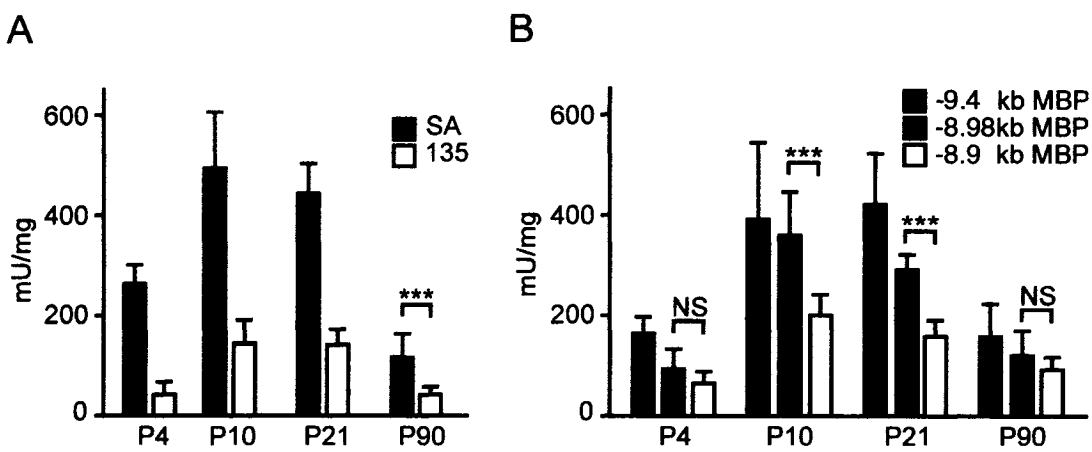
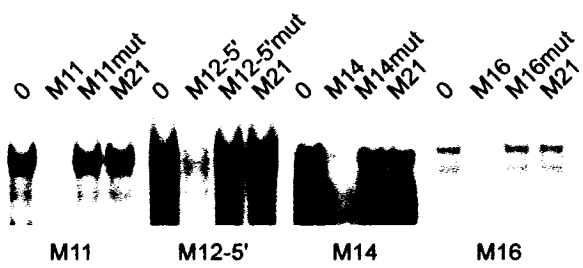
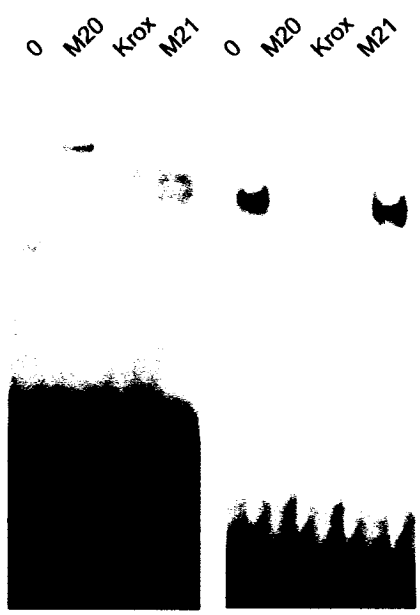

A
600
400
200
0
mU/mg
SA
135
***
P4
P10
P21
P90
B
600
400
200
0
mU/mg
-9.4 kb MBP
-8.98kb MBP
-8.9 kb MBP
NS
***
***
NS
P4
P10
P21
P90

Figure 6. A, Interaction of motifs M11, M12-5', M14 and M16 detected by EMSA. Labeled oligonucleotides were incubated with P10 sciatic nerve extracts. Competition was performed with the oligonucleotides indicated on top of each lane. Note that two specific complexes are formed with oligonucleotide M16. B, M20 was used in EMSAs with sciatic nerve extracts (left panel) or bacterially expressed Krox-20 (right panel). Competitions were performed with M20, a Krox control oligonucleotide or M21. C, β-galactosidase activity in sciatic nerve samples from mice bearing control constructs (135 and SA) or mutated constructs (135M16mut and SAM18mut and SAM20mut). The 135M14mut construct has no activity and is not displayed. Means +/- SD, *t*–test results are indicated as $P<0.05$: *; $P<0.001$: ***.

A


0
M11
M11mut
M21
0
M12-5'
M12-5'mut
M21
0
M14
M14mut
M21
0
M16
M16mut
M21
M11
M12-5'
M14
M16

B


0
M20
Krox
M21
0
M20
Krox
M21

C

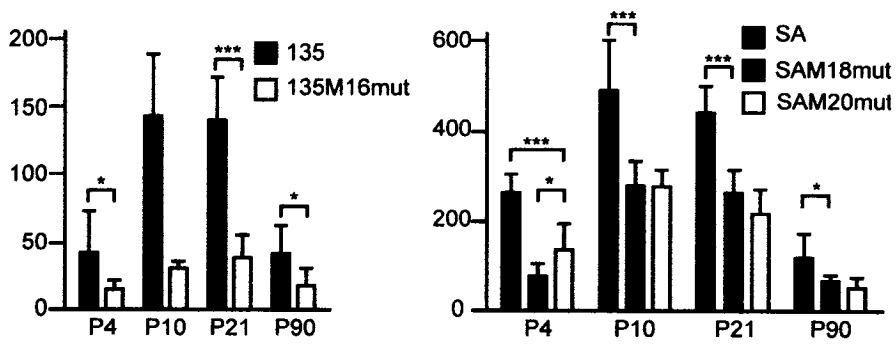

200
150
100
50
0
135
135M16mut
P4
P10
P21
P90
600
400
200
0
SA
SAM18mut
SAM20mut
P4
P10
P21
P90

Figure 7. VISTA plot of Mod4 sequence comparisons using a 20 bp window. Mouse and human (open) and mouse and chicken (filled) identities are displayed. Those sequences shown to be essential for Schwann cell targeting are shaded in blue while those conferring enhancing activity are white or shaded in grey (pre-weaning enhancing). The related Mod4 sequences included in reporter constructs are drawn below the plot.

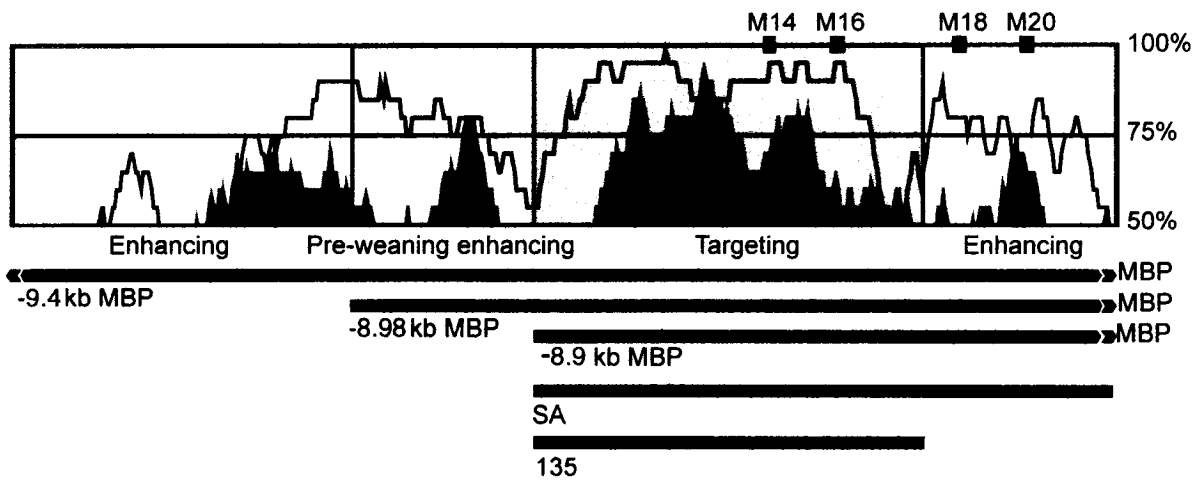

M14
M16
M18
M20
100%
75%
50%
Enhancing
Pre-weaning enhancing
Targeting
Enhancing
MBP
-9.4 kb MBP
MBP
-8.98 kb MBP
MBP
-8.9 kb MBP
SA
135

Table 1. MBP Mod4 motif 14 is present in all sequences known to be capable of conferring Schwann cell expression to reporter constructs. In these different Schwann cell targeting sequences, the M14 motif, shown in bold, is conserved in multiple mammalian species (shown for mouse, rat, human, and dog).

OCT-6 SCE1 (Mandemakers et al., 2000)	Mus Rat Hum Dog	TGACACA**AACA** **A**TCCTCAG TGACACA**AACA** **A**TCCTCAG TGACACA**AACA** **A**CCCTCAG AGGCACA**AACA** **A**CCCTTAG * ********* *** **	CCACAGA**AACAA** TGGGGGC CCACAGA**AACAA** TGGGGGC CCACAGG**AACAA** TGGGGGC CCACAGG**AACAA** TGGGGGC ****** **********	
Krox-20 MSE (Ghislain et al., 2002)	Mus Rat Hum Dog	CAGGAGT**TTGTT** GTTTCGT CAGAAGT**TTGTT** GTTTCAT CAGAAGT**TTGTT** GTTTCAT CAGAAGT**TTGTT** GTTTCAT *** ********** *	AAATGTT**AACAAT** TCAAAG AAATGCT**AACAAT** TCGAAG AAATGTT**AACAAT** CCAAAG AAATGTT**AACAAT** TCAAAG ***** ******* * ***	CAAATAT**TTGTT** CATTGCA CAAATAT**TTGTT** CATTGCA CAAATAT**TTGTT** CTTTGCA CAAATAT**TTGTT** CTTTGCA ********** *****

PMP22 CR1 (Maier et al., 2003)	Mus Rat Hum Dog	AGACCAT**AACA** **ATGAAGAG** AGACCAT**AACA** **ATGAAGAG** CTAGTCT**AACAA** TGAAGAG GATCTTC**AACAA** GGAAAAG ***** *** **	CCTAAGC**AACAAC** ACATTC CCTAAGC**AACAAC** ACACTC CCTAAGC**AACAAT** GCATTT CCTAAGC**AACAA** ACATTGG **********	
P0 (Feltri et al., 1999)	Mus Rat Hum Dog	AAGTGGG**AACA** **AATCTCTT** AAGTGGG**AACA** **AAGCTCTT** AAGTGGG**AACA** **AATCTCTT** AAGTGGG**AACA** **AATGTCTT** ********** ****	GCTGCTA**TTGTTC** CCAAAT TGGGCTA**TTGTTT** CCCAAA AGCGCTA**TTGTTT** CCCAGA AGTGCTA**TTGTTT** CCCAGA ********* ** *	

CHAPTER 4

PREFACE

The opportunity to use established transgenic lines as tools to expose the biology of myelinating glia was recognized early in the course of my graduate studies. An example is shown in this chapter on MBP-promoted transgene expression in heterozygote *Trembler-J* (Tr^J/+) Schwann cells. While wild-type mice continue to express transgenes containing the Schwann cell enhancer 1 sequence into adulthood, Tr^J/+ mice gradually shut down expression in the post-weaning period. Although this finding has not been fully reproduced in transgenic lines bearing HPRT docked constructs, a fundamental difference in the regulation of myelin protein gene expression is suggested between the primary myelinating and remyelinating glial differentiation states. Developmental phenotypes are currently being systematically illuminated using numerous crosses between transgenic mice and mice bearing mutations in myelin related genes.

MBP-LACZ TRANSGENE EXPRESSION IN JUVENILE AND ADULT *TREMBLER-J* MICE

Hooman F.Farhadi, Reza Forghani, Alan C. Peterson

Published in the Annals of the New York Academy of Sciences, 1999

INTRODUCTION AND DISCUSSION

Myelin basic protein (MBP) is expressed by both oligodendrocytes and Schwann cells. However, the regulatory elements in the *MBP* gene that drive expression in the two cell types are not the same. Transgenic mice bearing reporter constructs regulated by different lengths of MBP 5' flanking sequence have been derived to map their respective locations. The first few hundred base pairs of the promoter sufficient to target expression to oligodendrocytes, whereas an upstream enhancer element at approximately -10 kb is necessary for Schwann cell expression (Foran and Peterson, 1992; Goujet-Zalc et al., 1993; Gow et al., 1992) (also, Forghani *et al.*, manuscript submitted). Mice bearing constructs with MBP 5' flanking sequences extending up to and including the Schwann cell enhancer express ß-galactosidase in both oligodendrocytes and Schwann cells.

The *Trembler-J* (Tr^J) mutation in the PMP22 gene (Suter et al., 1992) precludes the maintenance of normal myelin in the mature PNS. Although myelination initiates and progresses in these mice, signs of myelin breakdown are obvious as early as the second postnatal week (Henry et al., 1983).Throughout maturity, Tr^J Schwann cells undergo

continuous rounds of abortive attempts at remyelination followed by division. In this investigation we asked whether the regulatory pathways that converge on the MBP Schwann cell enhancer are perturbed in Tr^J mice. Expression of the *MBP-lacZ* reporter construct containing the Schwann cell enhancer was compared in juvenile and mature normal and $Tr^J/+$ mice of the C57BL/6 strain. Whole mounts of the spinal cord and spinal roots were obtained from perfused animals and subjected to a histochemical assay for ß-galactosidase activity.

Typical of all normal mice bearing this transgene, both oligodendrocytes and Schwann cells in $Tr^J/+$ mice labeled in animals analyzed in the first few weeks of postnatal life. However, as these mice matured, transgene expression appeared to be downregulated specifically in the PNS; by three months of age, ß-galactosidase labeling could be detected only within the CNS (Fig. 1).

Our observations suggest that Schwann cells in mature $Tr^J/+$ mice are incapable of maintaining at least part of the program regulating myelin protein gene expression that they successfully access as juveniles. The basis of this unexpected difference could arise either (1) from changes at any level in the axon to Schwann cell signaling system, or (2) from intrinsic differences between juvenile and mature $Tr^J/+$ Schwann cells due, for instance, to the differentiation state either cell is able to achieve. Furthermore, this finding could extend to differences between cells undergoing primary myelination and those remyelinating axons in a mature host. These issues are currently under investigation.

Regardless of the level at which the change in transgene expression is orchestrated, our observations highlight a novel difference in the regulation of a normal myelin gene

during the juvenile phase when the elaboration of myelin normally occurs and that which is attempted in $Tr^J/+$ mice during remyelination in mature animals. The possibility that such maturation-related changes in gene regulation could contribute to the pathogenesis of CMT or additional peripheral neuropathies deserves further investigation.

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Figure 1. Gross microscopic views of the spinal cord and roots of an adult wild-type (left) and $Tr^J/+$ (right) mouse bearing the MBP-LacZ transgene (containing the Schwann cell enhancer). No labeling is evident in the PNS of the $Tr^J/+$ mouse.

WT

Tr-J

CHAPTER 5

GENERAL DISCUSSION

PREFACE

This chapter provides a general discussion of the investigations described in chapters two, three, and four, as well as a discussion of future directions and implications for glial biology and functional genomics research. By necessity, there is some redundancy between the content of this chapter and the individual discussions in previous chapters.

Axons exhibit an intimate anatomic and functional relationship with central and peripheral myelinating glia throughout development and maturity. Reciprocal axo-glial interplay occurs in a series of stages yielding phenotypically mature myelinated fibres. An important developmental milestone in the terminal differentiation of oligodendrocytes and Schwann cells is the formation of compact myelin. This is achieved, in part, through the coordinate up-regulation of genes encoding myelin structural proteins (Kidd et al., 1990; LeBlanc and Poduslo, 1990; McPhilemy et al., 1990).

The expression of reporter constructs in transgenic mice has been applied intensely to identify sequences from myelin protein genes that contain oligodendrocyte or Schwann cell specific targeting elements (Foran and Peterson, 1992; Gow et al., 1992; Gravel et al., 1998; Maier et al., 2002; Messing et al., 1992; Wight et al., 1993; Wrabetz et al., 1998). However, two general obstacles have prevented significant progress using this approach. First, regulatory sequences have been found to constitute only a small fraction of the non-coding portion of the mammalian genome. Also, the effects of elements that serve to modulate expression levels, rather than cell type specificity, are generally detectable only at low resolution in transgenic mice where constructs have inserted at random sites in the genome in variable numbers and configuration.

This thesis examines the transcriptional regulation of one representative myelin protein gene, *Myelin Basic Protein (MBP)*. To overcome the above limitations, I used a two-pronged approach combining interspecies non-coding sequence comparisons with targeted delivery of single-copy constructs at the ubiquitously expressed *hypoxanthine-guanine phosphoribosyltransferase* (*hprt)* locus.

Comparative human-mouse genomic analysis reveals four highly conserved modules

Comparison of orthologous sequences has emerged as a powerful tool in genome analysis. Such 'phylogenetic footprinting' (Pennacchio and Rubin, 2001) provides complementary data to computational, consensus sequence based predictions, as sequence conservation through evolution highlights segments in non-coding regions likely to play a functional role in the regulation of gene transcription.

As functional sequences are under selective pressure to be retained over moderate periods of evolution, the first challenge in phylogenetic footprinting is the choice of species to compare. An adequate balance between biological similarity and evolutionary distance optimizes the chances of identifying conserved sequences that appear to be evolving under evolutionary constraints in a background that has randomly diverged due to genetic drift.

In recent years, the availability of sequence from numerous species has allowed multiple interspecies comparisons to aid in calibrating the ideal evolutionary distance required for the optimal identification of functionally conserved sequences (Gottgens et al., 2002; Pennacchio and Rubin, 2001). The evolutionary distance between humans and mice places these species at strategic positions for the identification of shared functionally conserved sequences. Several studies have suggested that only a small portion (17-20%) of non-coding regions are conserved on average at this evolutionary distance (~ 80 million years) (Shabalina et al., 2001; Wasserman et al., 2000).

Due to a paucity of functional investigations, the significance of all such evolutionarily conserved non-coding sequences is yet to be fully determined. However,

the possibility that they play a gene regulatory role is being studied for an increasing number of loci. Reassuringly, functional sequences at several loci have been identified solely through the use of human-mouse genomic comparisons, further validating this assumption (Gottgens et al., 2000; Kappen and Yaworsky, 2003; Loots et al., 2000; Pennacchio et al., 2001).

Furthermore, similarity appears to be punctuated, with distinguishable segments of high similarity flanked by regions of apparently random sequence (roughly 33% nucleotide identity has been observed between random genomic sequences, with wide variations dependent upon the applied alignment algorithm, settings, and sequence characteristics (Duret and Bucher, 1997). This compartmentalized pattern of similarity is consistent with an emerging model in which multiple transcription factors bind in dense clusters termed regulatory modules (Arnone and Davidson, 1997); a circumstance which also suggests that contiguous blocks of sequence are required for transcriptional regulation.

Previous investigations, using randomly integrated reporter constructs in transgenic mice containing 1.9, 3.1, 5.8, and 8.9 kb of MBP 5' flanking sequence suggested that expression is primarily conferred through regulatory elements located within the 5' flanking region (Foran and Peterson, 1992; Forghani et al., 2001; Gow et al., 1992). These investigations also demonstrated that expression in oligodendrocytes and Schwann cells is mediated through different non-coding regions.

In an attempt to expand these observations and reveal the full complement of MBP regulatory elements, we decided to search for segments of preferential conservation in the MBP 5' flanking region by undertaking a comprehensive long-range comparison of

human and mouse genomic sequences. Four short, widely spaced, conserved modules were identified. Interestingly, modules 1, 2 and 4 overlapped (at least in part) with sequences previously implicated in oligodendrocyte or Schwann cell targeting function (Foran and Peterson, 1992; Forghani et al., 2001; Gow et al., 1992). However, module 3 represented a novel potential regulatory domain illuminated directly through comparative sequence analysis.

Identification of a complex integrated network of evolutionarily conserved MBP regulatory modules

Although phylogenetic footprints have the potential to significantly focus future investigations, genes such as *MBP*, which are regulated through complex axo-glial interactions, present a further challenge to experimental design and interpretation. This is in part due to the lack of an *in vitro* model that completely and robustly recapitulates the phenotype of myelinated fibers.

Consequently, I adopted an *in vivo* functional strategy with the potential to support high-resolution structure/function analyses. Specifically, (Bronson et al., 1996) introduced a strategy of transgenesis in which a single-copy construct is inserted in a controlled manner into a predetermined site in the 5' flanking sequence of the *hprt* locus. Thus, full control over the transgene genotype was provided and the opportunity to make direct and meaningful comparisons between the expression phenotypes of different constructs became available. As a first step, I set out to determine whether the chosen docking site is both permissive and neutral for MBP regulatory sequences. Representative

constructs, previously characterized in numerous independently derived random insertion transgenic lines, displayed predicted spatiotemporal expression patterns. Furthermore, a sensitive enhancer trap construct showed no expression in myelinating glia, thus suggesting that cell-specific enhancers for MBP were not significantly deregulated by enhancers associated with the ubiquitously expressed *hprt* locus itself. On the basis of the above results, I concluded that direct inter-construct regulatory program comparisons could be made reliably.

Constructs were then designed to interrogate the individual and combined regulatory roles conferred through the MBP modules. Throughout this analysis, *hprt* targeting allowed the use of interconstruct comparisons to assign precise spatiotemporal and quantitative phenotypes. A simple additive relationship amongst the regulatory sequences likely accounts for most of the quantitative interconstruct differences. However, summation of the expression levels derived from constructs regulated by individual modular sequences yields a value approximately one-half that observed for a construct regulated by -9.4 kb of contiguous MBP sequence. This observation may be related to either the exclusion of functionally relevant sequence from the predefined modules or to the influence of intermodular combinatorial relationships. It appears likely that, at least in part, the former explanation may be involved, especially when one considers the marked difference in expression observed for M3 alone as compared to M3 in the context of 2.3 kb of surrounding sequence.

As a second step in the analysis of the MBP regulatory program, I initiated the dissection of the modules themselves, first searching for the minimal sequence required to confer enhancer function and then to interrogate the contributions of individual motifs

with similarity to known transcription factor binding sites. Notably, M3, which appears necessary for mediating high-level expression to mature and newly myelinating adult oligodendrocytes, contains three high-affinity consensus sites for the homeodomain transcription factor Nkx6.2/Gtx (Awatramani et al., 1997). Although mutation of two of these core domains did not eliminate oligodendrocyte targeting, a significant reduction (~35%) was achieved in oligodendrocyte expression levels. Current investigations underway are examining the regulatory function of M3 subdomains and other putative transcription factor binding sites (Dionne et al., 2005).

In a gratifying extension of the earlier studies on MBP modular structure, we have now shown that similar combinatorial relationships exist at the level of elements within an individual module (Denarier et al., submitted). While in the M4 enhancer core, seven contiguous motifs cooperate to confer Schwann cell specificity, different combinations of flanking motifs engage, at different stages of Schwann cell maturation, to modulate expression levels. Mutation of a Krox-20 binding site reduces the level of reporter expression while mutation of a potential Sox element abrogates expression. The potential Sox motif also was found conserved in other Schwann cell enhancers suggesting that it contributes widely to regulatory function.

Taken together, these observations are of specific interest to myelin formation and disease. They provide the basis for a complex model in which the expression of MBP in myelinating glia is finely regulated throughout development, maturity, and following injury, through the integrated output of a network of regulatory sequences. Recently, further confirmation was provided for this combinatorial cell state dependent control of MBP expression. Using an oligodendrocyte precursor Oli-neu cell line (Gokhan et al.,

2005), transient co-transfection assays were undertaken with a luciferase reporter gene driven by -1.3 kb of MBP 5' flanking sequence. Transcription factors assayed included Olig1, Olig2, Mash1, and Sox10. Analysis of reporter gene levels revealed transcription factor expression profile and developmental stage dependent regulation, pointing to a combinatorial transcription factor interplay in the regulation of MBP expression.

Finally, from the vantage point of our current understanding, it seems probable that genes such as *MBP* are not regulated through interactions between private factors and elements but, rather, by factors and elements that are widely shared amongst the genes contributing to the myelin phenotype (Denarier et al., submitted). Thus, rather than magnifying a progressively more limited segment of glial biology, the identification of the specific regulatory elements and transcription factors that engage to regulate *MBP* appear to be opening a wide window onto the molecules and mechanisms controlling the overall myelination program.

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APPENDIX A

COMPLIANCE CERTIFICATES

4. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Check appropriate classification				Fellow
		Investigator	Technician & Research Assistant	Student		
				Undergraduate	Graduate	
Pierre Dumas	Molecular Oncology Group					x
Nancy Dionne	Molecular Oncology Group				x	
Melissa Beaudoin	Molecular Oncology Group		x			
Claire Tuascon	Molecular Oncology Group				x	
Liang Tao PhD	Molecular Oncology Group					x
Hana Friedman PhD	Molecular Oncology Group					x
Eric Denarier PhD	Molecular Oncology Group					x
Priscilla Valera	Molecular Oncology Group		x			
Samar Dib	Molecular Oncology Group				x	
Tess Fernandez	Molecular Oncology Group		x			
Christine Dy	Molecular Oncology Group		x			
Farnaz Forghani	Molecular Oncology Group				x	
Tanja Kuhlmann	Molecular Oncology Group					x

5. EMERGENCY: Person(s) designated to handle emergencies

Name: Dr. Alan Peterson Phone No: work: 934-1934 ext. 35846 home: (514) 937-3102

Name: Dr. Hana Friedman Phone No: work: 934-1934 ext. 35835 home: (514) 369-0786

6. Briefly describe:

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues) & designated biosafety risk group

We use E.coli K-12 for cloning with and propagating DNA plasmids - risk group 1

We use eukaryotic cell culture, mouse and rat cells. There are no pathogens used however it is recommended to treat all euk. cell culture as risk group 2.

We use human 293 liver transformed cell line.

We use mouse Embryonic stem cells to make transgenic mice

ii)the procedures involving biohazards 1) DNA cloning into E. coli K-12,

2) growth, transfection, RNA extraction from mouse/rat cell lines

3) Overexpression of transcription factors by transfection of human 293 liver cell line. Prodution of nuclear extracts from these cells.

4) Mouse ES cells are grown and introduced into mouse blastocysts to form transgenic mice.

iii) the protocol for decontaminating spills We would use the following protocol because we do not have large amounts of material that could spill: Small spills can be cleaned up immediately by lab personnel, provided that the organism does not pose a health risk (i.e., if the spill consists of low to moderate risk agents). Cover with a disinfectant-soaked towel (using a spray bottle for distributing the disinfectant generates aerosols and is to be avoided). Autoclave or discard contaminated material in a biomedical waste container.

7. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) which could increase the hazards of the infectious agent(s)?

NO

8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use? YES

9. What precautions are being taken to reduce production of infectious droplets and aerosols?

Euk cell culture is carried out in Class II cabinet

All material, including bacteria, disposed in biosafety disposal containers.

10. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
Hersey, Royal Vic Hospital	H5.31	Canadian Cabinets	BM4-2A-49	11925	July 25/03
Hersey, Royal Vic Hospital	H3.03d	Forma Scientific	ClassIIa/B3 1286	18160-235	July 25/03

4. Research Personnel and Qualifications

List the names of all individuals who will be in contact with animals in this study (including the Principal Investigator) and their employment classification (investigator, technician, research assistant, undergraduate/graduate student, fellow). If an undergraduate student is involved, the role of the student and the supervision received must be described. Indicate any animal related training received (e.g. workshops, lectures, etc.).

The PI certifies that all personnel listed here have suitable training and/or experience, or will be provided with the specific training which qualifies them to perform the procedures described in the protocol. The investigator is responsible for ensuring that his personnel obtain adequate training specific to the animals used and for documenting such training. The dates, place and trainer information needs to be included in the protocol. The Animal Care Committee has the authority to request additional training.

Each person listed in this section must sign to indicate that s/he has read this protocol. Emergency contacts listed in section 3 must be included here.

Space will expand as needed.

Name	Classification	Animal Related Training Information	Occupational Health & Safety Program *	Signature
Alan Peterson	Investigator	PhD mouse genetics		See attached for signatures
Tanja Kuhlmann	Investigator	MD Neuropathology		
Irene Tretjakoff	Technician	DEC in animal health		
Heidi Furtenbacher	Technician	DEC in animal health		
Hana Friedman	Res. Associate	PhD in molecular biology		
All members of the Peterson lab will take the Rodent course in Spring 2004				

*** indicate for each person, if participating in the local Occupational Health & Safety Program, see www.mcgill.ca/rgo/animal for details.**

5. Summary (in language that will be understood by members of the general public)

5 a) AIMS AND BENEFITS: Describe, in a short paragraph, the overall aim of the study and its potential benefit to human/animal health or to the advancement of scientific knowledge.

To study the protective and/or detrimental effects of cell-survival and cell-death related proteins in demyelinating diseases.

In this study the importance of the neurotrophin BDNF and key apoptosis related proteins in demyelinating disease will be investigated in vivo using transgenes in mice regulated by fully characterized regulatory sequences. We will determine the consequences of constituatively over expressing these agents to provide a framework for the development of future therapeutic strategies effective in promoting recovery in human demyelinating disease. Notably, both pro- and anti-apoptotic proteins are found in Multiple Sclerosis lesions. They are associated with cell death of oligodendroctyes (myelinating cells) and remyelination and recovery in the lesions. In cell culture experiments they variously demonstrate beneficial or detrimental effects on the survival and differentiation of oligodendrocytes.

5 b) SPECIFIC OBJECTIVES OF THE STUDY: Summarize in point form the primary objectives of this study.

We will determine if, and to what extent, overexpression of trophic factors and pro- and anti-apoptotic proteins in myelinating cell types and adjacent glia modulates the remyelination process and the fate of oligodendrocytes in demyelinating animal models. The course of demyelination and remyelination will be evaluated using a battery of quantitative and qualitative histological, immunocytochemical and ultrastructural assays.

The demyelination models to be applied are:
cuprizone demyelination
shiverer mouse mutant (central nervous sytem amyclination and peripheral nervous system hypomyelination)
trembler J mouse mutant (peripheral nervous system demyelination/remyelination)

APPENDIX B

COPYRIGHT TRANSFER AGREEMENTS

Hooman Farhadi, M.D., undertook his Ph.D. training under my supervision and is submitting his thesis in September 2005 to the Department of Neurology and Neurosurgery by Hooman F. Farhadi in partial fulfillment of the requirements of the degree of Doctor of Philosophy. The thesis is "Evolutionarily conserved non-coding sequences confer transcriptional regulation to the MBP gene" and, in conformity with the McGill Graduate program, he wishes to included both of the above manuscripts in their entirety as separate chapters in his thesis.

Thank you for this consideration,
Sincerely,

Alan C. Peterson.

------ End of Forwarded Message

APPENDIX C

SIGNED WAIVERS FROM CO-AUTHORS