Development/Plasticity/Repair

A Combinatorial Network of Evolutionarily Conserved *Myelin Basic Protein* Regulatory Sequences Confers Distinct Glial-Specific Phenotypes

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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 noncoding *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 intermodular sequences, were introduced by homologous recombination into the mouse *hprt* (hypoxanthine-guanine phosphoribosyltransferase) locus. The proximal modules M1 and M2 confer comparatively low-level oligodendrocyte expression primarily limited to early postnatal development, whereas the upstream M3 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. Mutation of putative Nkx6.2/Gtx sites within M3, although 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 noncoding *MBP* sequences and lead to a combinatorial model in which different regulatory modules are engaged during primary myelination, myelin maintenance, and remyelination.

Key words: **myelin basic protein; transcription; HPRT transgenesis; oligodendrocyte; Schwann cell; 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 compared with 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 (Topilko et al., 1994; Bermingham et al., 1996; Awatramani et al., 1997; Britsch et al., 2001; 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, myelin basic 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; Messing et al., 1992; Wight et al., 1993; Gravel et al., 1998; Wrabetz et al., 1998; Maier et al.,

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2002). 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* (hypoxanthine-guanine phosphoribosyltransferase) locus resolves these issues, allowing for higher-resolution interconstruct comparisons (Bronson et al., 1996; Vivian et al., 1999; Cvetkovic et al., 2000).

Regulatory sequences are thought to constitute a small fraction of the noncoding portion of the mammalian genome (Waterston et al., 2002). Computational sequence analysis and interspecies comparisons have been used to identify conserved noncoding sequences with potential gene regulatory properties. To date, however, evolutionarily conserved sequences associated with only a few loci have been assigned regulatory functions (Oeltjen et al., 1997; Gottgens et al., 2000; Loots et al., 2000; Pennacchio et al., 2001).

In this study, we combined human–mouse noncoding 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 after injury. 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 was validated with *Golli/MBP* exon-specific primers. PAC DNA was prepared from 2L cell cultures in LB-Broth containing 30 μg/ml kanamycin. DNA was isolated using a midi-prep kit following the instructions of the manufacturer (Qiagen, Hilden, Germany). 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, Beverly, MA). The fragments were gel purified twice to select for 2 kb fragments and ligated into an M13mp18 vector digested with *Sma*I. The reactions were then transformed into XL-2 competent cells (Stratagene, La Jolla, CA). Individual plaques of M13 subclones were grown for 16 hr 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 Biosystems, Foster City, CA) following the instructions of the manufacturer. Isolation of a mouse *MBP* genomic lambda DashII clone (~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, Foster City, CA). Sequencing reactions included ~400 ng of M13 DNA in 1.5 and 3 μl of reaction mixture for each of the four labeled primers. The thermal cycling parameters were as folows: 15 cycles at 96°C for 10 sec, 55°C for 5 sec, and 70°C for 1 min, followed by 15 cycles at 96°C for 10 sec 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 instructions of the manufacturer (Applied Biosystems). A portion of the mouse sequence (−12 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 (Accelrys, San Diego, CA) 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 (*Ehe*I), −300 bp (*Stu*I), −377 bp (*Apa*I), or −794 bp (*Bse*RI) of proximal promoter sequence driving *lacZ* expression. A −9.4 kb construct was derived by the addition of a 0.44 kb *Pvu*II–*Sac*II 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 *Kpn*I–*Xba*I fragment encompassing M3. Deletion of a 0.6 kb fragment containing M3 was performed by digestion with *Avr*II–*Bgl*II (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 included either the complete previously described Schwann cell enhancer 1 (SCE1) sequence (*Sac*II–*Sac*I fragment from −8.9 to −8.3 kb (Forghani et al., 2001) or truncated versions of 0.2 kb SCE1 (*Bst*EII–*Bgl*II from −8.8 to −8.6 kb), 0.5 kb SCE1 (*Sac*II–*Nae*I from −8.9 to −8.4 kb), or 0.44 kb of upstream SCE2 sequence (*Pvu*II–*Sac*II from −9.4 to −8.9 kb (Forghani et al., 2001), as shown in Fig. 1*C*.

Constructs containing M3 alone were generated by ligation of a 0.38 kb *Btg*I–*Ava*II fragment upstream of either −300 bp of proximal promoter sequence (as above) or a 0.3 kb *HSP68* (heat shock protein) minimal promoter ligated to *lacZ* (clone p610ZA; a gift from R. Kothary, University of Ottawa, Ottawa, Ontario, Canada). 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, Montreal, Quebec, Canada) (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 *Pvu*II–*Pvu*II fragment (spanning −9.4 to −8.3 kb), a mouse 0.4 kb *Bst*XI–*Avr*II fragment (spanning −9.1 to −8.7 kb), and a human 1.0 kb *Bss*SI–*Eco*RI 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, Hershey, PA).

Cell culture and electroporation. BK4-embryonic stem (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 (Invitrogen, Gaithersburg, MD) 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 leukemia inhibitory factor (Invitrogen). A quantity of $5–7 \times 10^6$ BK4-ES cells were electroporated with 40 μg of linearized DNA (250 V and 500 μF, Gene Pulser II; Bio-Rad, Hercules, CA). Homologous recombinants were selected on HAT-supplemented medium, containing 0.1 mM hypoxanthine, 0.0004 mM aminopterin, and 0.016 mM thymidine (Sigma, St. Louis, MO). HAT-resistant colonies were picked 14–21 d 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–L5 level with 0.3 μg of cholera toxin subunit B (CTB)-saporin (Advanced Targeting Systems, San Diego, CA) dissolved in sterile 0.9% saline. Tissues were analyzed after a 6 week 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 (two times for 10 sec each) at the midthigh level with a number 5 forceps. Sciatic nerves were analyzed at 14 or 21 d 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 and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. After postfixation for 1 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 (Leica, Wetzlar, Germany) and photographed with a Zeiss (Oberkochen, Germany) AxioCam HRc. Adjacent vibratome sections were embedded in Epon. After trimming, semithin (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 postnatal day 18 (P18) mice and homogenized with two 10 sec bursts of a Polytron homogenizer in 10 vol (0.7 ml) of lysis solution (100 mM potassium phosphate and 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 used 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, Chantilly, VA) was used following the instructions of the manufacturer for comparison of expression levels from independently derived transgenic lines bearing identical constructs (see Fig. 4*A*, inset). The fluorescent substrate DDAO galactoside [9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)galactopyranoside] (Molecular Probes, Eugene, OR) with quantitation using the Typhoon phosphorimager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA) was used for comparison of activity levels between different constructs (see Fig. 4*A*). Standard curves were generated by assaying serial dilutions of β-galactosidase (Roche Diagnostics, Hertfordshire, UK) 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 1*A* 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 β-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 to 369 bp (Fig. 1*B*) and demonstrate percentage identities of 87, 78, 79, and 82% (M1 to M4, respectively). All intermodular sequences are longer in human than in mouse, with additional 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 whether regulatory function is conferred by the conserved noncoding *MBP* sequences, we applied a controlled transgenesis strategy permitting direct interconstruct comparisons of both qualitative and quantitative regulatory phenotypes. Reporter constructs containing different configurations of modular and intermodular sequence were inserted, in single-copy and in a known orientation, at a predetermined site at the *hprt* locus (Bronson et al., 1996; Vivian et al., 1999; Cvetkovic et al., 2000) (Fig. 1*C*). 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 (Fig. 2*A*,*B*,*E*,*F*), whereas stronger promoters (−9.4 kb, Fig. 2*C*,*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. 2*D*,*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 additional features of the *hprt*-docking site chromatin and regulatory environment remain to be elucidated, this circumstance supports the use of direct interconstruct regulatory program comparisons made throughout this investigation.

Multiple pathways mediate oligodendrocyte targeting during development

The design of reporter constructs analyzed here was based on 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. 3*F*, 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. 3*A*), which

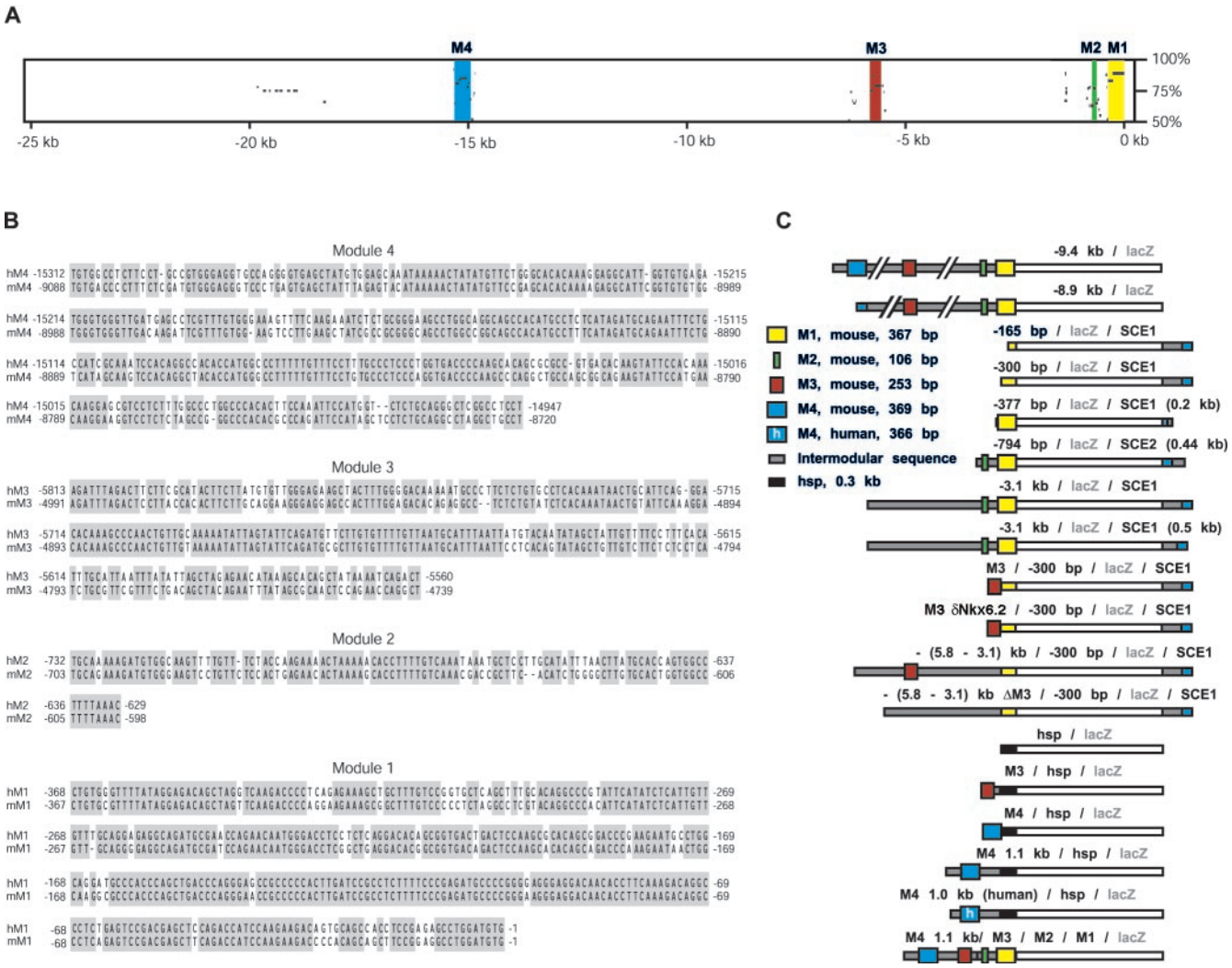

Figure 1. Human–mouse sequence comparison identifies putative regulatory modules evaluated in reporter constructs. *A*, Alignment of human (25 kb) and mouse (12 kb) *MBP* 5′ flanking sequences using the PIPMaker software. The human genomic sequence is represented on the *x*-axis, and its percentage identity to the mouse sequence (from 50 to 100%) is represented 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 *A*, correspond to the conserved modules in their entirety. Intermodular *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.

shuts off by approximately 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. 3*B*, see below) and extends the period of expression (Fig. 3*C*) (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 3 months of age, only a small oligodendrocyte subpopulation 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. 3*D*,*E*). Furthermore, an identical CNS expression pattern is observed when either the 0.38 or 2.7 kb (from −5.8 to −3.1 kb) M3-containing sequence is ligated to the nontargeting (minimal) −300 bp *MBP* promoter (Fig. 3*F–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. 3*I*).

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 on the stage of CNS maturation, M1, M2, and M3 all contribute to oligodendrocyte expression. Whereas 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. Among *MBP*-regulated constructs that express in oligodendrocytes during primary development, the −3.1 kb regulated construct fails to be reactivated in such newly myelinating adult brain oligodendrocytes, whereas a −8.9 kb regulated construct is expressed robustly (B. Finsen and A. C. Peterson, unpublished observations). Using a novel spinal cord demyelination strategy (Jasmin et al., 2000), we show here that neither the −3.1 kb (Fig. 4*A*,*C*) nor the −794 bp sequence (Fig. 4*B*,*D*) drive expression in newly myelinating oligodendrocytes analyzed at 6 weeks after 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. 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.

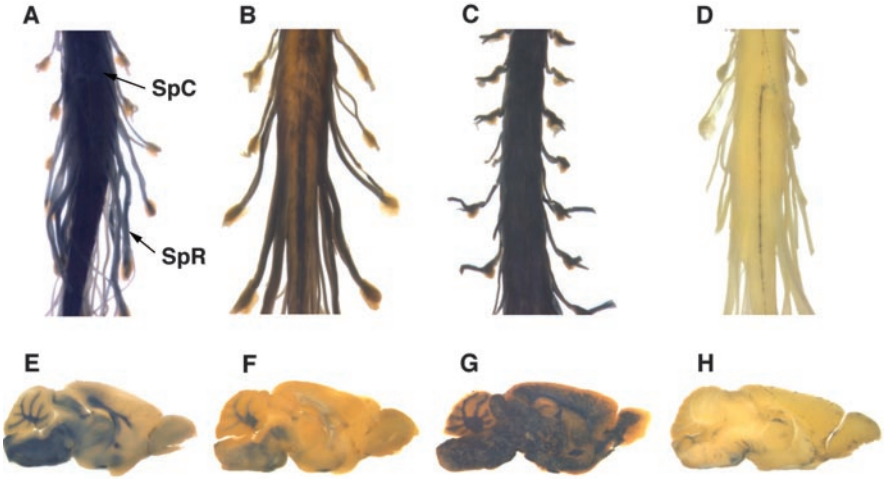

Figure 2. The *hprt*-docking site is permissive and does not deregulate *MBP* targeting in myelinating glia. *A*, *E*, As predicted from pronuclear lines (Foran and Peterson, 1992; Forghani et al., 2001), a *lacZ* reporter construct driven by −3.1 kb of proximal promoter sequence and SCE1 is expressed 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, whereas high-level SCE1-mediated Schwann cell expression is maintained (shown at 6 weeks). *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 weeks). *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 weeks).

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 after sciatic nerve crush (Fig. 5*A*,*B*,*F*). Furthermore, 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. 5*C*,*D*).

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 weeks), 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 weeks, respectively). *F*, A truncated version of M1 (−300 bp) does not drive oligodendrocyte expression (shown at P18). *G*, *H*, Addition of either 0.38 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).

Our combined data provide strong evidence that major MBP regulatory subprograms 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; Gow et al., 1992; Forghani et al., 2001). Thus, it was unexpected that a construct bearing only M3 sequences would drive expression in Schwann cells. Nonetheless, when isolated from adjacent intermodular sequences, M3 drives robust, albeit transient, Schwann cell expression during both development and sciatic nerve remyelination (Fig. 5*E*,*G*,*H*). Because the control *HSP68* construct is not expressed in either developing or remyelinating sciatic nerve Schwann cells (Fig. 5*I*), this activity is attributable to M3 alone.

Additional evidence for combinatorial regulatory control was revealed for M4-containing constructs. When divorced from additional *MBP* sequence, M4-containing constructs express

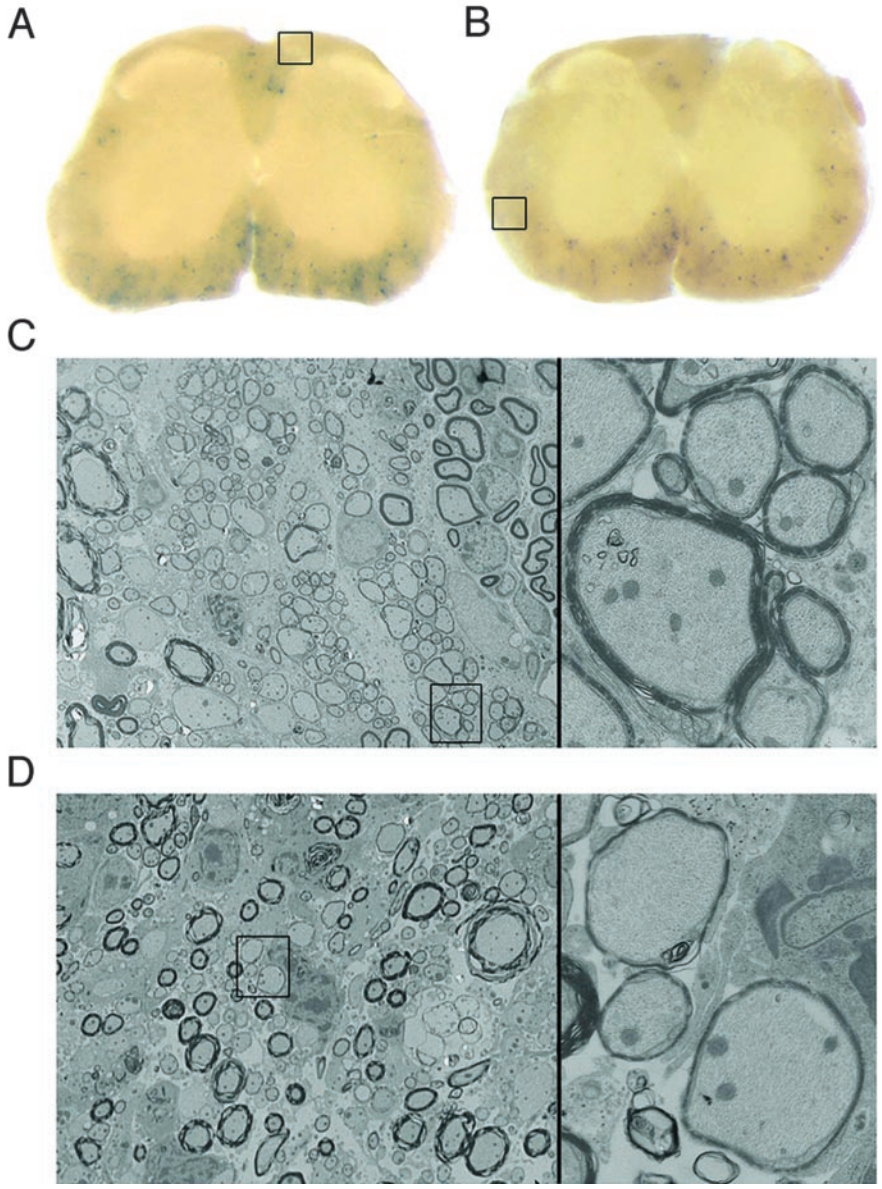

Figure 4. M1 and M2 do not drive expression in newly myelinating oligodendrocytes in adults. *A*, *B*, *lacZ* reporter constructs bearing −3.1 kb/SCE1 (0.5 kb) or −794 bp/SCE2 (0.44 kb), respectively, fail to express in newly myelinating adult oligodendrocytes 6 weeks after spinal cord demyelination 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* and *B* sampled in *C* and *D*, respectively. Variable β-galactosidase labeling is observed across the section, but areas devoid of labeling contain profiles typical of newly myelinating oligodendrocytes (boxed areas at 900× shown at 6300× on right).

ectopically in satellite glia of dorsal root ganglia (Fig. 5*A–D* and data not shown), whereas the −9.4 kb construct shows no such expression (Fig. 2*C*). 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 unmasked M3 Schwann cell enhancer activity and the M4-mediated satellite cell targeting suggest that either intermodular or modular sequences can serve to restrict the cell-specific enhancer function contained in modular sequences. Because 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 (Yuh et al., 1998, 2001; Bolouri and Davidson, 2002), quantitative functions have been shown to play a major role in the overall *cis*-regulatory program. To determine whether *MBP* regulatory sequences similarly control 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 β-galactosidase. Figure 7 (inset) shows that pairs of transgenic lines derived from independent embryonic stem cell clones bearing −3.1 kb/SCE1, −8.9 kb, or −9.4 kb constructs display similar β-galactosidase activity levels (across the broad range of quantitative phenotypes predicted from the histochemical preparations). Thus, we conclude that differences in interconstruct β-galactosidase activity levels can be reliably ascribed to the complement of regulatory elements contained within each construct.

Our investigations reveal extensive quantitative modulation because mice bearing the various constructs demonstrated a wide range of oligodendrocyte β-galactosidase activity levels (Fig. 7). There is an ~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 β-galactosidase activity levels approximately eightfold that of −794 bp/SCE2 (0.44 kb) and 36% that of the −9.4 kb line. In addition, certain intermodular sequences also appear to be associated with significant quantitative activity; the (−5.8 to −3.1 kb)/−300 bp/SCE1 transgenic line displays ~2.1-fold β-galactosidase activity compared with 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, because 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., 1997, 2000). 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., 1997, 2000). 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 β-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.

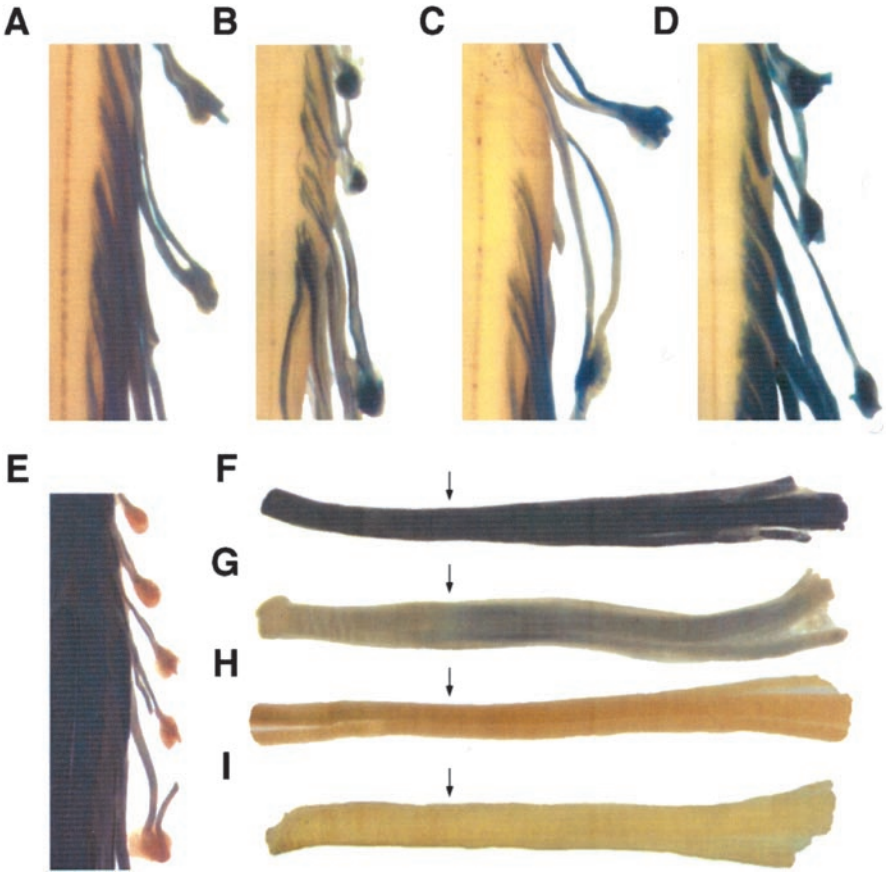

Figure 5. M3 and M4 individually target expression to myelinating Schwann cells. *A–C*, Reporter constructs M4 1.1 kb/*hsp*, M4/*hsp*, and M4 1.0 kb (human)/*hsp* all demonstrate high-level expression in spinal root Schwann cells throughout primary development and in the adult (all shown at 12 weeks). *D*, A randomly inserted M4 1.0 kb (human)/hsp reporter construct similarly results in high-level Schwann cell expression (shown at 12 weeks). *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 21 dpc). *G*, *H*, The M3/*hsp* construct also drives expression in Schwann cells during the initial phase of remyelination after 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).

Discussion

Multiple modules differentially regulate *MBP* expression in oligodendrocytes

In this investigation, we 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* noncoding sequences identified four widely spaced conserved noncoding 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. 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, whereas modular and intermodular 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 (Turnley et al., 1991; Gow et al., 1992; Miskimins et al., 1992; Goujet-Zalc et al., 1993; Wrabetz et al., 1998). Other studies, using similar promoters, have revealed ectopic expression in a number of nonneural tissues (Yoshioka et al., 1991; Asipu et al., 2001). 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, Fig. 3*F*; −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 approximately eightfold 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 expres-

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 weeks).

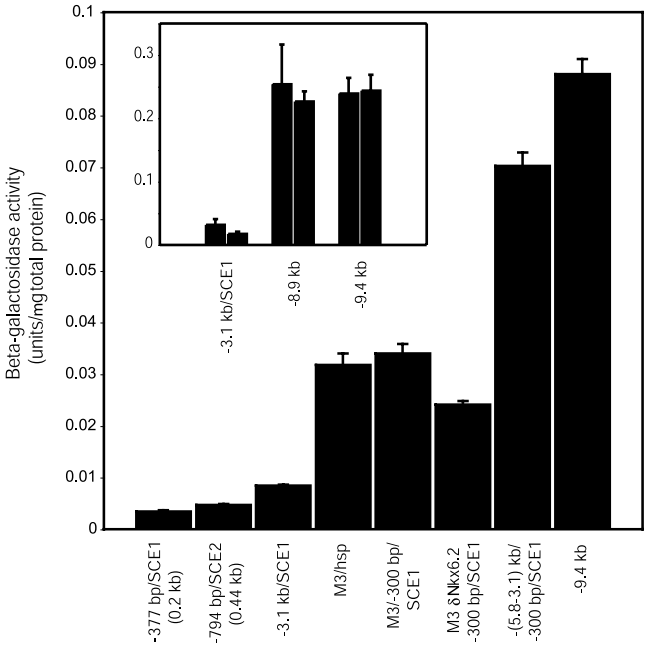

Figure 7. Multiple comparisons of β-galactosidase activity levels between transgenic lines validates quantitative analysis of *hprt*-docked reporter genes. Inset demonstrates that independently derived transgenic lines bearing the same construct at the *hprt* locus accumulate similar levels of β-galactosidase (units per micrograms of 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 three oligodendrocyte targeting modules.

sion 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

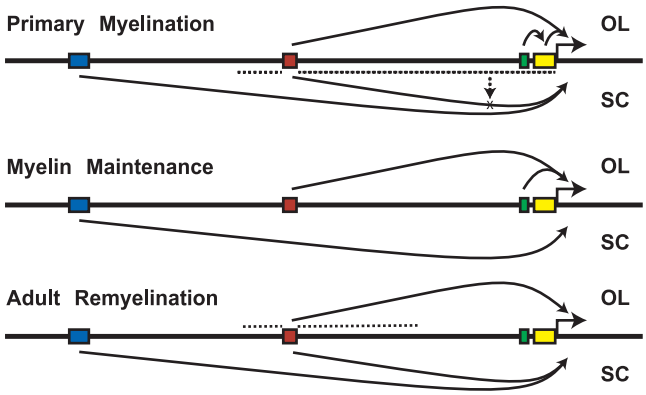

Figure 8. Modular organization of the *MBP cis*-regulatory system. Schematic representation of recognized regulatory programs conferred by *MBP* modular sequences. Targeting activity is represented by solid arrows in oligodendrocytes (OL) and Schwann cells (SC). Potential intermodular regulatory activity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

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). Unpredicted effects of copy number and integration site or the presence of introns could account for this difference. Alternatively, the −1.9 kb promoter contains a unique combination of enhancer and repressor elements not revealed by the current analysis. We consider the latter scenario less likely because 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, *hprt* targeting made it possible to use interconstruct comparisons to assign precise quantitative phenotypes. Analysis of these quantitative results suggests that most of the differences may derive from a simple additive relationship among the regulatory sequences. Notably, addition of the activities derived from constructs regulated by individual modular sequences results in a value approximating one-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 an additional 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 *proteolipid protein* proximal promoters, although none of the *MBP* putative sites previously examined encode a high-affinity TAAT core (Awatramani et al., 1997, 2000). 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). Although 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 after a demyelinating insult. Although M1 and M2 drive expression during development in the CNS, they neither maintain expression in the adult nor reinitiate expression after 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 compared with 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. Although 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 predetermined 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 after 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 additional model loci, new insights into genome organization and the structure of regulatory networks appear likely.

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