A Distal Upstream Enhancer from the *Myelin Basic Protein* Gene Regulates Expression in Myelin-Forming Schwann Cells

Reza Forghani, Lorella Garofalo, David R. Foran, Hooman F. Farhadi, Pierre Lepage, Thomas J. Hudson, Irene Tretjakoff, Priscila Valera, and Alan Peterson

Laboratory of Developmental Biology, Department of Neurology and Neurosurgery, Molecular Oncology Group H-5, McGill University, Montreal, Quebec, Canada, H3A 1A1

In peripheral nerves, large caliber axons are ensheathed by myelin-elaborating Schwann cells. Multiple lines of evidence demonstrate that expression of the genes encoding myelin structural proteins occurs in Schwann cells in response to axonal instructions. To gain further insight into the mechanisms controlling myelin gene expression, we used reporter constructs in transgenic mice to search for the DNA elements that regulate the *myelin basic protein* (*MBP*) gene. Through this *in vivo* investigation, we provide evidence for the participation of multiple, widely distributed, positive and negative elements in the overall control of *MBP* expression. Notably, all constructs bearing a 0.6 kb far-upstream sequence, designated

Schwann cell enhancer 1 (SCE1), expressed at high levels in myelin-forming Schwann cells. In addition, robust targeting activity conferred by SCE1 was shown to be independent of other *MBP* 5' flanking sequence. These observations suggest that SCE1 will make available a powerful tool to drive transgene expression in myelinating Schwann cells and that a focused analysis of the SCE1 sequence will lead to the identification of transcription factor binding sites that positively regulate *MBP* expression.

Key words: *myelin basic protein*; Schwann cells; myelination; *MBP* regulation; *MBP* regulated transgenes; sensory and motor fibers; *Krox-20*

Schwann cells arise from a common pool of neural crest cells and differentiate into myelinating or nonmyelinating glia (Mirsky and Jessen, 1996). Axonal signals control the decision to myelinate, quantitative features of the sheath, and are required for its subsequent maintenance (Aguayo et al., 1976a,b; Berthold, 1978). These axonal instructions operate, in part, by controlling the expression of the genes encoding myelin structural proteins. In PNS myelin these include protein zero (P0), peripheral myelin protein 22 (PMP22), and myelin basic protein (MBP). High-level expression of these genes coincides with myelin elaboration, continues throughout myelinogenesis, and ceases should the axon be disrupted (Gupta et al., 1988; Trapp et al., 1988; Lamperth et al., 1990; LeBlanc and Poduslo, 1990; Stahl et al., 1990; Snipes et al., 1992). Molecules implicated in the control of Schwann cell maturation and myelin formation include hormones such as progesterone, the second messenger cAMP, the zinc finger transcription factor *Krox-20*, and the POU domain transcription factor *Tst-1/SCIP/Oct-6* (*SCIP*) (Lemke and Chao, 1988; Monuki et al.,

1989, 1990; He et al., 1991; Morgan et al., 1991; Topilko et al., 1994; Koenig et al., 1995; Weinstein et al., 1995; Bermingham et al., 1996; Jaegle et al., 1996; Zorick and Lemke, 1996). However, neither the molecules that transduce axonal instructions nor the Schwann cell signaling pathways that they control are known. Similarly, the most downstream components of such pathways, the transcription factors, and DNA regulatory elements that confer expression to myelin genes remain to be elucidated.

Previously, the expression of reporter genes promoted by one to several kilobases of 5' flanking sequences from the *P0*, *MBP*, and *CNPase* genes were investigated in transgenic mice. The constructs incorporating *P0* or *CNPase* sequence expressed in Schwann cells indicating the presence of positive Schwann cell targeting elements (Messing et al., 1992; Gravel et al., 1998). In contrast, constructs incorporating similar lengths of *MBP* 5' flanking sequence were expressed only in oligodendrocytes, the myelin-forming cell type in the CNS, leaving the location of *MBP*-related Schwann cell targeting elements unknown (Foran and Peterson, 1992; Gow et al., 1992; Miskimins et al., 1992; Goujet-Zalc et al., 1993; Stankoff et al., 1996). Here, using the same approach, we searched within previously uncharacterized regions of the *MBP* locus for Schwann cell regulatory functions. Sequences contributing both positively and negatively to the *MBP* expression phenotype were found widely distributed across 12 kb of *MBP* 5' flanking sequence. We show that one of these, a 0.6 kb Schwann cell enhancer 1 (SCE1), confers high-level reporter gene expression to myelin-forming Schwann cells. Furthermore, we demonstrate that SCE1 is a classic enhancer of transcription, capable of conferring expression through a heterologous promoter and in an orientation- and position-independent manner. Because SCE1 functions robustly in isolation from further *MBP* promoter sequence, the search for *cis*-regulatory elements that positively

Received Jan. 9, 2001; revised March 2, 2001; accepted March 13, 2001.

This work was supported by the Ludwig Institute (D.R.F.), the Canadian Neuroscience Network (L.G.), and the Medical Research Council of Canada (MRC) (R.F., H.F.F.). The Canadian Neuroscience Network, the Multiple Sclerosis Society of Canada, and the MRC supported parts of this investigation. We are grateful for the technical assistance provided by C. Artigas and J. Tremblay.

R.F. and L.G. contributed equally to this investigation.

Correspondence should be addressed to A. Peterson, Laboratory of Developmental Biology, Department of Neurology and Neurosurgery, Molecular Oncology Group H-5, McGill University, 687 Pine Avenue West, Montreal, Quebec, Canada, H3A 1A1. E-mail: alan@devbiol2.molonc.mcgill.ca.

L. Garofalo's present address: Health Canada, 1600 Scott Street, Ottawa, Ontario, Canada K1A 1B8.

D. R. Foran's present address: Department of Forensic Sciences, The George Washington University, 2036 H Street, North West, Washington, DC 20052.

P. Lepage's and T. Hudson's present address: Montreal Genome Centre, McGill University Health Center, 1650 Cedar Avenue, Room L3-401, Montreal, Quebec, Canada, H3G 1A4.

Copyright © 2001 Society for Neuroscience 0270-6474/01/213780-08\$15.00/0

regulate *MBP* expression in Schwann cells can be significantly focused within this 0.6 kb.

MATERIALS AND METHODS

Generation of reporter constructs. A 15 kb *Bam*HI *MBP* genomic fragment, containing ~12.0 kb of *MBP* 5' flanking sequence, was obtained by screening a lambda DASH-129 mouse genomic library (J. Rossant, Mount Sinai, Toronto, Ontario, Canada). This sequence was subcloned in the *Bam*HI site of pSK (B clone). A *Sac*II (–9.0 kb)/*Xba*I (–3.1 kb) fragment from B clone was inserted into the respective sites in clone pm12 (3.1 kb *MBP* 5' flanking sequence in *Xba*I/*Xma*I sites of pSK–; Foran and Peterson, 1992) to generate the –9.0 kb *MBP* promoter (clone 8). B clone was digested with *Sac*II followed by intramolecular ligation of the *Sac*II ends to generate the –12.0 kb (*Bam*HI) to –9.0 kb (*Sac*II) *MBP* 5' flanking sequence in pSK (B subclone 1A) (all vectors other than pm12 from Stratagene, La Jolla, CA; restriction enzymes from New England Biolabs, Mississauga, Ontario, Canada).

To generate reporter constructs, d10 *lacZ* (Foran and Peterson, 1992) was released from a pUC18 subclone with *Sal*I and cloned in the *Sal*I site of clone 8 (3' to the –9 kb *MBP* promoter). This clone contained a double insert of *lacZ*, and the second insert was released by *Bam*HI digestion followed by intramolecular ligation (clone 5). Constructs containing 9.0 kb (*Sac*II), 8.5 kb (*Nae*I), 7.0 kb (*Sph*I), or 6.0 kb (*Kpn*I) of *MBP* promoter were obtained by restriction digestion of clone 5 and agarose (Boehringer Mannheim, Laval, Quebec, Canada) gel purification (0.5% Tris-Acetate-EDTA). A *Kpn*I/*Bam*HI fragment of clone 5 (containing –6.0 kb *MBP-lacZ*) was cloned into the respective sites in B subclone 1A to generate a clone containing the –12.0 to –9.0 5' *MBP* fragment at the 3' end of *lacZ* in 5' to 3' orientation. This clone was digested with *Kpn*I/*Sac*II and the construct was gel purified as described above. Two constructs were used to test the position and orientation independence of SCE1. SCE1 (0.6 kb; *Sac*II/*Sac*I) was isolated from clone 5 by *Sac*I digestion and cloned in the *Sac*I site of pSK+ (clone 6). We isolated –6.0 kb *MBP-lacZ* from clone 5 (*Kpn*I/*Bam*HI) and cloned it into the same sites in clone 6 to generate –6.0 *MBP-lacZ-5'*(SCE1)3'. The construct was obtained by linearizing with *Kpn*I. To test the reverse orientation, SCE1 was isolated from clone 5 (*Sac*I) and cloned in the *Sac*I site of pBS (clone 7). We isolated –6.0 *MBP-lacZ* from clone 5 with *Kpn*I and cloned it into the *Kpn*I site of clone 7 to generate –6.0 *MBP-lacZ-3'*(SCE1)5'. The construct was released with *Sph*I/*Sac*II for pronuclear injection. A clone containing SCE1 and –3.1 kb *MBP-lacZ* also was generated. We isolated 3.1 kb *MBP-lacZ* from clone pm12 with *Xba*I and cloned it into the *Xba*I site of clone 7, resulting in a clone containing 5'(SCE1)3'–3.1 kb *MBP-lacZ*. The construct was released with *Sac*II/*Sph*I.

To generate SCE1-hsp-*lacZ* constructs, the minimal 0.3 kb hsp68 promoter (*Hind*III/*Nco*I) ligated to *lacZ* (clone p610ZA; R. Kothary, University of Ottawa, Ottawa, Ontario, Canada) was used. The 0.6 kb SCE1 (*Sac*II/*Sac*I) was blunted (Klenow; Boehringer Mannheim) and inserted in the *Eco*RV site of pKS+. In clone KS-SCE 8A, the 5' end of the enhancer is closest to the *Sac*II site of the pKS+ multiple cloning site (and the 3' end closest to the *Kpn*I site). We isolated 0.3 kb hsp68-*lacZ* from clone p610ZA (*Hind*III/*Kpn*I) and cloned it into the same sites in KS-SCE 8A to generate SCE-hsp 2G (5'(SCE1)3'-hsp68-*lacZ* in pKS+). The construct was released by *Sma*I digestion and purified as above. To generate a construct having the SCE1 in 3' to 5' orientation, the 0.6 kb SCE1 (*Sac*II/*Sac*I) was cloned in the *Sac*II/*Sac*I sites of pSK+ (clone SK-SCE). We isolated 0.3 kb hsp68-*lacZ* from p610ZA (*Hind*III/*Kpn*I) and cloned it into the same sites in SK-SCE (clone SCE-hsp 1B: 3'(SCE1)5'-hsp68-*lacZ*). This construct was linearized with *Kpn*I.

To test whether the Krox-20 site within SCE1 is essential for enhancer function, a 5 bp mutation that abolishes Krox-20 binding [(–) GCGTGGGTG→GCGGTTTCG; Chavrier et al., 1990; Forghani et al., 1999] was introduced into SCE1, and the mutated SCE1 (δKrox-20-SCE1) was ligated to the 3' end of the *lacZ* reporter gene driven by the 6 kb *MBP* promoter. To generate δKrox-20-SCE1, the 0.6 kb SCE1 (*Sac*II/*Sac*I) was blunted with Klenow (Boehringer Mannheim) and inserted in the blunted (Klenow) *Xba*I site of pKS+. In clone KS-SCE 11A, the 5' end of the enhancer is closest to the *Kpn*I site of the pKS+ multiple cloning site (and the 3' end closest to the *Sac*II site). To introduce a Krox-20 mutation into SCE1, complementary oligonucleotides encoding SCE1 sequence flanked by *Mlu*I and *Sbf* I sites and bearing the mutated Krox-20 site (custom made by Sheldon Biotechnology Centre, McGill University, Montreal, Quebec, Canada) were annealed and cloned into their respective sites within clone KS-SCE 11A,

Table 1. Transgenic mice used for the functional analysis of the *MBP* promoter

Construct	Trenic line (#) or 1 [°]	Postnatal expression	
		CNS	PNS
–6.0 kb <i>MBP</i> + <i>lacZ</i>	#2	+	–
	#5	+	–
–7.0 kb <i>MBP</i> + <i>lacZ</i>	#11	+	–
	#18	+	–
	#26	+	–
–8.5 kb <i>MBP</i> + <i>lacZ</i>	#4	+	–
–9.0 kb <i>MBP</i> + <i>lacZ</i>	#7	–	–
	#17	+ ^b	+
	#24	+	+
	#32	+	+
SCE1 (5'→3') + –3.1 kb <i>MBP</i> + <i>lacZ</i>	#12	+	+
	#28	+	+
	#32	–	–
–6.0 kb <i>MBP</i> + <i>lacZ</i> + SCE1 (3'→5')	#9	+	+
	#10	+	+
	#16	+	+
–6.0 kb <i>MBP</i> + <i>lacZ</i> + SCE1 (5'→3') + pSK	#7	+	+
–6.0 kb <i>MBP</i> + <i>lacZ</i> + SCE2 (5'→3')	1 [°]	+	+
SCE1 (5'→3') + hsp + <i>lacZ</i>	#17	^b	+
	#18	–	+
	#20	–	–
	#21	–	–
	#40	^b	+
	#42	^b	+
	#46	–	–
	#49	–	+
	#54	^b	+
SCE1 (3'→5') + hsp + <i>lacZ</i> + pSK	1 [°]	^b	+
–6.0 kb <i>MBP</i> + <i>lacZ</i> + δKrox-20-SCE1 (5'→3')	#4	+	+

The designations + (indicates reporter gene expression) and – (no expression) were made based on the β-galactosidase histochemical assay, as described in Materials and Methods. Designations were made based on comprehensive analysis that included samples from the period of maximal myelin gene expression in early postnatal development (P3–P21). Constructs designated (+) were defined by obvious staining that contrasted to the failure to stain in littermates processed during the same experiment.

^a1[°] indicates that a primary transgenic mouse, rather than mice from a transgenic line, was analyzed for a given construct.

^bLines known to show unique patterns of expression outside the PNS attributed to enhancer trapping at the site of transgene insertion.

replacing the wild-type sequence and resulting in a mutant 0.6 kb SCE1 (δKrox-20-SCE1; confirmed by sequencing of both strands). Then, the final clone was generated by isolating the –6.0 kb *MBP-lacZ* from clone 5 (*Kpn*I/*Bam*HI) and cloning it into the *Kpn*I/*Bam*HI sites of pKS-δKrox-20-SCE1. The construct was released by *Kpn*I/*Sac*II digestion.

Derivation of transgenic mice. Transgenic mice were derived by injection of DNA into the pronuclei of B6C3F2 zygotes as previously described (Foran and Peterson, 1992). Injected zygotes were transplanted into the oviducts of B6C3F1 females rendered pseudopregnant by mating with vasectomized males. Litters were delivered either spon-

To determine how closely the expression conferred by SCE1 tracks the expression phenotype of the endogenous *MBP* gene, we analyzed prenatal and postnatal transgenic mice bearing SCE1-containing constructs. The endogenous *MBP* locus is expressed at low but detectable levels in the developing PNS of prenatal mice (Bachnou et al., 1997). Transgenic mice bearing SCE1-hsp68-*lacZ* constructs similarly express low but detectable levels of β -galactosidase activity in fetal peripheral nerves from embryonic

Figure 2. β -galactosidase labeling in transgenic mice bearing *MBP*-promoted, SCE-containing constructs. In transgenic mice bearing *MBP* 5' flanking sequences that include SCE1, both oligodendrocytes and Schwann cells label intensely with incubation times varying from minutes to hours. *a*, Lumbar spinal cord and attached roots from a 21-d-old -9 kb line 17 mouse. *Inset*, A 1- μ m-thick cross section of L4 ventral root embedded in plastic subsequent to whole-mount labeling. Blue-gal reaction product is associated with the majority of the myelin and Schwann cell cytoplasmic profiles. *b*, A 21-d-old mouse bearing a -6.0 kb *MBP-lacZ* construct with SCE2 ligated to the 3' end of *lacZ* expresses β -galactosidase in both oligodendrocytes and Schwann cells. *Inset*, A 1- μ m-thick cross section of whole-mount-stained L4 root demonstrates labeling of a subpopulation of myelin and Schwann cell profiles in this mosaic, primary transgenic mouse. *c*, Reporter constructs bearing the -6 kb *MBP* promoter target expression to oligodendrocytes but not Schwann cells. *d*, Teased fiber preparation from an SCE1-bearing transgenic mouse shows β -galactosidase reaction product accumulated in the cytoplasmic compartments of the Schwann cell, including the perinuclear cytoplasm (*asterisk*), Schmidt-Lantermann incisures (*closed arrowhead*), and paranodal loops (*open arrowhead*). *e*, A 12- μ m-thick cryostat cross section from a sciatic nerve of a transgenic

mouse bearing the SCE1-containing -9 kb promoter shows labeling on many myelin and Schwann cell profiles. Because the intra-Schwann cell distribution of the reaction product is not uniform along the internode (*d*), not all such profiles should be labeled. Scale bar: *a-c*, 3.1 mm; *a*, *b*, *insets*, 0.05 mm; *c*, 0.05 mm; *d*, 0.17 mm; *e*, 0.05 mm.

day 14 (E14) through birth (Fig. 4*a,b*). Also reflecting the endogenous expression program, high-level expression of β -galactosidase appeared in Schwann cells coincident with myelin formation when accumulation of endogenous MBP mRNA markedly increases. As predicted by the temporally discordant myelination programs in dorsal and ventral spinal roots of mice (Baron et al., 1994), ventral roots began to label intensely 1–2 d before dorsal roots (Fig. 4*c,d*). During the first postnatal week of mouse development, the number of myelinating Schwann cells greatly increases (Bray et al., 1977), and the apparent level of β -galactosidase accumulation, assessed by the rapidity and intensity of histochemical labeling, increased in parallel. Because similar expression programs were observed for SCE1 containing constructs promoted either by hsp68 or proximal *MBP* sequence, we conclude that this short enhancer sequence is capable of directing the major features of the endogenous *MBP* expression program during Schwann cell maturation.

Despite the close relationship between the expression in peripheral nerves of the transgene and the endogenous *MBP* locus, an apparent discrepancy was observed during midfetal development. Of the three SCE1-hsp68 lines examined (17, 18, and 42), all three consistently revealed transient β -galactosidase expression in a small subpopulation of cells in the neural tube, brain, and retina (data not shown). Because constructs bearing SCE1 with the 6 kb *MBP* promoter are not similarly expressed (our unpublished observations), putative negative regulatory elements serving to repress *MBP* expression in these cell populations appear to be located within the first 6 kb of *MBP* 5' flanking sequence. Further ectopic expression attributable to elements within SCE1 was not observed.

Throughout maturity, a stable level of MBP mRNA accumulates in peripheral nerves (LeBlanc and Poduslo, 1990; Stahl et al., 1990; Snipes et al., 1992). Reflecting this endogenous program, SCE1-hsp68-*lacZ* lines (17, 49, and 54) maintain expression throughout maturity (examined up to 7–9 months of age). In contrast, whereas intense labeling was observed in lines 18 and 42 during preweaning development, by 3 months of age only faint staining could be elicited. Such differential regulation could arise from unusual effects of transgene integration sites. Alternatively, comparison of transgene expression in mice from ongoing C57BL/6 and C3H backcross programs consistently revealed greater PNS labeling intensity in those backcrossed to C57BL/6 (data not shown). Consequently, the unique genetic background of each B6C3F1 derived transgenic line might be a contributing factor in maintaining or downregulating the mature expression phenotype.

As mice bearing the 9.0 kb promoted construct matured, there was a modest decline in β -galactosidase labeling intensity throughout their mixed nerves (lines 17 and 32) and in the mosaic line 24, expression ceased. Surprisingly and consistently, in mice >3 months of age from lines 17 and 32, dorsal roots continued to label, but a further precipitous decline in transgene expression was observed in ventral roots (Fig. 4*f*). In line 17 mice, expression appeared to be shut off, whereas in line 32, ventral root downregulation was not absolute, but labeling intensity was clearly weaker than that observed in dorsal roots. A similarly dramatic difference in dorsal and ventral root labeling intensity was observed in one line of SCE1-hsp68-*lacZ* mice (also designated 17). These combined observations suggest a novel level of heteroge-

Figure 3. SCE1 targets Schwann cell expression in the context of a heterologous promoter. *a*, SCE1 was ligated to a 0.3 kb hsp68 minimal promoter in either orientation, and each construct was used to derive transgenic mice. The sequence represented by the *striped box* in 3'(SCE1)5'-hsp68-lacZ is from the vector, pSK+. *b*, Dorsal view of a whole-mount histochemical β -galactosidase preparation from a 7-d-old SCE1-hsp68-lacZ (line 18) mouse showing the intense labeling of fibers in lumbar spinal roots but no expression in oligodendrocytes in the CNS. *c*, *d*, Cranial nerves and cervical spinal roots also demonstrate intense labeling, whereas neither oligodendrocytes nor other cell types label in the brain. Note that this specimen was a B6C3F1 derivative and was pigmented accounting for the black pigmentation, and not reaction product, observed in the eyes. Scale bar: *b*, 1.6 mm; *c*, *d*, 4.4 mm.

Figure 4. Developmental expression of transgenes bearing SCE1. Low-level transgene expression is detected in some regions of the PNS in an SCE1-hsp68-lacZ line 18 fetus at E15.5. Mixed nerves exiting the spinal column have detectable but unevenly deposited β -galactosidase reaction product (*a*), whereas the trigeminal nerve is more obviously and uniformly labeled (*b*). High-level expression, detectable in whole-mount preparations, initiates only in the postnatal period. *c*, Whole-mount preparation of a 2-d-old -9 kb MBP-lacZ mouse (line 17) shows labeling in ventral but not dorsal spinal roots, reflecting the relative developmental delay in the myelination program of dorsal roots. *d*, Electron micrograph prepared from whole-mount-labeled tissue reveals β -galactosidase reaction product deposited adjacent to developing myelin sheaths in the L4 ventral root. At this stage of development, whereas some ventral root fibers have initiated sheath formation, the unlabeled dorsal roots (data not shown) do not contain any myelin profiles. *e*, Whole-mount preparation of roots and dorsal root ganglia from a preweaning -9 kb MBP-lacZ transgenic mouse. Uniform and intense staining is observed in both dorsal and ventral roots, whereas neither the neurons nor non-Schwann cell glia in the dorsal root ganglia label. *f*, In 3-month-old -9 kb line 17 MBP-lacZ transgenic mice, Schwann cells ensheathing motor axons in ventral roots no longer label, whereas lacZ expression continues, at readily detectable levels, in Schwann cells ensheathing sensory axons in dorsal roots (root modality was identified by the spinal cord insertion site). Scale bar: *a*, *b*, 0.18 mm; *c*, 1.14 mm; *d*, 4 μ m; *e*, 1.25 mm; *f*, 1.6 mm.

neity among Schwann cells that coincides with the modality (sensory versus motor) of the innervating axon. Although a striking finding in the multiple lines where it was observed, this phenomenon was not encountered in all SCE1-bearing lines. Notably, both dorsal and ventral roots in mice from the SCE1-hsp68-lacZ lines 49 and 54 continued to label at an apparently similar level throughout maturity.

Because our studies demonstrated that the 0.6 kb SCE1 sequence contained the elements necessary for targeting high-level Schwann cell expression during maturation, we evaluated the sequence for known regulatory elements that could control MBP expression. We searched for such elements using MacVector software, as well as the online TFSEARCH program supported by TRANSFAC databases (Heinemeyer et al., 1998). Among the

Figure 5. Sequence analysis reveals a Krox-20 site that is neither essential nor sufficient for Schwann cell expression. *a*, Sequence analysis reveals multiple potential regulatory elements within SCE1, and only a few are shown. Among these is a Krox-20 site [(-)GCGTGGGTG; Sham et al., 1993]. This site lies near the 3' end of SCE1, and consequently, was included in the 8.5 kb promoted construct that expressed only in oligodendrocytes, demonstrating that it is not sufficient for conferring Schwann cell expression. *b*, To determine whether the Krox-20 site is essential for SCE1-mediated expression, a construct bearing a mutation within the Krox-20 site was generated [(-)GCGTGGGTG→GCGGTTTCG, δ Krox-20-SCE1]. The mutated SCE1 was ligated to the 3' end of *lacZ* driven by 6 kb of *MBP* promoter. *c*, A P30 transgenic mouse bearing the δ Krox-20-SCE1 construct demonstrates high-level reporter gene expression in both Schwann cells and oligodendrocytes, indicating that the Krox-20 site is not essential for SCE1 targeting function. Scale bar: *c*, 4.4 mm.

>150 putative elements recognized in the 0.6 kb sequence, one with a perfect match to a Krox-20 binding site was encountered near the 3' end of SCE1 [(-)GCGTGGGTG; Sham et al., 1993; Fig. 5*a*]. In mice homozygous for null Krox-20 alleles, Schwann cells ensheath axons but fail to elaborate myelin (Topilko et al., 1994), demonstrating that Krox-20 plays an essential role in Schwann cell maturation or myelination. Because the Krox-20 element within SCE1 is located within the 0.1 kb region of overlap with the 8.5 kb promoter and transgenic animals bearing the 8.5 kb promoted construct do not express in Schwann cells (Table 1), we conclude that the Krox-20 element is not sufficient for conferring Schwann cell expression. To determine whether it is necessary for SCE1 enhancer activity, perhaps through interactions with additional SCE1 elements, we introduced a 5 bp mutation [(-)GCGTGGGTG→GCGGTTTCG that abolishes Krox-20 binding; Chavrier et al., 1990; Forghani et al., 1999]. A construct-bearing SCE1 with this mutant Krox-20 site (δ Krox-20-SCE1) at the 3' end of *lacZ* and driven by the 6 kb *MBP* promoter was generated (Fig. 5, Table 1). To date, transgenic mice from one line bearing the δ Krox-20-SCE1 construct have been analyzed [at postnatal day 30 (P30) and P160] and in both, high-level β -galactosidase expression was observed in Schwann cells throughout the PNS.

DISCUSSION

In this investigation, the expression of reporter constructs in transgenic mice was used to map DNA regulatory domains that control *MBP* expression in Schwann cells. One robust Schwann

cell enhancer was located and characterized, and we encountered evidence for additional enhancer activity in a further 5' sequence. Our investigation also revealed activity of a developmentally regulated cell-specific repressor located in the more proximal *MBP* promoter. Furthermore, SCE1, in combination with the heterologous hsp promoter, was found to drive expression in Schwann cells but not in oligodendrocytes, whereas *MBP* promoted constructs lacking the SCEs expressed only in oligodendrocytes. Despite the common role of *MBP* as a structural protein in both CNS and PNS myelin, the promoter organization we define here suggests that the *MBP* gene may be regulated, in large measure or entirely, through different transcription factors and regulatory elements in these two cell types.

The SCE sequences described here were found to direct reporter gene expression to Schwann cells in a position- and orientation-independent manner, consistent with their role as classic enhancers of transcription. Based on histochemical labeling intensity, SCE1 is maximally active during myelin elaboration and, like the endogenous *MBP* locus, SCE1-bearing reporter constructs are expressed at lower levels in mature mice. Although the developmental expression programs conferred by SCE1-containing constructs reflect major features of that reported for the endogenous *MBP* gene, no construct yet examined expresses in a manner that fully reflects normal *MBP* transcriptional output. Notably, differences in expression levels were encountered between sensory and motor fibers in mature mice in some SCE1-bearing lines, and some transgenes that were expressed robustly during myelination were shut off in mature animals. Consequently, additional Schwann cell regulatory sequence might remain to be located. Alternatively, a more normalized expression phenotype might be achieved by constructs in which contiguous SCE1 and SCE2 sequences are included.

Our investigations support a model in which the transcriptional regulation of *MBP* is achieved through multiple regulatory sequences sharing fundamental features with better characterized loci (Yuh et al., 1998). This organization would provide significant opportunity for the coordinately expressed myelin genes to share common regulatory components. A 1.1 kb 5' flanking sequence from the P0 gene confers a program of expression to reporter constructs with similarities to that observed here for *MBP* SCE1 (Messing et al., 1992). In addition, 4 kb of 5' flanking sequence from the CNPase gene drives expression in both oligodendrocytes and Schwann cells (Gravel et al., 1998). Consequently, it is plausible that elements within these promoters may bind identical transcription factors that are themselves regulated through shared signaling pathways. However, unless ordered and spaced similarly, the frequently short sequences that constitute functional transcription factor-binding sites would be difficult to identify by direct sequence comparisons. To date, no convincing sequence homology between SCE1 and the regulatory domains of other myelin genes has been identified.

Despite the clear role of axonal signals in the regulation of myelin genes, candidate molecules that could be implicated in the relevant signaling pathways are not abundant. Compounds and experimental conditions known to modulate myelin gene expression in Schwann cells and oligodendrocytes include progestins, which potentiate myelination both *in vivo* and *in vitro*, and electrical stimulation, which modulates the level of *MBP* expression in cultured Schwann cells (Koenig et al., 1995; Stevens et al., 1998). It also is well established that elevation of cAMP levels in cultured Schwann cells results in the upregulation of myelin gene expression (Lemke and Chao, 1988; Morgan et al., 1991). More

recently, a role for myelin-associated glycoprotein (MAG) in bidirectional transduction of axon-Schwann cell signals has been proposed based on the observation that homozygous MAG null animals express both axonal and myelin anomalies (Li et al., 1994; Yin et al., 1998). However, a putative downstream effector of MAG, Fyn tyrosine kinase, does not appear to be required for PNS myelination (Fujita et al., 1998; Osterhout et al., 1999).

Within Schwann cells, one molecule with an essential role in myelinogenesis is the zinc finger transcription factor Krox-20. In mice bearing null Krox-20 alleles, Schwann cells make appropriate associations with axons, but subsequent myelin formation and the associated upregulation of myelin genes generally fails (Topilko et al., 1994). Krox-20 could effect its action by directly binding elements in the promoters of individual myelin genes, but our initial findings indicate that the one Krox-20 site recognized within SCE1 is neither sufficient nor necessary for Schwann cell targeting. Although this observation does not rule out a direct role for Krox-20 in modulating levels of myelin gene expression, it suggests that any targeting function mediated through Krox-20 would be achieved indirectly. The latter interpretation also is consistent with our inability to recognize Krox-20 binding sites within the 1.1 kb PO promoter, as well as the previous demonstration that during development, high-level Krox-20 expression precedes upregulation of myelin genes by several days (Murphy et al., 1996).

Last, the POU domain transcription factor SCIP has been implicated in the control of myelin gene expression. It is down-regulated immediately before myelination, and both *in vitro* myelin gene promoter studies and a dominant negative transgenic model are consistent with it playing a role in repressing genes essential for Schwann cell maturation (Monuki et al., 1989, 1990; He et al., 1991; Weinstein et al., 1995). However, homozygous null SCIP mutants do myelinate, albeit in a delayed manner (Birmingham et al., 1996; Jaegle et al., 1996), leaving the precise role of SCIP unclear. Analysis of the SCE1 sequence did not reveal any SCIP sites.

Differential expression of SCE1-regulated constructs in those Schwann cells myelinating motor and sensory fibers of mature mice was unanticipated. Although this phenomenon was not detected in mice from lines in which histochemical labeling was particularly rapid and intense, it was a prominent feature of several moderately expressing lines. Therefore it is possible that significant differences may exist in all lines but be obscured in high-expressing lines. Differences recognized previously between myelinated motor and sensory fibers include the patterns of action potential trafficking and acquisition of the post-translational L2/HNK-1 epitope on several proteins in Schwann cells innervated by motor axons (Bowe et al., 1985; Martini et al., 1994). Because *MBP* is expressed constitutively by all myelin-bearing Schwann cells (Peterson and Bray, 1984), the striking difference in expression seen in motor and sensory fibers of mature mice from multiple SCE1 bearing transgenic lines appears to diverge from the normal expression pattern of the endogenous *MBP* locus. This difference, presumably arising from differential use of elements within SCE1, suggests a novel level of Schwann cell heterogeneity directed by the innervating axon. Consequently, it is an intriguing possibility that the unequal susceptibility recognized for motor and sensory fiber types in certain human neuropathies and experimental animal models (Martini et al., 1995; Wrabetz et al., 2000) may arise at this level of Schwann cell heterogeneity.

Among the lines of transgenic mice established for this inves-

tigation, some consistently express the lacZ reporter in Schwann cells at exceptionally high levels and thus provide unique opportunities to introduce sensitive Schwann cell markers into culture, transplant, and regeneration preparations. In addition, some lines of mice bear reporter constructs containing limited numbers of *MBP* regulatory elements, and these should provide novel opportunities to investigate the Schwann cell signaling pathways that control myelination (Farhadi et al., 1999). The robust expression observed in numerous lines bearing SCE1-regulated constructs suggests that SCE1 will provide a powerful tool for promoting gene expression in myelinating Schwann cells for both biological investigations and potential therapeutic interventions. Last, the 0.6 kb SCE1 defines a realistic target size within which the organization and function of the DNA regulatory elements of the *MBP* gene, along with their related transcription factors, can be investigated (Bronson et al., 1996; Forghani et al., 1999).

REFERENCES

- Aguayo AJ, Charron L, Bray GM (1976a) Potential of Schwann cells from unmyelinated nerves to produce myelin: a quantitative ultrastructural and radiographic study. *J Neurocytol* 5:565–573.
- Aguayo AJ, Epps J, Charron L, Bray GM (1976b) Multipotentiality of Schwann cells in cross-anastomosed and grafted myelinated and unmyelinated nerves: quantitative microscopy and radioautography. *Brain Res* 104:1–20.
- Bachnou N, Schwartz Z, Farhadi HF, Peterson AC (1997) Developmental programming of a myelin gene revealed at high resolution in *MBP* knock-in mice. *Frontiers of Myelin Biology Conference Abstracts VIII*: 79.
- Baron P, Shy M, Kamholz J, Scarlato G, Pleasure D (1994) Expression of P0 protein mRNA along rat sciatic nerve during development. *Brain Res Dev Brain Res* 83:285–288.
- Birmingham Jr JR, Scherer SS, O'Connell S, Arroyo E, Kalla KA, Powell FL, Rosenfeld MG (1996) Tst-1/Oct-6/SCIP regulates a unique step in peripheral myelination and is required for normal respiration. *Genes Dev* 10:1751–1762.
- Berthold CH (1978) Morphology of normal peripheral axons. In: *Physiology and pathobiology of axons* (Waxman SG, ed), pp 3–63. New York: Raven.
- Bowe CM, Kocsis JD, Waxman SG (1985) Differences between mammalian ventral and dorsal spinal roots in response to blockade of potassium channels during maturation. *Proc R Soc Lond B Biol Sci* 224:355–366.
- Bray GM, Perkins S, Peterson AC, Aguayo AJ (1977) Schwann cell multiplication deficit in nerve roots of newborn dystrophic mice. A radioautographic and ultrastructural study. *J Neurol Sci* 32:203–212.
- Bronson SK, Plaehn EG, Kluckman KD, Hagaman JR, Maeda N, Smithies O (1996) Single-copy transgenic mice with chosen-site integration. *Proc Natl Acad Sci USA* 93:9067–9072.
- Chavrier P, Vesque C, Galliot B, Vigneron M, Dolle P, Duboule D, Charnay P (1990) The segment-specific gene Krox-20 encodes a transcription factor with binding sites in the promoter region of the Hox-1.4 gene. *EMBO J* 9:1209–1218.
- Farhadi HF, Forghani R, Peterson AC (1999) *MBP-lacZ* transgene expression in juvenile and adult Trembler-J mice. *Ann NY Acad Sci* 883:538–539.
- Foran DR, Peterson AC (1992) Myelin acquisition in the central nervous system of the mouse revealed by an *MBP-Lac Z* transgene. *J Neurosci* 12:4890–4897.
- Forghani R, Nesbitt J, Snipes J, Shooter EM, Peterson A (1999) Preparation of nuclear extracts from myelinating Schwann cells. *J Neurosci Methods* 89:129–132.
- Fujita N, Kemper A, Dupree J, Nakayasu H, Bartsch U, Schachner M, Maeda N, Suzuki K, Popko B (1998) The cytoplasmic domain of the large myelin-associated glycoprotein isoform is needed for proper CNS but not peripheral nervous system myelination. *J Neurosci* 18:1970–1978.
- Goujet-Zalc C, Babinet C, Monge M, Timsit S, Cabon F, Gansmuller A, Miura M, Sanchez M, Pournin S, Mikoshiba K, et al (1993) The proximal region of the *MBP* gene promoter is sufficient to induce oligodendroglial-specific expression in transgenic mice. *Eur J Neurosci* 5:624–632.
- Gow A, Friedrich VL Jr, Lazzarini RA (1992) Myelin basic protein gene contains separate enhancers for oligodendrocyte and Schwann cell expression. *J Cell Biol* 119:605–616.
- Gravel M, Di Polo A, Valera PB, Braun PE (1998) Four-kilobase sequence of the mouse *CNP* gene directs spatial and temporal expression of *lacZ* in transgenic mice. *J Neurosci Res* 53:393–404.

- Gupta SK, Poduslo JF, Mezei C (1988) Temporal changes in P0 and MBP gene expression after crush-injury of the adult peripheral nerve. *Brain Res* 464:133–141.
- He X, Gerrero R, Simmons DM, Park RE, Lin CJ, Swanson LW, Rosenfeld MG (1991) Tst-1, a member of the POU domain gene family, binds the promoter of the gene encoding the cell surface adhesion molecule P0. *Mol Cell Biol* 11:1739–1744.
- Heinemeyer T, Wingender E, Reuter I, Hermjakob H, Kel AE, Kel OV, Ignatieva EV, Ananko EA, Podkolodnaya OA, Kolpakov FA, Podkolodny NL, Kolchanov NA (1998) Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL. *Nucleic Acids Res* 26:362–367.
- Jaegle M, Mandemakers W, Broos L, Zwart R, Karis A, Visser P, Grosveld F, Meijer D (1996) The POU factor Oct-6 and Schwann cell differentiation. *Science* 273:507–510.
- Koenig HL, Schumacher M, Ferzaz B, Thi AN, Ressouches A, Guennoun R, Jung-Testas I, Robel P, Akwa Y, Baulieu EE (1995) Progesterone synthesis and myelin formation by Schwann cells. *Science* 268:1500–1503.
- Lamperth L, Manuelidis L, Webster HD (1990) P0 glycoprotein mRNA distribution in myelin-forming Schwann cells of the developing rat trigeminal ganglion. *J Neurocytol* 19:756–764.
- LeBlanc AC, Poduslo JF (1990) Axonal modulation of myelin gene expression in the peripheral nerve. *J Neurosci Res* 26:317–326.
- Lemke G, Chao M (1988) Axons regulate Schwann cell expression of the major myelin and NGF receptor genes. *Development* 102:499–504.
- Li C, Tropak MB, Gerlai R, Clapoff S, Abramow-Newerly W, Trapp B, Peterson A, Roder J (1994) Myelination in the absence of myelin-associated glycoprotein. *Nature* 369:747–750.
- Martini R, Schachner M, Brushart TM (1994) The L2/HNK-1 carbohydrate is preferentially expressed by previously motor axon-associated Schwann cells in reinnervated peripheral nerves. *J Neurosci* 14:7180–7191.
- Martini R, Zielasek J, Toyka KV, Giese KP, Schachner M (1995) Protein zero (P0)-deficient mice show myelin degeneration in peripheral nerves characteristic of inherited human neuropathies. *Nat Genet* 11:281–286.
- Messing A, Behringer RR, Hammang JP, Palmiter RD, Brinster RL, Lemke G (1992) P0 promoter directs expression of reporter and toxin genes to Schwann cells of transgenic mice. *Neuron* 8:507–520.
- Mirsky R, Jessen KR (1996) Schwann cell development, differentiation and myelination. *Curr Opin Neurobiol* 6:89–96.
- Miskimins R, Knapp L, Dewey MJ, Zhang X (1992) Cell and tissue-specific expression of a heterologous gene under control of the *myelin basic protein* gene promoter in transgenic mice. *Brain Res Dev Brain Res* 65:217–221.
- Monuki ES, Weinmaster G, Kuhn R, Lemke G (1989) SCIP: a glial POU domain gene regulated by cyclic AMP. *Neuron* 3:783–793.
- Monuki ES, Kuhn R, Weinmaster G, Trapp BD, Lemke G (1990) Expression and activity of the POU transcription factor SCIP. *Science* 249:1300–1303.
- Morgan L, Jessen KR, Mirsky R (1991) The effects of cAMP on differentiation of cultured Schwann cells: progression from an early phenotype (04+) to a myelin phenotype (P0+, GFAP-, N-CAM-, NGF-receptor-) depends on growth inhibition. *J Cell Biol* 112:457–467.
- Murphy P, Topilko P, Schneider-Maunoury S, Seitanidou T, Baron-Van Evercooren A, Charnay P (1996) The regulation of Krox-20 expression reveals important steps in the control of peripheral glial cell development. *Development* 122:2847–2857.
- Osterhout DJ, Wolven A, Wolf RM, Resh MD, Chao MV (1999) Morphological differentiation of oligodendrocytes requires activation of Fyn tyrosine kinase. *J Cell Biol* 145:1209–1218.
- Peterson AC, Bray GM (1984) Normal basal laminas are realized on dystrophic Schwann cells in dystrophic in equilibrium shiverer chimera nerves. *J Cell Biol* 99:1831–1837.
- Sham MH, Vesque C, Nonchev S, Marshall H, Frain M, Gupta RD, Whiting J, Wilkinson D, Charnay P, Krumlauf R (1993) The zinc finger gene Krox20 regulates HoxB2 (Hox2.8) during hindbrain segmentation. *Cell* 72:183–196.
- Snipes GJ, Suter U, Welcher AA, Shooter EM (1992) Characterization of a novel peripheral nervous system myelin protein (PMP-22/SR13). *J Cell Biol* 117:225–238.
- Stahl N, Harry J, Popko B (1990) Quantitative analysis of myelin protein gene expression during development in the rat sciatic nerve. *Brain Res Mol Brain Res* 8:209–212.
- Stankoff B, Demerens C, Goujet-Zalc C, Monge M, Peyron F, Mikoshiba K, Zalc B, Lubetzki C (1996) Transcription of *myelin basic protein* promoted by regulatory elements in the proximal 5' sequence requires myelination. *Mult Scler* 2:125–132.
- Stevens B, Tanner S, Fields RD (1998) Control of myelination by specific patterns of neural impulses. *J Neurosci* 18:9303–9311.
- Topilko P, Schneider-Maunoury S, Levi G, Baron-Van Evercooren A, Chennoufi AB, Seitanidou T, Babinet C, Charnay P (1994) Krox-20 controls myelination in the peripheral nervous system. *Nature* 371:796–799.
- Trapp BD, Hauer P, Lemke G (1988) Axonal regulation of myelin protein mRNA levels in actively myelinating Schwann cells. *J Neurosci* 8:3515–3521.
- Weinstein DE, Burrola PG, Lemke G (1995) Premature Schwann cell differentiation and hypermyelination in mice expressing a targeted antagonist of the POU transcription factor SCIP. *Mol Cell Neurosci* 6:212–229.
- Wrabetz L, Feltri ML, Quattrini A, Imperiale D, Previtali S, D'Antonio M, Martini R, Yin X, Trapp BD, Zhou L, Chiu SY, Messing A (2000) P(0) glycoprotein overexpression causes congenital hypomyelination of peripheral nerves. *J Cell Biol* 148:1021–1034.
- Yin X, Crawford TO, Griffin JW, Tu P, Lee VM, Li C, Roder J, Trapp BD (1998) Myelin-associated glycoprotein is a myelin signal that modulates the caliber of myelinated axons. *J Neurosci* 18:1953–1962.
- Yuh CH, Bolouri H, Davidson EH (1998) Genomic cis-regulatory logic: experimental and computational analysis of a sea urchin gene. *Science* 279:1896–1902.
- Zorick TS, Lemke G (1996) Schwann cell differentiation. *Curr Opin Cell Biol* 8:870–876.