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TRANSCRIPTIONAL REGULATION OF THE MYELIN BASIC PROTEIN GENE IN SCHWANN CELLS

Reza Forghani

Department of Neurology and Neurosurgery

McGill University, Montreal

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

This thesis is dedicated to my mother, Ashraf Masoudi Forghani, for her inexhaustible love and support

In honor of Harvey Cushing, Santiago Ramón y Cajal, and all those who have endeavored to advance the understanding and treatment of neurological disease and the frontiers of basic neuroscience

Authoritarian pronouncements about real phenomena must be tested against the evidence given us in nature

Galileo Galilei

"He who climbeth on the highest mountains, laugheth at all tragic plays and tragic realities"

Friedrich Nietzsche

Thus Spake Zarathustra I, *7 Reading and Writing*

ACKNOWLEDGMENTS

I would like to express my gratitude to my supervisor and mentor, Dr. Alan Peterson. Notwithstanding the fact that he provided me with a superbly stimulating research environment and tremendous resources for my pursuits, Alan has changed my way of thinking forever. Alan has always been around, either during the countless hours we spent in scientific work and discussion, or for advice about personal matters. I am most fortunate to have had the opportunity to work with him.

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I am indebted to the members of my thesis advisory committee, Dr. J. Snipes, Dr. P. Gros, and Dr. D. Kaplan, for their valuable advice and insight, and to members of the MD/PhD committee for their help and support. In particular, Dr. B. Collier, Dr. J. Trasler, and Dr. A. Tenenhouse were always available and ready to help. Although I have been fortunate to be surrounded by individuals with a strong commitment to academic pursuits and teaching, I have to mention Dr. Collier's inexhaustible dedication to teaching and student support. In addition to always being there for a helpful chat and advice, Dr. Collier was well known among my MD/PhD colleagues as the one person who would

never miss a Thursday seminar. Of course, his excellent taste in beer did not go unnoticed either.

It is needless to say that I truly appreciate the help and support of friends, laboratory colleagues and collaborators, and administrators who have supported my endeavors. I am particularly grateful to our excellent senior technicians, P. Valera and I. Tretjakoff, and all other technician members of our team for their help and assistance in technical matters as well animal care. Also, I am grateful to N. Dionne for her help with the translation of the Abstract to French and to my long-time colleague and collaborator H. Farhadi. Monique Ledermann, administrator in the Department of Neurology and Neurosurgery, was always available for help and a great advocate for students. Unfortunately, I cannot list all the names on this page. However, their help is appreciated and will not be forgotten.

Last but not least, I am very grateful for the love, support, and encouragement from the members of my family. Most notably, I am grateful for the never ending support of my mother who has conquered all and provided the basis that has enabled me and my siblings to get to their highest potential.

Financial support for this work was provided by the Medical Research Council of Canada, the Multiple Sclerosis Society of Canada, and the Canadian Neuroscience Network.

ABSTRACT/RÉSUMÉ

ABSTRACT

Myelination of large caliber axons is an essential step in the development of the vertebrate nervous system. In peripheral nerves, axons instruct ensheathing Schwann cells to elaborate and maintain myelin sheaths, and this is achieved in part through the coordinate up-regulation of genes encoding myelin structural proteins. In order to gain insight into the transcriptional regulation of one such gene, Myelin Basic Protein (MBP), I have investigated MBP 5' flanking sequence using a combination of sequence analysis, in vitro DNA-protein interaction assays, and functional analysis in transgenic mice including a high-resolution preparation in which a single copy of a reporter construct is inserted into the HPRT locus. These investigations have located a robust 0.6 kb Schwann cell enhancer designated SCE1 that corresponds to a highly conserved human regulatory module. A method to prepare transcription factor containing Schwann cell nuclear extracts was developed and using electrophoretic mobility shift assays with such peripheral nerve extracts, multiple candidate regulatory elements were identified. SCE1 contains all the necessary elements for conferring reporter gene expression to myelinating Schwann cells in vivo and is composed of multiple functional sub-domains with independent targeting capabilities and one sub-domain that appears to positively modulate the level of expression. The 3 kb sequence immediately upstream of SCE1 also can target Schwann cell expression and there is indirect evidence for repressor activity in the proximal promoter that serves to limit expression to Schwann cells during embryonic development. In summary, these investigations demonstrate that the expression of MBP in Schwann cells is achieved through the integrated output of multiple interacting

regulatory sequences and the techniques established during the course of these investigations will support future higher resolution in vivo studies of myelin gene regulation. The conserved Schwann cell enhancer described here also should have multiple applications in driving high-level expression of genes in Schwann cells both for biological investigations and gene therapy.

RÉSUMÉ

La myélinisation est un étape essentielle au développement du système nerveux des vertébrés. Dans les nerfs périphériques, les axones instruisent les cellules de Schwann à élaborer et maintenir les gaines de myéline. Ceci se produit en partie par la régulation coordonnée des gènes codant pour les protéines structurales de la myéline. Afin d'obtenir un aperçu de la régulation de la transcription d'un des ces gènes, codant pour les protéines basiques de la myéline (MBP), j'ai investigué la séquence adjacente à MBP en 5', en utilisant une combinaison d'analyses de séquence, d'essais d'interaction ADN/protéines *in vitro*, et d'analyses de fonction *in vivo* avec des modèles transgéniques incluant entre autres une stratégie de haute résolution avec laquelle une seule et unique copie d'un construit est insérée dans le locus HPRT. Ces investigations ont mené à la localisation d'un robuste *enhancer* des cellules de Schwann, désigné SCE1, que correspondent à un module régulateur fortement conservé chez l'humain. Une méthode de préparation d'extraits nucléaires de cellules de Schwann contenant les facteurs de transcription a été développée et utilisée pour des extraits de nerf qui ont servi à faire des essais de mobilité électrophorétique. Ceci a mené à l'identification de plusieurs potentiels éléments régulateurs: SCE1 contient tous les éléments essentiels pour conférer l'expression d'un gène reporter aux cellules de Schwann qui produisent la myéline, et est par ailleurs constitué de plusieurs sous-domaines fonctionnels ayant des capacités de ciblage différentes, dont un en particulier module positivement le niveau d'expression. Une séquence 3kb immédiatement en amont de SCE1 cible aussi l'expression aux cellules de Schwann et d'indirectes évidences suggèrent une activité répressive de le promoteur

proximal durant le développement embryonnaire. En résumé, ces investigations démontrent que l'expression de mbp dans les cellules de Schwann se produit par l'interaction de plusieurs séquences régulatrices qui interagissent. De plus les techniques établies au cours de ces investigations pourront supporter de futures expérimentations *in vivo* de haute résolution des gènes de la myéline. L'*enhancer* des cellules de Schwann décrit ici pourra avoir de multiples applications pour induire l'expression de gènes dans les cellules de Schwann et ce, à la fois pour les investigations biologiques et la thérapie génique.

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CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

As stated in the "Guidelines for Thesis Preparation", Faculty of Graduate Studies and Research, McGill University:

Elements of the thesis that are considered to constitute original scholarship and an advancement of knowledge in the domains in which the research was conducted must be clearly indicated. This requirement is mandatory for doctoral theses.

Chapter 2

1. Identification of two MBP enhancers, designated Schwann cell enhancer 1 (SCE1; 0.6 kb) and 2 (SCE2; 3 kb) that control expression in Schwann cells in vivo using reporter constructs in transgenic mice.
2. SCE1 contains sufficient elements for conferring Schwann cell expression independently from the proximal MBP promoter.
3. Characterization of the developmental expression programming conferred by SCE1.
4. Differential expression of some SCE1 driven transgenes in motor and sensory fibers.
5. Contribution of the proximal 6 kb of MBP 5' flanking sequence to the cell specificity conferred by SCE1 through repression during embryogenesis.
6. Sequencing and submission of the 0.6 kb SCE1 and 3 kb SCE2 to GenBank.
7. Subsequence analysis of SCE1 and identification of potential regulatory elements, including a Krox-20 site.

8. The demonstration that the Krox-20 site within SCE1 is neither sufficient nor essential for SCE1 activity in vivo.

Chapter 3

1. Systematic derivation and establishment of an effective protocol for obtaining nuclear factors from myelinating Schwann cells of peripheral nerves.

Chapter 4

1. There is no significant homology between the 0.6 kb SCE1 and the 1.1 kb P0 promoter (using PIP analysis).
2. SCE1 bears 3 ER half sites, among many other potential regulatory elements.
3. SCE1 bearing transgenic lines continue reporter gene expression on the ROR-α null background.
4. SCE1 bearing transgenic lines continue reporter gene expression on the NGFI-B null background.
5. EMSAs using peripheral nerve extracts and sequences spanning part of the 0.6 kb SCE1 (–8.791 to –8.682 kb; 110 bp) reveal two prominent bands, designated complex I and II.
6. A 15 bp contiguous sequence (5' CCAGGTGACCCCAAG 3') containing an ER half site (underlined; GGTCA on the minus strand) mediates the binding activity represented by complexes I and II based on competition assays with mutant oligonucleotides in EMSAs.

7. Bases 5'CCAGG3' are required for complex II formation but not complex I.
8. Excess oligonucleotides encoding the AP-1 recognition site effectively compete complex I.
9. Excess oligonucleotides encoding recognition sites for many members of the nuclear receptor family (NBRE, ERE, H-2RII, COUP, CF-1) effectively compete complex II. Those bearing an ERRE site are not very effective at competing complex II or I.
10. A 3 bp mutation at the core of the ER half site (GGTCA → GGAAT on the minus strand) abolishes binding by both complex I and II.
11. SCE1 (0.6 kb) bearing the above 3 bp mutation continues to confer reporter gene expression in Schwann cells of transgenic mice derived by pronuclear injection.
12. SCE1 consists of two independent targeting sub-domains in vivo.
13. In the absence of the 3' 73 bp sub-domain, the probability that SCE1 targeting sub-domains confer Schwann cell expression to reporter constructs in transgenic mice by pronuclear injection (hence random integration) is significantly reduced.
14. SCE1 and targeting sub-domains continue to confer appropriate Schwann cell expression when inserted into the HPRT docking site.
15. The 510 bp sub-domain of SCE1 containing both targeting sub-domains but lacking the 73 bp modulatory sequence continues to confer Schwann cell expression in vivo when inserted into the HPRT locus, albeit at a reduced level compared to the 0.6 kb SCE1. Hence the 73 bp sub-domain enhances the expression level conferred by the targeting sub-domains.

16. Mutation of the Krox-20 site within SCE1 results in a decline in the expression level conferred to Schwann cells by reporter constructs inserted in the HPRT locus (under further investigation).
17. Reporter constructs bearing –3.1 kb proximal MBP 5' flanking sequence continue to confer oligodendrocyte expression when inserted into the HPRT docking site.

CONTRIBUTIONS OF AUTHORS

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As stated in the "Guidelines for Thesis Preparation", Faculty of Graduate Studies and Research, McGill University:

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis.

The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following: (a) a table of contents; (b) an abstract in English and French; (c) an introduction which clearly states the rational and objectives of the research; (d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper); (e) a final conclusion and summary.

As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

Contributions of the authors on co-authored papers

All the investigations described in this thesis where performed in the laboratory of Dr. A. Peterson, my Ph.D. supervisor, and therefore he is senior author on all the manuscripts (chapters 2, 3, and 4). The role of additional authors on each manuscript is discussed below. These descriptions include a brief overview of my own role for maximal clarity.

Chapter 2

A DISTAL UPSTREAM ENHANCER FROM THE MYELIN BASIC PROTEIN GENE REGULATES EXPRESSION IN MYELIN FORMING SCHWANN CELLS

R. Forghani*, L. Garofalo*, D. R. Foran, H. F. Farhadi, P. Lepage, T. Hudson, I. Tretjakoff, P. Valera, and A. Peterson

(* These authors contributed equally to this investigation.)

J Neurosci: in press

R. Forghani: I made the following constructs and oversaw the derivation of transgenic mice and their analysis: SCE1 (5'→3') + hsp + lacZ, SCE1 (3'→5') + hsp + lacZ + pSK, -6.0 kb MBP + lacZ + δKrox-20-SCE1 (5'→3'), and -6.0 kb MBP + lacZ + SCE2 (5'→3'). I performed subsequence analysis leading to the Krox-20 mutation experiment (above) as well as PIP analysis comparing SCE1 with the P0 promoter. Finally, I was responsible for the packaging of experiments and preparation (text + figures) of this manuscript leading to its acceptance for publication.

L. Garofalo: Dr. Garofalo is a former post-doctoral fellow at the Peterson laboratory. She made the following three constructs SCE1 (5'→3') + -3.1 kb MBP + lacZ, -6.0 kb MBP + lacZ + SCE1 (3'→5'), and -6.0 kb MBP + lacZ + SCE1 (5'→3') + pSK and oversaw the derivation and analysis of transgenic lines derived from these constructs.

D. R. Foran: Dr. Foran is a former post-doctoral fellow at the Peterson laboratory. He made the -9.0 kb MBP + lacZ construct and oversaw its injection and the injection of its digests leading to the -6.0 kb MBP + lacZ, -7.0 kb MBP + lacZ, and -8.5 kb MBP + lacZ constructs for the derivation of transgenic mice.

H. F. Farhadi: Mr. Farhadi is a graduate student at the Peterson laboratory. He helped with the analysis of mice from the SCE1 (5'→3') + -3.1 kb MBP + lacZ line in whole mount preparations.

P. Lepage and T. Hudson: Dr. Lepage is a post-doctoral fellow at the Hudson laboratory. He did part of the sequencing of the 3 kb SCE2 and was responsible of submission of SCE1 and SCE2 sequence to GenBank.

I. Tretjakoff: Ms. Tretjakoff is a senior technician at the Peterson laboratory. She provided technical support with the pronuclear injection of constructs for the derivation of transgenic animals, as well as transplantation of zygotes and maintenance of the colony.

P. Valera: Ms. Valera is a senior technician at the Peterson laboratory. She provided technical assistance in the preparation of whole mount and tissue sections, as well as with the typing of animals by PCR.

Chapter 3

PREPARATION OF NUCLEAR EXTRACTS FROM MYELINAITNG SCHWANN CELLS

R. Forghani, J. Nesbitt, J. Snipes, E. M. Shooter, and A. Peterson

J Neurosci Methods 89 (1999): 129 - 132

R. Forghani: I have designed and performed all experiments described in this manuscript, including all dissections, extract preparations, and EMSAs.

J. Nesbitt, J. Snipes, and E. M. Shooter: As a starting point for my experiments, I used a protocol that was started at the Shooter laboratory by two post-doctoral fellows, Dr. J. Nesbitt and Dr. J. Snipes. That protocol was important in providing a starting point for my strategy.

Chapter 4

MYELIN BASIC PROTEIN EXPRESSION IN SCHWANN CELLS IS CONTROLLED THROUGH THE INTEGRATED OUTPUT OF MULTIPLE REGULATORY SUB-DOMAINS

R. Forghani, H. F. Farhadi, W. Orfali, G. J. Snipes, and A. Peterson

(In preparation)

R. Forghani: I have designed all experiments, performed all EMSAs, made over 10 of the constructs (exceptions are noted below), and oversaw the derivation and analysis of transgenic animals from these construct (with direct participation in whole mount preparations, along with a senior technician in our laboratory, P. Valera).

H. F. Farhadi: Mr. Farhadi is a graduate student at the Peterson laboratory. He was responsible for a significant part of the cloning of the following two constructs (part of the initial cloning was done by myself): SCE1-*HPRT* #1, SCE1-*HPRT* #2, and δKrox-20-SCE1-*HPRT* targeting vectors.

W. Orfali and J.G. Snipes: Dr. Orfali is a graduate student at the Snipes laboratory. He was responsible for part of the cloning and derivation of the following construct (part of the initial cloning was done by myself): Δ1-SCE1-*HPRT* targeting vector.

CHAPTER 1

CHAPTER 1

GENERAL INTRODUCTION

PREFACE

One of the fundamental goals in axon-glia biology has been to elucidate the mechanism underlying glial cell differentiation and maturation. In the PNS, there is significant evidence supporting a model in which axons instruct Schwann cells to elaborate and maintain myelin sheaths in part through the coordinate up-regulation of genes encoding myelin structural proteins. However, the signaling pathways and the downstream cis DNA elements regulating myelin gene expression are poorly understood. The central objective of this thesis is to gain further insight into the transcriptional regulation of one of the myelin genes, the Myelin Basic Protein (MBP), in Schwann cells. This chapter starts with a review of the literature and provides the rationale and objectives for the investigations presented in chapters two, three, and four.

I. OVERVIEW OF MYELINATION IN THE PERIPHERAL AND CENTRAL NERVOUS SYSTEM

(a) General overview of peripheral and central myelin: origin and function

Myelination is an essential step in the maturation of fast-conducting vertebrate axons. Myelin is a lipidic substance that ensheathes many axons in the peripheral (PNS) and central nervous system (CNS) of most vertebrates (Waehneldt, 1990). Although elaborated by two distinct cell lineages, Schwann cells in the PNS and oligodendrocytes in the CNS, peripheral and central myelin function similarly to increase the axonal conduction velocity, while simultaneously reducing energy expenditure all without an excessive increase in fiber diameter (Bray et al., 1981; Lemke, 1988; Hildebrand et al., 1993). The essential role of myelination for the appropriate functioning of the nervous system becomes readily evident when this process is disrupted, resulting in the severe disturbances seen in dysmyelinating and demyelinating neuropathies in both animal models and human diseases (Roach et al., 1983; Roach et al., 1985; Giese et al., 1992; Suter et al., 1992; Adlkofer et al., 1995; Martini et al., 1995b; Suter and Snipes, 1995; Anzini et al., 1997; Martini et al., 1998; Quattrini et al., 1999; Martini, 2000; Previtali et al., 2000).

(b) General overview of myelinated fibers: fundamental structural features underlying function

Consistent with their similar functional characteristics, peripheral and central myelinated fibers have similar structural features. In both cases, the length of the axon is covered by adjacent sheaths of myelin elaborated by either the neural crest derived Schwann cells (PNS) or the neural tube derived oligodendrocytes (CNS). As required of this arrangement, there are regular interruptions between adjacent myelin sheaths. These interruptions are referred to as nodes of Ranvier, and myelin segments between consecutive nodes are called internodes. In a process referred to as saltatory conduction, an action potential traveling along the length of an axon is propagated from node to node, where there are high concentrations of voltage gated sodium channels. In this manner, action potential velocity is accelerated compared to an unmyelinated fiber of similar diameter. Furthermore, as relatively fewer ions per unit length are required to move in order to generate an action potential in a myelinated fiber, the rapid conduction is achieved at a significantly lower net metabolic cost (Peters et al., 1991; Hille, 1992).

(c) Basic ultrastructural features of the myelin sheath

The microscopic organization of peripheral and central myelin is similar. The myelin sheath results from the wrapping of multiple layers of the glial cell plasma membrane around the axon in a lamellar fashion. Then, these wraps are compacted providing a highly lipidic sheath on the internodal segments of axons. In electron

micrographs, the myelin sheath appears as spirally wrapped lamellae composed of major dense lines, composed by the apposition of the cytoplasmic faces of the same pair of plasma membranes, and intraperiod lines, arising from the apposition of the outer faces of adjacent plasma membranes (Peters et al., 1991). However, despite the similarities between peripheral and central myelin, there are significant differences in the molecular components of peripheral and central myelin sheaths, and these differences will be explored later in this chapter.

(d) Differences in the interaction between axons and glial cells in the peripheral and central nervous system

There are significant differences between myelinating glia and the way they interact with axons in the PNS and CNS. Whereas a single oligodendrocyte may myelinate up to 50 different axons, a myelinating Schwann cell associates with a single axon. Furthermore, while there is substantial evidence for axon-glia interactions in both the peripheral and central nervous system, such interactions are particularly tightly controlled in the PNS. Specifically, the correlation between the caliber of the innervating axon and myelination is much tighter in the PNS than in the CNS (Aguayo et al., 1977; Peters et al., 1991). Further, when Schwann cells are isolated from the innervating axon, either through denervation in vivo or isolation in culture, they revert to a non-myelinating phenotype and down-regulate the genes for the major myelin associated proteins (Gupta et al., 1988; Lemke and Chao, 1988; Trapp et al., 1988; LeBlanc and Poduslo, 1990; Snipes et al., 1992). Isolated oligodendrocytes, on the other hand, continue to extend their

processes, make myelin figures, and express major myelin associated genes at significant levels (Abney et al., 1981; Poduslo et al., 1982; Bradel and Prince, 1983; Zeller et al., 1985; Rome et al., 1986).

II. DEVELOPMENT OF PERIPHERAL FIBERS AND CELL-CELL INTERACTIONS LEADING TO THEIR MATURATION

(a) Overview of axon-Schwann cell interactions and the regulation of myelinogenesis and myelin maintenance

It has been well established that in the PNS, the differentiation of Schwann cells into myelinating cells is controlled by the innervating axon (Aguayo et al., 1976a; Aguayo et al., 1976b; Aguayo et al., 1977). In addition to determining whether a Schwann cell myelinates, various quantitative aspects of the myelin sheath, such as its thickness, are determined by the axon (Friede and Samorajski, 1967; Friede and Martinez, 1970; Berthold, 1978). Furthermore, following transection of a peripheral nerve, the Schwann cells in the distal stump shed their myelin and only if re-innervated by appropriate regenerating axons will they re-elaborate myelin, demonstrating that continued axonal contact is required for the maintenance of the myelin sheath (Gupta et al., 1988; Trapp et al., 1988; LeBlanc and Poduslo, 1990; Snipes et al., 1992). Lastly, such axon-Schwann cell signaling is not unidirectional; it has been well established that Schwann cells influence the axonal phenotype (Aguayo et al., 1977; de Waegh and Brady, 1990; de Waegh et al., 1992; Cole et al., 1994), consistent with a model in which

(c) Multi-potentiality of Schwann cells and the regulation of Schwann cell differentiation by axons

Light microscopic studies starting in the early 20th century suggested that the differentiation of Schwann cells into myelinating cells is directed by the innervating axon (Langley and Anderson, 1903; Simpson and Young, 1945; Hillarp and Olivecrona, 1946; Aguayo et al., 1976b). However, the final unequivocal evidence emerged from pivotal investigations by Aguayo and colleagues (Aguayo et al., 1976b; Aguayo et al., 1977). These studies involved cross-anastomosis and grafting experiments, taking advantage of the ability of a peripheral nerve to regenerate following injury in order to evaluate the plasticity of Schwann cells. Following a nerve disrupting injury, a myelinated axon distal to the site of a lesion degenerates and Schwann cells adopt a non-myelinating phenotype and remain within their basal laminae. If continuity is maintained with the distal stump, peripheral axons can regenerate and will be remyelinated once they establish contact with Schwann cells in the distal stump (Fawcett and Keynes, 1990; Bunge, 1993). In one of the original studies evaluating axon-Schwann cell interactions leading to myelination (Aguayo et al., 1976a), the phrenic nerve, which contains a majority of myelinated fibers, was cross-anastomosed to the cervical sympathetic trunk (CST), a nerve consisting mostly of unmyelinated fibers. Two to six months later, many myelinated fibers were seen in the distal CST stump. In contrast, following CST $\rightarrow$ phrenic cross-anastomosis, the fibers in the phrenic segment became largely unmyelinated. Furthermore, grafting of a segment of CST between the cut ends of the sural nerve, which contains myelinated and

unmyelinated fibers, resulted in the appearance of myelinated fibers in the graft. Similar to earlier light microscopic studies, these phase and electron microscopic studies suggested that the axon determines whether a Schwann cell becomes a myelinating cell.

To establish that the changes in Schwann cell morphology truly reflected the plasticity of grafted Schwann cells and were not observations confounded by the migration of Schwann cells from the proximal stump, ^{3}H-thymidine labeling was used to assess Schwann cell proliferation (Aguayo et al., 1976a). First, these investigations demonstrated that the labeling indices in the distal stumps following cross-anastomosis, self-anastomosis, or in isolated segments increased to 15-20% and in most cases fell to less than 3% one week after injury, decreasing to approximately 1% in cross-anastomosed nerves 1, 2, 4, or 6 months following injury. In order to assess the potential migration of proximal stump Schwann cells into the distal stump, a segment of a sural nerve was resected, followed by the intra-peritoneal injection of ^{3}H-thymidine 2 days later when there is intense Schwann cell proliferation in these injured nerves. Twenty-four hours later, when presumably most of the labeled thymidine had been metabolized, an autologous segment of the CST was grafted into the previously injured sural nerve. Given that at the CST had not been injured at the time of ^{3}H-thymidine administration, any labeling would presumably result from migration of already labeled Schwann cells from the proximal stump. Despite labeling indices in the 15-37% range in the proximal stump, the labeling indices in the mid-portion of the CST graft were less than 3%. Some labeling in the proximal 1 mm of the graft was seen and was attributed to cells bridging the gap between the two nerves. Overall, these observations suggested that there is no substantial

migration of Schwann cells into the graft. Subsequent grafting studies between the dysmyelinating mouse mutant Trembler and normal mice confirmed the absence of significant migration of Schwann cells into grafted nerve segments (Aguayo et al., 1976a), supporting earlier observations on the role of the axon in instructing Schwann cells to myelinate.

(d) Regulation of quantitative features of the myelin sheath by the innervating axon

In addition to controlling the differentiation of Schwann cells, axons also appear to control quantitative features of the myelin sheath, as both the length of the myelin internode and the thickness of the myelin sheath increase in direct proportion to the caliber of the innervating axon (Friede and Samorajski, 1967; Friede and Martinez, 1970; Berthold, 1978). Interestingly, experimental perturbation leading to neurofilament-deficient axons results in smaller caliber axons that are relatively hypermyelinated in the PNS, suggesting that the signaling mechanism controlling myelin sheath thickness operates independently of the actual axon diameter achieved (Eyer and Peterson, 1994). In contrast, the same investigation reveals that CNS axons with similarly reduced diameters are not hypermyelinated, suggesting that a different signaling mechanism is used to regulate the thickness of the myelin sheath elaborated by oligodendrocytes. Perhaps the association of a myelinating oligodendrocyte with multiple axons requires such differential regulatory mechanism.

(e) Modulation of the axonal phenotype by ensheathing Schwann cells

While axonal regulation of Schwann cell differentiation has been extensively documented, several lines of investigation suggest that such inter-cellular signaling is not unidirectional. Notably, myelination results in an increase in axonal diameter. In the Trembler mouse, a spontaneous mutant that bears a mutation in the gene encoding the major structural myelin protein PMP-22 (peripheral myelin protein 22; Suter et al., 1992), there is severe hypomyelination of peripheral nerves that is accompanied by a general decrease in axon caliber (Low and McLeod, 1975; de Waegh and Brady, 1990). When Trembler nerve segments are grafted into normal sciatic nerves, axons from the normal proximal stump re-innervate Trembler Schwann cells within the grafted segment and there is hypomyelination, reduction in axon caliber, and associated changes in the neurofilament cytoskeleton that are restricted to part of the axon ensheathed by Trembler Schwann cells (Aguayo et al., 1977; de Waegh et al., 1992). These observations suggest that myelinating Schwann cells modulate axon caliber, and do so by modulating the neurofilament cytoskeleton.

Such apparent regulation of axonal features by myelinating Schwann cells is not limited to the mutant model Trembler. Targeted expression of diphteria toxin A (DT-A) or a mutated form of SV40 large T antigen to Schwann cells in transgenic mice also results in a hypomyelinating phenotype in the PNS (Messing et al., 1992; Messing et al., 1994) and the degree of hypomyelination in such mice is reflected in the smaller caliber of their axons and changes in the axonal neurofilament cytoskeleton, similar to

observations in the Trembler (Cole et al., 1994). Studies of normal nerves provide additional supporting evidence for the observations made in mutant models. Specifically, in sensory fibers of normal animals, there is a local reduction in axon diameter and neurofilament spacing in the non-myelinated stem processes and at the nodes of Ranvier relative to the myelinated internodes (Hsieh et al., 1994). In vitro, bare neurites in dorsal root ganglia-Schwann cell co-cultures have a reduced median diameter compared to their myelinated counterparts (Windebank et al., 1985). Indeed, Schwann cell-directed changes in the axonal cytoskeleton may also extend to microtubules (Kirkpatrick and Brady, 1994), all consistent with extensive signaling and regulation of axonal features by myelinating Schwann cells.

(f) Axon-Schwann cell interactions in the PNS: summary

Overall, the investigations described above demonstrate that extensive axon-Schwann cell interactions contribute to the development and maturation of peripheral fibers. In the next two sections, I review the events leading to the myelination of peripheral axons at the molecular level, starting with a discussion of the molecular components of myelin.

III. MYELINATION OF PERIPHERAL AXONS: MAJOR MYELIN STRUCTURAL PROTEINS

(a) Composition of the myelin sheath: the molecular perspective

With technological advances in biochemistry and molecular biology, the interactions leading to the differentiation of peripheral fibers have been re-evaluated in order to elucidate the molecular components of myelin and the signaling pathways that regulate them. Among these, the genes encoding myelin structural proteins and their function have been particularly well studied. This section will examine the major biological features of three major structural proteins of peripheral myelin, myelin basic protein (MBP), myelin protein zero (P0), and peripheral myelin protein 22 (PMP-22). This limited discussion is designed to provide the essential background for the following section on the regulation of myelination in the PNS.

(b) The GOLLI-MBP locus, myelin basic protein, and its contribution to the formation of the major dense line

Myelin basic protein (MBP) is a cytoplasmic protein that is present both in PNS and CNS myelin and accounts for 5 – 15 % and 30 – 40 % of their total myelin protein content, respectively (Greenfield et al., 1973; Milek et al., 1981; Greenfield et al., 1982; Hahn et al., 1987; Lemke, 1988 - *NB: many early studies did not distinguish MBP from the minor myelin protein P2*). The murine MBP maps to chromosome 18 and consists of 7

exons that give rise, through alternative splicing, to protein products ranging from 14 to 21.5 kDa in molecular mass (Barbarese et al., 1978; de Ferra et al., 1985; Roach et al., 1985; Takahashi et al., 1985). Interestingly, the MBP gene is part of a much larger transcriptional unit that includes the gene GOLLI (gene expressed in the oligodendrocyte lineage) at its 5' end, and the first three MBP exons and the proximal MBP promoter actually contribute to translated GOLLI exons (Campagnoni et al., 1993; Pribyl et al., 1993). Although the precise function of GOLLI remains to be elucidated, recent investigations suggest a role in oligodendrocyte differentiation (Campagnoni et al., Abstract 30.16, 30th Annual Meeting of the Society for Neuroscience, New Orleans, 2000). However, GOLLI is not expressed in Schwann cells and based on their very distinct expression programming, the transcriptional regulation of MBP and GOLLI appears to occur through distinct and independent regulatory sequences.

The major role of MBP in myelination is its contribution to the formation of the major dense line in central and peripheral myelin, consistent with its localization on the cytoplasmic aspect of the plasma membrane. Insight into the function of MBP was first gained through the analysis of the Shiverer mutant mouse that bears a partially deleted MBP gene and has very little if any MBP mRNA (Roach et al., 1983; Roach et al., 1985). Homozygous mutant Shiverer mice display a generalized action tremor, convulsions, and premature death (Chernoff, 1981) and analysis of their CNS reveals widespread absence of myelin. Furthermore, where present, Shiverer myelin is abnormal, containing normal intraperiod lines but uncompacted major dense lines (Privat et al., 1979; Kirschner and Ganser, 1980). The myelination defect is corrected in transgenic MBP-expressing

shiverer mice demonstrating that it is directly related to the absence of MBP (Readhead et al., 1987).

In contrast to the marked abnormalities seen in their CNS myelin, compact myelin is formed in the PNS of Shiverer mice and their PNS myelin appears for the most part normal when examined by light microscopy (Kirschner and Ganser, 1980). Mild abnormalities are only seen upon close examination of Shiverer PNS myelin and include a slight hypomyelination and multiple interlamellar gaps (Rosenbluth, 1980; Peterson and Bray, 1984). Because of this, it was initially assumed that P0, the most abundant myelin protein in the PNS, mediates myelin compaction alone. However, studies of P0 deficient mice and mice deficient in both P0 and MBP have now clarified that there is redundancy among certain functions of these two proteins. Notably, both P0 and MBP contribute to the formation of the major dense line and myelin compaction (Giese et al., 1992; Martini et al., 1995a).

(c) The PNS-specific myelin protein zero and its extensive contribution to myelin formation

Myelin protein zero (P0) is the most abundant peripheral myelin protein, accounting for approximately 50% of its total protein content, and is not present in CNS myelin (Greenfield et al., 1973; Lemke and Axel, 1985; Lemke, 1988). Protein zero is a transmembrane glycoprotein member of the immunoglobulin superfamily (Lemke et al., 1988) and it plays a pivotal role in the formation of compact myelin, including significant

demonstrated by experiments in mice doubly mutant for P0 and MBP (Martini et al., 1995a).

(d) Peripheral myelin protein 22

Peripheral myelin protein 22 (PMP-22) is another protein that is present in peripheral, but not central, myelin where it constitutes 2 to 5% of the total protein content (Snipes et al., 1999). Initially named gas-3 (growth arrest specific mRNA 3) because its expression was negatively correlated with cellular growth in cultured fibroblasts, this protein was later shown to be expressed predominantly in Schwann cells where it is localized to the compact portion of myelin. Furthermore, molecular studies revealed that the gene encoding PMP-22 is mutated in the spontaneous dysmyelinating mutant Trembler, as discussed in earlier sections (Welcher et al., 1991; Suter et al., 1992).

In Trembler mice, peripheral nerves are severely hypomyelinated and as these animals mature, their peripheral nerves experience continuous rounds of demyelination, followed by Schwann cell division and attempted remyelination. While the peripheral nerve abnormalities in Trembler mice have been shown to be secondary to a Schwann cell defect and not an axonal defect (Aguayo et al., 1977; Pollard and McLeod, 1980), the precise role of PMP-22 has so far been elusive. However, possible roles in intracellular adhesion, cellular growth arrest, and apoptosis have all been proposed based on circumstantial evidence (Snipes et al., 1999).

(e) Major myelin structural proteins: summary

In this section, the role of three major structural proteins in peripheral myelin, MBP, P0, and PMP-22, was examined, laying the background for the discussion of the molecular regulation of myelination in the next section. A more detailed discussion of the current state of knowledge regarding each of these proteins is available in recent review articles (Filbin and Tennekoon, 1992; Brophy et al., 1993; Lemke, 1993; Snipes et al., 1993; Snipes and Suter, 1995; Martini, 1997; Martini and Schachner, 1997; Martini, 1999; Niemann et al., 1999; Schmidt, 1999; Snipes et al., 1999; Vance, 1999; Kamholz et al., 2000; Muller, 2000; Campagnoni and Skoff, 2001). It is noteworthy that these three structural proteins are only a few among many myelin genes that are important in peripheral nervous system development and/or myelination, including myelin associated glycoprotein (Montag et al., 1994; Carenini et al., 1998; Yin et al., 1998; Schachner and Bartsch, 2000), connexin 32 (Bennett, 1994; Anzini et al., 1997; Martini, 1997; Balice-Gordon et al., 1998; Neuberg et al., 1998; Scherer et al., 1998; Fischbeck et al., 1999; Neuberg and Suter, 1999; Scherer et al., 1999; Kamholz et al., 2000), and periaxin (Gillespie et al., 1994; Scherer et al., 1995; Gillespie et al., 2000), among others. The investigations reviewed here demonstrate the intriguingly complex and sometimes overlapping roles of individual myelin components.

IV. REGULATION OF MYELINATION IN THE PERIPHERAL NERVOUS SYSTEM

(a) Regulation of myelination: overview

The preceding sections have provided an overview of the axon-Schwann cell interactions leading to the development of myelinated peripheral fibers and key components and features of the myelin sheath. With technological advances in molecular biology, the early observations describing axon-Schwann cell interactions leading to myelination have been re-evaluated in order gain insight into the molecular pathways regulating key events in the differentiation of peripheral fibers. As noted previously, axons instruct Schwann cells to myelinate, and this response is achieved in part through the coordinate up-regulation of myelin genes such as those encoding MBP, P0, and PMP-22. In this section, the current state of knowledge on developmental expression of structural myelin proteins and the candidate molecules regulating their expression are examined.

(b) Myelin gene expression during development and regeneration following axonal injury

In the rodent PNS, myelinogenesis begins in the peri-natal period and coincident with the initiation of myelin formation, Schwann cells coordinately up-regulate myelin specific genes such as MBP, P0, and PMP-22. Expression of these genes reaches maximal

levels by approximately 3 weeks postnatally, and thereafter, lower steady state levels are maintained throughout maturity. Should the axon be disrupted, as in experimental models of axonal injury, Schwann cells in the distal stump rapidly down-regulate myelin gene expression and pre-existing myelin sheaths are shed. In such "denervated" Schwann cells, up-regulation of myelin genes and elaboration of new myelin sheaths occurs only when Schwann cells re-establish contact with regenerating axons (Gupta et al., 1988; Trapp et al., 1988; Lamperth et al., 1990; LeBlanc and Poduslo, 1990; Stahl et al., 1990; Snipes et al., 1992; Snipes et al., 1999). Likewise, if Schwann cells are isolated in tissue culture preparations, myelin gene expression down-regulates under most conditions (Lemke and Chao, 1988; Morgan et al., 1991), emphasizing the importance of continuous Schwann cell association with the axon for myelin gene expression. The mechanism underlying such axon to Schwann cell signaling is not clear, but likely requires contact and may involve diffusible molecules (Bolin and Shooter, 1993).

(c) In vitro studies of myelination and the role of cyclic AMP in myelin gene regulation

As discussed before, myelin gene expression either ceases or is markedly down-regulated in isolated Schwann cells in culture under most conditions. However, addition of agents that raise the intracellular level of cyclic AMP (cAMP), such as forskolin, partly restores the expression of myelin genes such as MBP and P0 (Lemke and Chao, 1988; Morgan et al., 1991), suggesting that cAMP is a candidate signaling molecule regulating myelin gene expression. Furthermore, the expression of myelin genes under such conditions depends on the position of Schwann cells in the cell cycle, as expression is

negatively correlated with the presence of serum and a proliferative state (Morgan et al., 1991). Based on these observations, a model of Schwann cell maturation has been proposed. In this model, increased cAMP levels, followed by a secondary permissive signal such as withdrawal from the cell cycle, results in the induction of myelination during development (Morgan et al., 1991). Although this model is attractive, its application to myelination in vivo and the precise role of cAMP are at present unclear. For example, while there is some reduction of cAMP in sciatic nerves distal to the site of nerve transection, there is a significant delay in the rise in cAMP levels compared to the rise in P0 mRNA during regeneration (Poduslo et al., 1995). Furthermore, in the same study, addition of forskolin to distal stumps of transected nerves did not increase myelin gene expression. Therefore, additional investigations are required to elucidate the precise role of cAMP in myelination.

In apparent contrast to some of the observations described above, Schwann cell cultures derived and maintained in defined serum-free medium constitutively express P0 and MBP in the absence of forskolin (Cheng and Mudge, 1996). In this context, expression is suppressed by the addition of agents such as glial growth factor (GGF) and these investigators have suggested that prior to myelination in vivo, myelin gene expression by Schwann cells may be inhibited by negative regulatory signals and "release" from such inhibition may play a part in the up-regulation observed later during myelination. In addition to the surprising observations above, Cheng and Mudge also demonstrate that dividing Schwann cells can express myelin genes, suggesting that

withdrawal from the cell cycle in not required for myelin gene expression under all circumstances.

While the observations of Cheng and Mudge are interesting and challenge some of the conventional thinking in the field, their hypothesis requires significant additional investigation and cannot fully account for all the major aspects of myelin gene regulation in vivo. For example, distal to the site of a transection were the axon has degenerated, myelin-specific genes remain down-regulated and are only up-regulated if the axon is allowed to regenerate. The cessation of myelin gene expression by these Schwann cells is inconsistent with the hypothesis presented, unless the assumption is made that these Schwann cells are different from Schwann cells that have never been in contact with axons. In addition, low-level myelin gene expression occurs in vivo in Schwann cells prior to myelination and the expression described may simply reflect that and not the major up-regulation that occurs coincident with myelination. Additional investigations are necessary to address these issues and reconcile existing data from in vitro studies. Ultimately, the relevance of these observations to myelin gene regulation in vivo needs to be established.

(d) DNA sequences regulating myelin gene expression: in vitro and in vivo investigations

Despite the significant advances made in the understanding of the molecular regulation of myelination over the past decade, our knowledge of the transcriptional

regulation of myelin genes remains rudimentary. One approach has been to study the promoters of myelin genes in vitro in order to identify *cis*-acting elements which in turn could be used to identify transcription factors regulating myelination (Monuki et al., 1989; Monuki et al., 1990; He et al., 1991; Monuki et al., 1993; Li et al., 1994b; Shy et al., 1996; Brown and Lemke, 1997; Desarnaud et al., 1998; Peirano et al., 2000). As for the studies implicating cAMP in myelin gene regulation, important regulatory candidates, such as the POU domain transcription factor SCIP (described below), have been investigated in such preparations. However, since no in vitro model completely recapitulates the phenotype of myelinating Schwann cells in vivo, and given the apparent importance of axon-Schwann cell interactions in regulating myelin gene expression, the relevance of such in vitro findings is somewhat controversial. Consequently, the promoters of myelin genes have also been analyzed in vivo in transgenic preparations. Despite caveats such as position variegation and different copy numbers, this is a traditionally powerful approach for identifying and characterizing the expression programming conferred by DNA regulatory sequences. Using this approach, the expression of reporter genes promoted by one to several kbs of 5' flanking sequences from the P0, MBP and CNPase genes have been investigated in transgenic mice. For example, 1.1 kb of 5' flanking sequence from the P0 gene has been shown to contain sufficient regulatory elements to target reporter gene expression to myelinating Schwann cells (Messing et al., 1992), although the expression conferred to reporter constructs appears to be significantly more effective and robust when additional P0 intragenic and/or 3' flanking sequence is present (Feltri et al., 1999). In addition, 4 kb of 5' flanking sequence from the CNPase gene targets expression to both Schwann cells (albeit only

during early postnatal development) and oligodendrocytes (Gravel et al., 1998). In contrast, constructs regulated by similar lengths of MBP 5' flanking sequence express only in oligodendrocytes, the myelin forming cell type in the central nervous system, 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; Wrabetz et al., 1998).

(e) Krox-20: an essential transcription factor for the maturation of Schwann cells into myelinating cells

Another approach in investigating myelin gene regulation involves the disruption or mutation of specific transcription factors. Using such an approach, it has been demonstrated that the zinc finger transcription factor Krox-20/Egr2 (Krox-20; Chavrier et al., 1988; Joseph et al., 1988) is essential for the maturation of Schwann cells into myelinating cells. In mice bearing homozygous null Krox-20 alleles, Schwann cells associate with axons but do not myelinate them (Topilko et al., 1994). However, it remains to be shown whether Krox-20 is required for the maturation of Schwann cells to a myelinating competent state or whether it directs the myelination program per se. Because expression of Krox-20 significantly precedes myelin gene up-regulation, any role in the direct regulation of myelin genes would require interaction with co-regulators.

(f) The role of the transcription factor SCIP in Schwann cell maturation

Another transcription factor of importance for peripheral nerve maturation is SCIP (suppressed cAMP-inducible POU). SCIP is also known as Oct-6 and Tst-1 and is a POU domain transcription factor that is expressed in CNS and PNS glia (He et al., 1989; Monuki et al., 1989; Suzuki et al., 1990). In the PNS, SCIP expression transiently peaks prior to the peak expression of myelin specific genes both during development and during regeneration following axonal injury (Monuki et al., 1989; Monuki et al., 1990; He et al., 1991; Scherer et al., 1994). The developmental expression of SCIP at an apparently specific stage of Schwann cell maturation aroused interest and further investigations were conducted in cultured Schwann cells. In these investigations, forskolin treatment induced SCIP expression and this expression temporally preceded the induction of the myelin gene expression (Monuki et al., 1989).

In order to investigate a direct role of SCIP in myelin gene expression, in vitro co-transfection assays were conducted. When SCIP expressing plasmids were co-transfected with reporter constructs driven by the P0 (1.1 kb) or MBP (1.4 kb) promoter, reporter gene activity was repressed (Monuki et al., 1990; He et al., 1991). Based on the in vivo spatio-temporal pattern of SCIP expression and the in vitro promoter studies, the following model was proposed. SCIP expression is regulated by axons and when SCIP is expressed in Schwann cells they are competent to ensheathe axons, but the expression of myelin genes and entry into the myelinating stage is inhibited (Monuki et al., 1990; Monuki et al., 1993; Scherer et al., 1994). The expression of SCIP itself is under axonal

control and when SCIP is down-regulated in response to axonal signals, Schwann cells are capable of entering a myelinating program. It is noteworthy that the 1.4 kb MBP promoter used in co-transfection studies does not confer expression to Schwann cells in vivo (Foran and Peterson, 1992; Gow et al., 1992; Miskimins et al., 1992). However, additional evidence supporting the proposed model emerged from studies of a SCIP dominant negative transgenic model (Weinstein et al., 1995). In these mice, a truncated form of SCIP that can bind DNA but cannot repress or transactivate in co-trasfection assays, acting as a dominant negative factor in vitro, was targeted to Schwann cells. The transgenic mice generated (Δ-SCIP) show multiple abnormalities, including premature myelination and hypermyelination in the adult, consistent with the proposed role of SCIP as a repressor of Schwann cell maturation into myelinating cells.

More recently, two groups have generated SCIP null mutant mice (Bermingham et al., 1996; Jaegle et al., 1996) and interestingly, the phenotype is the opposite of that anticipated from previous work. Interpretation is rendered even more difficult since while the phenotypes of the two SCIP null mutant mice are similar, they are not identical. The majority of homozygous null SCIP mice generated by both groups died shortly after birth, and only the rare pups that survived (Jaegle et al. report 2-4 %) were analyzed. There are two major differences between the phenotypes reported by the two groups. First, Bermingham et al. report that rare pups survived up to 22 days and that axons in their sciatic nerves at P3 were mostly unmyelinated. However, Jaegle et al. were able to analyze pups that were up to 90 days old, and while they reported myelin deficiency in the second week of postnatal development, in the adult, myelination was apparently

complete. The discrepancy can perhaps be explained by the longer survival of the animals generated by the latter group, which may in turn be related to their genetic background. It is likely that the general failure of myelination reported by the first group simply reflects the fact that their animals did not live long enough for their peripheral nerves to become myelinated on a SCIP null background. Regardless, it is clear that myelination is delayed in the absence of SCIP, inconsistent with the previously proposed function for SCIP as a repressor of the myelinating state.

The second major difference between the two reports relates to the expression of major late myelin-associated genes in the two mutants and is harder to reconcile. Bermingham et al. report nearly absent MBP and P0 protein in the homozygous null mutants but comparable MBP and P0 mRNA to controls by in situ hybridization. Jaegle et al., on the other hand, report reduced MBP (among others) mRNA by semiquantitative PCR. The observations are important in relation to the transcriptional regulation of myelin-associated genes by SCIP. The discrepancies may be related to the ages examined and the assays used as discussed elsewhere (Zorick and Lemke, 1996).

Regardless of the differences between the two mutants, their phenotypes are not consistent with the proposed model, based on co-transfection assays and Δ-SCIP transgenic mice, that SCIP represses myelin-specific genes in vivo. A thorough discussion of the differences between the phenotypes of Δ-SCIP animals and SCIP knock-outs and potential explanations for the discrepancies can be found elsewhere (Zorick and Lemke, 1996). However, one possible explanation is that Δ-SCIP, for unknown and

unpredictable reasons, is acting as a dominant positive instead of dominant negative mutant in vivo (Zorick and Lemke, 1996). Indeed, the octamer motif is known to mediate both transcriptional activation and repression (Lenardo et al., 1989; Scholer et al., 1989). Future investigations should clarify the precise role of this intriguing transcription factor in PNS maturation.

(g) Molecules regulating peripheral nerve maturation and myelination: summary

In this section, key aspects of some important candidate molecules regulating PNS maturation and/or myelination were reviewed. Perhaps the most striking candidate is the transcription factor Krox-20, in the absence of which PNS axons do not become myelinated. However, even in the case of Krox-20, its exact role in PNS maturation and whether or not it directly regulates myelin gene expression remain to be established. Another transcription factor with an essential role in peripheral nervous system development is Sox10, as Schwann cells fail to develop from neural crest cells in mice bearing null Sox10 alleles (Kuhlbrodt et al., 1998; Britsch et al., 2001). Sox10 has also been proposed to regulate myelin gene expression (Peirano et al., 2000), but conclusive evidence supporting such a role in vivo is still lacking.

Along with the molecules discussed above, other compounds and experimental conditions known to modulate myelin gene expression in Schwann cells and oligodendrocytes include progestins, that potentiate myelination both in vivo and in vitro, and electrical stimulation, that modulates the level of MBP expression in cultured

Schwann cells (Koenig et al., 1995; Jung-Testas et al., 1996; Stevens et al., 1998). A role for myelin associated glycoprotein (MAG) in bi-directional transduction of axon-Schwann cell signals has also been proposed based on the observation that homozygous MAG null animals express both axonal and myelin anomalies (Li et al., 1994a; 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). Finally, the current knowledge of the cis DNA elements regulating myelin gene expression in vivo is rudimentary. Although significant advances have been made with the identification of transcription factors Krox-20 and SCIP, the exact mechanism controlling myelin gene expression remains unknown.

V. OBJECTIVES AND RATIONALE FOR THE INVESTIGATIONS IN THIS THESIS

As discussed above, our understanding of the transcriptional regulation of myelin gene expression in vivo is rudimentary. In order to gain further insight into the transcriptional regulation of myelination in Schwann cells, I examined the regulation of one of the myelin structural protein genes, MBP. I used a combination of sequence analysis, in vitro DNA-protein interaction assays, and in vivo functional assays to investigate MBP regulation. The design of all the experiments takes into full account the complexities of myelin gene regulation and the requirement for close interaction between axons and Schwann cells. Therefore, all functional promoter assays have been performed in vivo in transgenic mice. In addition, I derived a protocol for obtaining extracts

containing transcription factors from myelinating Schwann cells in sciatic nerves in order to enable the application of powerful in vitro DNA-protein interaction assays. Given their source, these extracts are most likely to contain factor(s) that reflect the in vivo regulation of myelin genes. Using such an approach, the objectives of my investigations were:

1. To identify and characterize enhancer(s) regulating MBP expression in Schwann cells *in vivo*.
2. To locate and characterize, to the extent possible, the regulatory sequences underlying the activity of MBP Schwann cell enhancer(s).

Towards these objectives, chapter 2 describes the identification of MBP enhancers and the characterization of the developmental expression programming of a robust 0.6 kb Schwann cell enhancer (SCE1). Sequence analysis and a preliminary mutagenesis study of SCE1 are also described in this chapter. In the next chapter (3), the derivation of the protocol for obtaining extracts from mouse sciatic nerves that contain transcription factors from myelinating Schwann cells is described. Then, chapter 4 provides a functional in vivo breakdown of the sequences underlying SCE1 function and the use of electrophoretic mobility shift assays using peripheral nerve extracts to identify candidate elements for mutagenesis studies. In addition to describing a complex regulatory network controlling MBP expression in Schwann cells, the investigations presented in this chapter describe a high resolution in vivo method for studies of MBP expression in a quantitative manner. A general discussion follows in chapter 5.

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CHAPTER 2

ABSTRACT

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 0.6kb 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.

Previously, the expression of reporter genes promoted by one to several kbs 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 myelinating 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 expressed only in oligodendrocytes, the myelin forming cell type in the central nervous system, 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. Further, 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 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 BamH I MBP genomic fragment, containing approximately 12.0 kb of MBP 5' flanking sequence, was obtained by screening a lambda DASH - 129 mouse genomic library (J. Rossant, Mount Sinai, Toronto). This sequence was subcloned in the BamHI 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 (BamH I) 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, ON).

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 –9kb MBP promoter). This clone contained a double insert of lacZ and the second insert was released by BamH I digestion followed by intramolecular ligation (clone 5). Constructs containing 9.0 kb (Sac II), 8.5 kb (Nae I), 7.0 (Sph I), or 6.0 kb (Kpn I) of MBP promoter were obtained by restriction digestion of clone 5 and agarose (Boehringer Mannheim, Laval, PQ) gel purification (0.5%/TAE). A Kpn I/BamH I 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). -6.0 kb MBP-lacZ was isolated from clone 5 (Kpn I/BamH I) and cloned in 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). -6.0 MBP-lacZ was isolated from clone 5 with Kpn I and cloned in 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. -3.1 kb MBP-lacZ was isolated from clone pm12 with Xba I and cloned in 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) was used. The 0.6 kb SCE1 (Sac II/Sac I) was blunted (Klenow (Boehringer Mannheim, Laval, PQ)) and inserted in the EcoR V 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). 0.3 kb hsp68-lacZ was isolated from clone p610ZA (Hind III/Kpn I) and cloned in 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). 0.3 kb hsp68-lacZ was isolated from p610ZA (Hind III/Kpn I) and cloned in 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, Laval, PQ) 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) were annealed and cloned into their respective sites within clone KS-SCE 11A, 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/BamH I) and cloning it into the Kpn I/BamH I sites of pKS-δKrox-20-SCE1. The construct was released by Kpn I/Sac II digestion.

slides and viewed either directly or after staining with toluidine blue. Additional tissue was cryoprotected by immersion in 30% sucrose prior to freezing and 12 μm cryostat sections were made and subsequently incubated in stain containing 0.8 mg/ml X-gal (Gibco/BRL, Burlington, Ontario). To investigate prenatal expression, we applied a histochemical technique capable of detecting low-level ß-galactosidase activity in sections. Fetuses were recovered and immersion fixed for 1 hour in the same aldehyde mix at 4^0 C. To cryoprotect, they were then incubated at 4^0 C in 30% sucrose overnight prior to freezing in isopentane pre-cooled in liquid nitrogen. Cryostat sections, 12 μm thick, were picked up on slides and dried for 30 min at room temperature and 20 min at 37^0 C. Sections were then post-fixed by immersion in 4% formalin and 7.5% sucrose in 0.1 M phosphate buffer for 20 min at 4^0 C, washed for 5 min in buffer alone 3 times, then transferred to ß-galactosidase stain (X-gal at 0.8 mg/ml) and incubated at 37^0 C overnight. Slides were then cover-slipped using 10% glycerol as the mounting medium.

RESULTS

In a search for the cis-regulatory sequences that control MBP expression in Schwann cells, we analyzed the expression conferred to MBP regulated reporter constructs in transgenic mice. In the initial constructs we evaluated, lacZ was promoted by approximately 6, 7, 8.5 or 9 kb of MBP 5' flanking sequence (Fig. 2.1). Constructs containing flanking sequences extending to -8.5 kb expressed only in oligodendrocytes while the construct regulated by the 9.0 kb sequence also expressed in Schwann cells

(Figs. 2.1 & 2.2) indicating that one or more Schwann cell targeting elements are located in the sequence between -9 and -8.5 kb.

To assess whether this distal sequence had functions characteristic of an enhancer, we generated constructs in which the slightly longer –9 to –8.4 kb sequence was ligated, in both orientations, to the 3' end of the lacZ reporter promoted by 6 kb of MBP 5' flanking sequence (Fig. 2.1). In another construct, it was ligated immediately upstream of 3.1 kb of MBP 5' flanking sequence driving lacZ. Consistent with classic enhancer function, robust expression was observed in the PNS of multiple lines bearing all three constructs (Table 2.1). The Schwann cell targeting conferred by this 0.6 kb MBP sequence was assigned the interim designation, Schwann Cell Enhancer 1 (SCE1).

To search for further sequences capable of conferring Schwann cell expression, the 3 kb sequence immediately upstream of SCE1 (-12.0 to –9.0) was ligated to the 3' end of the lac Z reporter gene, driven by 6 kb of MBP 5' flanking sequence (Fig. 2.1). So far, one transgenic mouse that failed to transmit the transgene has been analyzed. Although expression of the transgene was mosaic, those Schwann cells that labeled did so intensely (Fig. 2.2; Table 2.1). As the MBP sequence in this construct was ligated 3' of lacZ, its provisional targeting ability also appeared to be mediated through enhancer activity and it was assigned the interim designation, Schwann Cell Enhancer 2 (SCE2). The sequence extending from -12 kb to -8.4 kb (including both SCE1 and SCE2) has been deposited to GenBank (accession number AF277397).

The observation that SCE1 functions in a position and orientation independent manner was made in the context of 6kb or 3.1 kb of proximal MBP 5' flanking sequence. Consequently, Schwann cell targeting activity could require interaction between SCE1 and additional elements contained within the proximal MBP promoter. To investigate this possibility, the -9.0 to -8.4 kb sequence was ligated, in both orientations, 5' of a heterologous promoter (0.3 kb hsp68) driving the lacZ reporter gene. Transgenic mice bearing this SCE1-hsp68-lacZ construct were derived and seven of ten independent transgenic lines, including those bearing SCE1 in either orientation, expressed ß-galactosidase in myelin forming Schwann cells (Fig. 2.3; Table 2.1). Because these constructs were expressed at high levels in the myelinating Schwann cells of multiple lines, SCE1 contains robust Schwann cell enhancer activity that is sufficient to target Schwann cell expression.

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., ISN Satellite and UConn-Kroc Symposium, Mystic, 1997). Transgenic mice bearing SCE1-hsp68-lacZ constructs similarly express low but detectable levels of ß-galactosidase activity in fetal peripheral nerves from E14 through birth (Fig. 2.4, a, b). Also reflecting the endogenous expression program, high-level expression of ß-galactosidase appeared in Schwann cells coincident with myelin formation when accumulation of endogenous MBP mRNA markedly increases. As predicted by the temporally discordant myelination

endogenous program, SCE1-hsp68-lacZ lines (17, 49, and 54) maintain expression throughout maturity (examined up to 7 - 9 months of age). In contrast, while intense labeling was observed in lines 18 and 42 during pre-weaning development, by three 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 down regulating the mature expression phenotype.

As mice bearing the 9.0 kb promoted construct matured, there was a modest decline in ß-galactosidase labeling intensity throughout their mixed nerves (lines 17 and 32) and in the mosaic line 24, expression ceased. Surprisingly and consistently, in mice over 3 months of age from line 17 and 32, dorsal roots continued to label but a further precipitous decline in transgene expression was observed in ventral roots (Fig. 2.4d). In line 17 mice, expression appeared to be shut off while in line 32, ventral root down regulation 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 heterogeneity amongst 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-lac Z lines 49 and 54 continued to label at an apparently similar level throughout maturity.

Since 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 more than 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. 2.5a). 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. As 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 2.1), 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 (δKrox-20-SCE1) at the 3' end of lacZ and

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. 1.1 kb of 5' flanking sequence from the protein zero (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 myelinating 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, that potentiate myelination both *in vivo* and *in vitro*, and electrical stimulation, that 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 up-regulation of myelin gene expression (Lemke and Chao, 1988; Morgan et al., 1991). More recently, a role for myelin associated glycoprotein (MAG) in bi-directional 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 up-regulation 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. While 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 up-regulation of myelin genes by several days (Murphy et al., 1996).

Lastly, the POU domain transcription factor SCIP has been implicated in the control of myelin gene expression. It is down-regulated immediately prior to 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; Monuki et al., 1990; He et al., 1991; Weinstein et al., 1995). However, homozygous null SCIP mutants do myelinate, albeit in a delayed manner (Bermingham 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). As 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.

Amongst the lines of transgenic mice established for this investigation, 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. Lastly, 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).

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

Table 2.1. Transgenic mice used for the functional analysis of the MBP promoter: The designations + (indicates reporter gene expression) and – (no expression) were made based on the β-galactosidase histochemical assay as described in Materials and Methods.

Figure 2.1. MBP-promoted lacZ reporter constructs used to map Schwann cell enhancers and repressors in transgenic mice: 12 kb of MBP 5' flanking sequence was cloned from a lambda DASH-129 mouse genomic library. Constructs incorporating various lengths of these MBP sequences were then used to derive transgenic mice and transgene expression was assessed by the β-galactosidase histochemical assay. 5' flanking sequences extending to -6, -7, -8.5 and -9 kb were ligated to the lacZ reporter gene and the sequence lying between -9 and -8.5 kb was found to be required for Schwann cell expression. To evaluate potential effects of position and orientation, the slightly longer 0.6 kb fragment extending from -9.0 to -8.4 kb, designated Schwann cell enhancer 1 (SCE1), was ligated to constructs promoted by -3.1 or -6 kb MBP sequence. A second Schwann cell targeting activity was detected in the further upstream sequence (-12 to - 9 kb) and, as it also functions as an enhancer when ligated 3' of the lacZ reporter, it was designated SCE2. The striped region in the -6 kb MBP-lacZ-5'(SCE1)3' construct designates the pSK+ vector included in the injected construct.

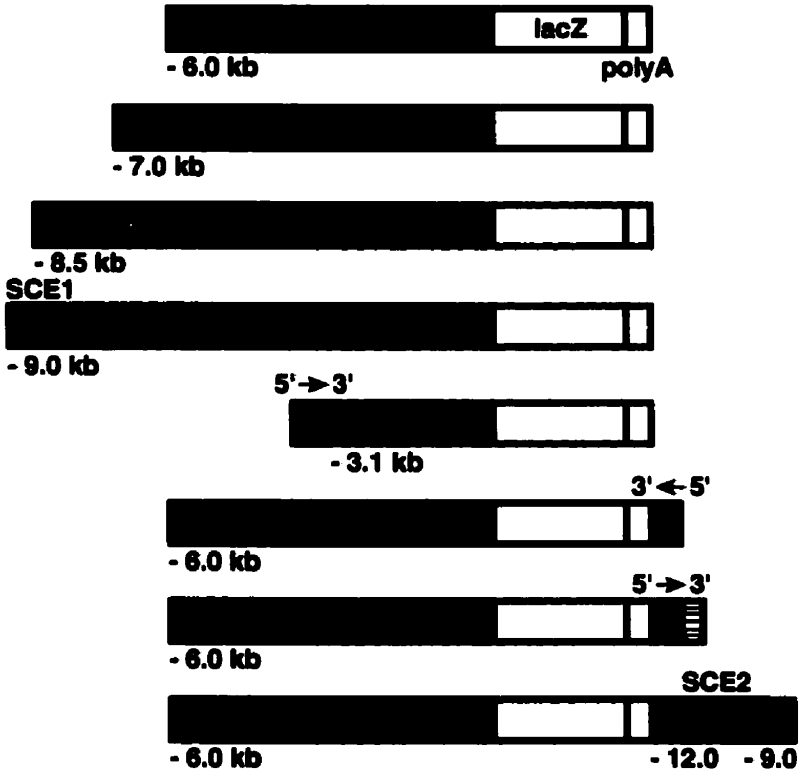

lacZ
- 6.0 kb
polyA
- 7.0 kb
- 8.5 kb
SCE1
- 9.0 kb
5'→3'
- 3.1 kb
3'←5'
- 6.0 kb
5'→3'
- 6.0 kb
SCE2
- 6.0 kb
- 12.0
- 9.0

Figure 2.2. ß-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 day old -9 kb line 17 mouse. Inset, 1 µm thick cross section of L4 ventral root embedded in plastic subsequent to whole mount labeling. Bluo-gal reaction product is associated with the majority of the myelin and Schwann cell cytoplasmic profiles. (b) 21 day 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, 1 µm thick cross section of whole mount stained L4 root demonstrates labeling of a sub-population 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 a SCE1-bearing transgenic mouse shows ß-galactosidase reaction product accumulated in the cytoplasmic compartments of the Schwann cell including the perinuclear cytoplasm (asterix), Schmidt-Lantermann incisures (closed arrowhead), and paranodal loops (open arrowhead). (e) 12 µm 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. As the intra-Schwann cell distribution of the reaction product is not uniform along the internode (see panel d), not all such profiles should be labeled. **Calibration: a, b, c = 3.1mm; insets in a, b = 0.05mm; c = 0.05mm; d = 0.17mm; e = 0.05 mm.**

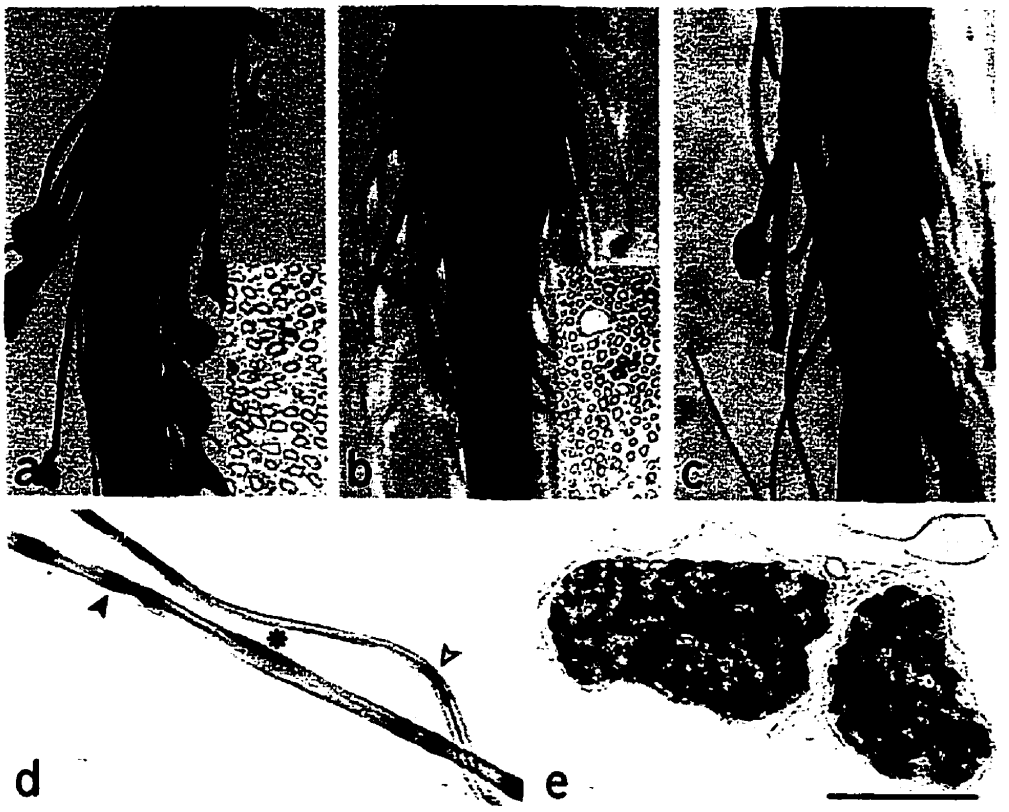
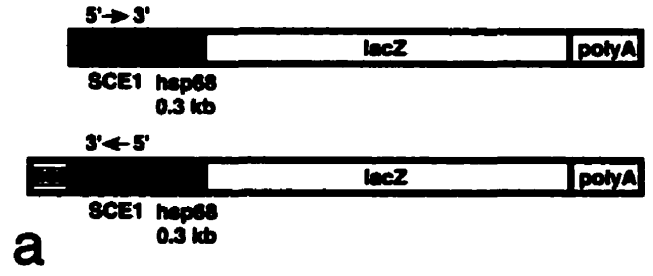

a
b
c
d
e

Figure 2.3. 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'-hsp-lacZ is from the vector, pSK+. (b) Dorsal view of a whole mount histochemical β-galactosidase preparation from a 7 day 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 while 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. **Calibration: b = 1.6mm; c & d = 4.4 mm.**



a

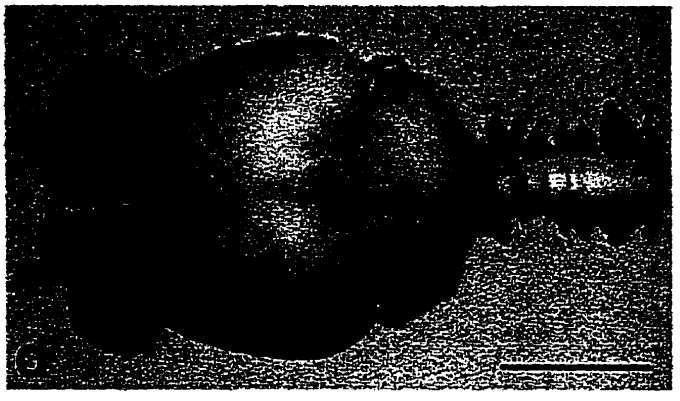


Figure 2.4. Developmental expression of transgenes bearing SCE1: Low-level transgene expression is detected in some regions of the PNS in a SCE1-hsp68-lacZ line 18 fetus at E.15.5. Mixed nerves exiting the spinal column have detectable but unevenly deposited ß-galactosidase reaction product (a) while the trigeminal nerve is more obviously and uniformly labeled (b). High-level expression, detectable in whole mount preparations, initiates only in the post-natal period. (c) Whole mount preparation of a 2 day 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, while some ventral root fibers have initiated sheath formation, the unlabeled dorsal roots (not shown) do not contain any myelin profiles. (e) Whole mount preparation of roots and dorsal root ganglia from a pre-weaning -9 kb MBP-lacZ transgenic mouse. Uniform and intense staining is observed in both dorsal and ventral roots while neither the neurons nor non-Schwann cell glia in the DRG label. (f) In 3 month old -9 kb line 17 MBP-lacZ transgenic mice, Schwann cells ensheathing motor fibers in ventral roots no longer label while 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). **Calibration: a = 0.18 mm; b = 0.18 mm; c = 1.14 mm; d = 4 um; e = 1.25 mm; f = 1.6 mm.**

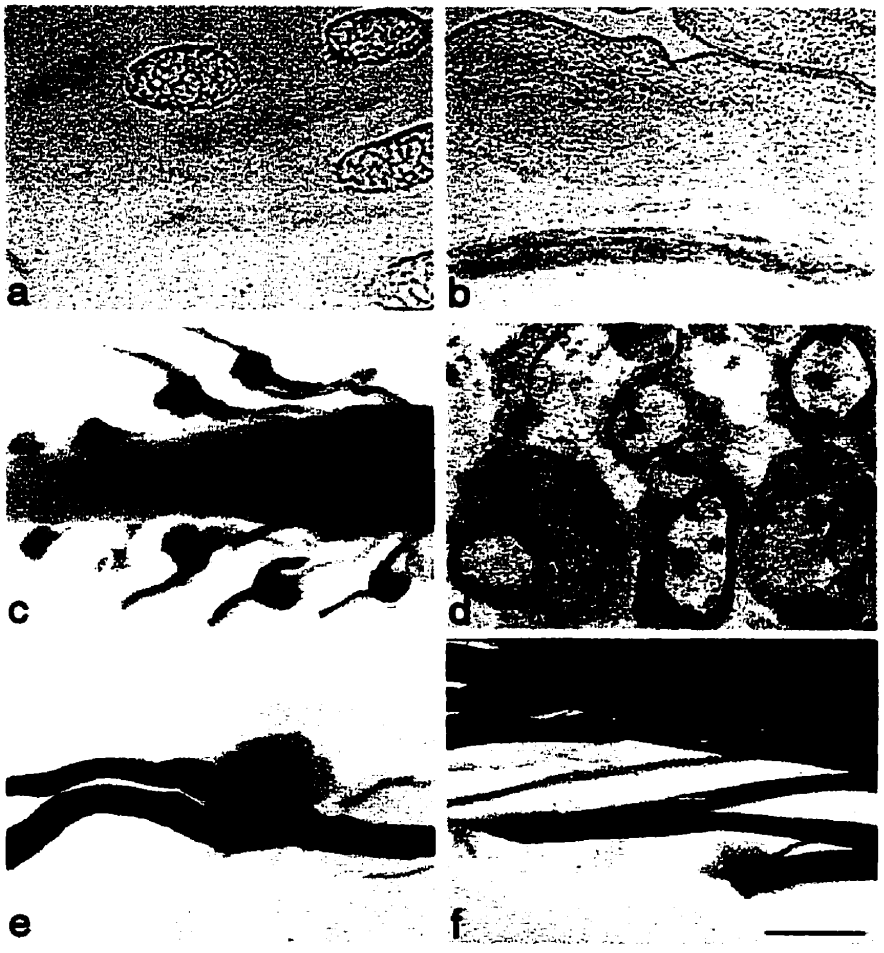
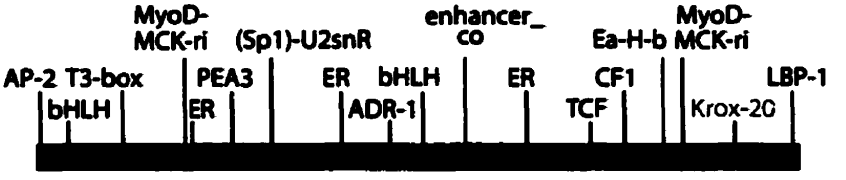

a
b
c
d
e
f

Figure 2.5. 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), δKrox-20-SCE1). The mutated SCE1 was ligated to the 3' end of lacZ driven by 6 kb of MBP promoter. (c) P30 transgenic mouse bearing the δKrox-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. **Calibration (c): 4.4 mm.**


MyoD-
MCK-ri
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MyoD-
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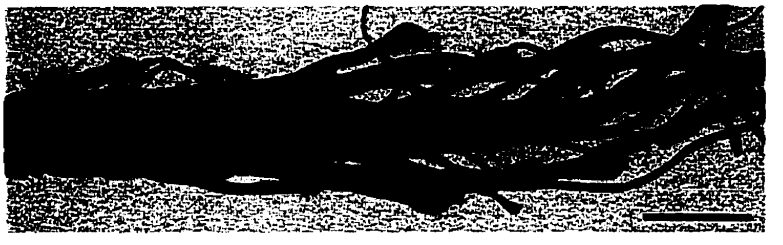
bHLH
ER
bHLH
ER
CF1
LBP-1
bHLH
ER
ADR-1
TCF
Krox-20

a


SCE
- 6 kb 5' MBP
lacZ
(∂Krox-20)

b



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CHAPTER 3

CHAPTER 3

PREPARATION OF NUCLEAR EXTRACTS FROM MYELINAITNG SCHWANN CELLS

R. Forghani, J. Nesbitt, J. Snipes, E. M. Shooter, and A. Peterson

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PREFACE

One obstacle to identifying cis elements and trans factors regulating myelin gene expression has been the lack of an effective approach for obtaining transcription factors from myelinating Schwann cells. This problem stems from the unique anatomical configuration of peripheral nerves that interferes with traditional methods of obtaining nuclear extracts from tissues. The aim of this study was to derive an effective protocol for obtaining extracts containing nuclear factors from myelinating Schwann cells in mouse sciatic nerves. Binding assays for the transcription factor Krox-20 were used as a positive control in order to monitor the effectiveness and guide the development of the protocol.

INTRODUCTION

Myelination in the peripheral nervous system requires contact between Schwann cells and the axon, coordinate up-regulation of numerous genes encoding myelin-specific proteins, as well as extensive lipid synthesis (Lemke, 1988). Despite the importance of myelin in nervous system function and the significant disease burden that occurs when the process of myelination is interrupted or mature myelin is destroyed, neither the signaling pathways nor the transcription factors regulating myelin genes are well understood. One experimental strategy that could lead to a better understanding of the pathways underlying axon-Schwann cell signaling and myelin gene regulation is to characterize the cis elements and the transcription factors that regulate the expression of those myelin genes that are controlled by axonal contact.

The promoters of two myelin genes expressed in the periphery, myelin protein zero (P0) and myelin basic protein (MBP), have been extensively studied in both functional assays and in vitro DNA-protein interaction assays (Monuki et al., 1989; Monuki et al., 1990; He et al., 1991; Morgan et al., 1991; Foran and Peterson, 1992; Gow et al., 1992; Messing et al., 1992; Goujet-Zalc et al., 1993; Monuki et al., 1993). Moreover, certain culture conditions are known to promote myelin gene expression in isolated primary Schwann cells. While these and other studies (Lemke and Chao, 1988) have provided candidate molecules that may participate in potential signaling mechanisms controlling myelination, high level expression of myelin genes by Schwann

cells is tightly controlled by axonal signals and isolated primary Schwann cells do not fully differentiate into myelinating cells. Consequently, both the validation of the in vitro models and the identification of additional transcription factor(s) controlling myelin gene expression would be greatly facilitated if the regulatory elements that confer expression to myelin genes in vivo could be identified. Traditionally, electrophoretic mobility shift assays (EMSA) and DNA footprinting techniques have been used to reach similar objectives with other cell types. Relevant nuclear extracts are a prerequisite of this strategy and these are readily available from cells in culture and the majority of tissues. However, the lack of an effective method for obtaining Schwann cell nuclear extracts from peripheral nerves has precluded this approach for myelin genes.

Anatomic features unique to peripheral nerves make simple nuclear preparation strategies ineffective and interfere with traditional methods of extracting nuclear proteins. Peripheral nerve fibers are invested with three separate connective tissue sheaths which normally serve to limit the flow of macromolecules (Peters et al., 1991). Moreover, each Schwann cell elaborates a basal lamina and the space between fibers in mature nerves contains abundant collagen. We now have overcome the problems presented by this unique anatomical configuration and report a reliable protocol to make transcription factor containing nuclear extracts from myelinating Schwann cells. Consequently, both the molecules regulating myelin gene expression and the axon signals that control the process should become significantly more accessible.

MATERIALS AND METHODS

Tissue source and preparation

We developed the method using mouse sciatic nerves since they contain a significant population of myelinating Schwann cells and their development is well characterized. As excess collagen interferes with homogenization and the collagen content in peripheral nerves increases with age, we focused our attempts to prepare nuclear extracts to young mice between postnatal days 3 and 10. During this period of maturation, active myelin elaboration initiates on the majority of axons destined to become myelinated. Consequently, a large proportion of Schwann cells have begun to express myelin genes at high levels.

Mouse pups in the P3 - P10 age range were killed by cervical dislocation and their sciatic nerves were recovered and immediately frozen in a small mortar pre-cooled on dry ice. The mortar was covered by plastic wrap during dissection to prevent excessive frost formation. The pestle, also wrapped in plastic film, was pre-cooled on dry ice. After accumulating sufficient nerves (typically > 200) they were ground to a powder with the pre-cooled pestle. Then, using a pre-cooled spatula, the powder was accumulated at the bottom of the bowl, and ground again. The powder was transferred to a 1 ml Wheaton® homogenizing tube on ice and extraction was performed using a simple two-step protocol, all on ice at 4°C in the presence of protease inhibitors (added to the buffer immediately before extraction from frozen stocks).

Preparation of nuclear extracts

In the first step, the powder was homogenized (25 strokes, pestle B) against buffer A (10 mM Hepes, pH 7.9, 150 mM NaCl, 0.6% NP-40, 1 mM EDTA, 1 mM DTT, 4 mM benzamidine, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM PMSF). The tissue to buffer ratio was ≈ 100 nerves/200 μl buffer A. The homogenate was centrifuged at 2500 x *g* for 5 min and the supernatant (cytoplasmic fraction) was removed. To reduce the likelihood of nuclear lysis, the pelleted nuclei/debris were first resuspended in low salt buffer Bl (20 mM Hepes, pH 7.9, 0.2 M KCl, 1.2 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 4 mM benzamidine, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM PMSF, 25% glycerol). The tissue to buffer ratio at this step was ≈ 100 nerves/50 μl buffer Bl. The suspension was transferred to a micro homogenizing tube and then, buffer B2 (same as Bl, except 1.4 M KCl) was added (1 volume of B2 for two volumes of Bl) slowly while homogenizing (as above), to obtain a final KCl concentration of 0.6 M. The homogenate was incubated for 45 min and homogenized as above at 15 min intervals. Then, it was centrifuged at 16000 x *g* for 20 min. The supernatant was aliquoted, snap frozen on ethanol/dry ice, and stored at -80 °C. Protein concentration in the final supernatant was typically between 4 and 8 μg/μl (Bradford, Bio-Rad protein Assay).

Electrophoretic mobility shift assays

To monitor the effectiveness of each modification, we used EMSAs with an oligonucleotide probe containing the binding site for Krox-20 (Egr-2) (Chavrier et al.,

1988; Joseph et al., 1988; Chavrier et al., 1990; Nardelli et al., 1991). In peripheral nerves, this transcription factor is specifically expressed in myelinating Schwann cells (Topilko et al., 1994). Complementary oligonucleotides gatcCTTG-TACGCGGGGGCGGTTAGT and ctagACTAACCGCCCCCGCGTACAAG were annealed and purified from 5% non-denaturing polyacrylamide gel and radioactive probes were generated by end filling with Klenow. Reactions (40 µl) were carried out at room temperature with probe alone (Fig. 3.1, lane 1) or by incubating 20 µg of nuclear extract (Fig. 3.1, lanes 2-6) with 2 µg poly(d*I* - d*C*) · poly(d*I* - d*C*) (Pharmacia) in 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.25 µg/µl BSA, 5% glycerol for 5 min at room temperature. Then 1 ng of probe (± 100-fold cold competitor) was added and incubation continued for 15 min. Competitions were done with cold Krox-20 oligos (Fig. 3.1, lane 5, 100x) or control (consensus AP-1 site) oligos (Fig. 3.1, lane 6). For supershifts, 2 µg affinity purified rabbit polyclonal Krox-20 antibody (BABCO) (Fig. 3.1, lane 3) or control (rabbit Met antiserum) (Fig. 3.1, lane 4) was added and incubation continued for another 30 min. Reactions were run in a 5% polyacrylamide gel (0.5 x Tris - Boric acid – EDTA) at room temperature. Gels were dried and exposed overnight with an intensifying screen.

RESULTS

To assess the effectiveness of this protocol, extracts were tested in EMSAs (below). During protocol development, extraction buffer content, EMSA binding buffer content, and technical aspects of tissue handling were optimized in 19 extract

preparations. When extracts were dialyzed (1 or 11 h in Sartorius micro-collodion bags) or filtered (Millipore microcon microconcentrators of 3 or 10 kd cut-off), we did not see an improvement in EMSAs with either Krox-20 or an uncharacterized Schwann cell enhancer as a probe (data not shown). One critical component identified during protocol refinement was the KCl concentration in the high salt extraction step. The final KCl concentrations tested include 0.4, 0.6, 0.8 and 1.2 M. Zero point six molar gave the best results, although 0.4 M was moderately effective. If a concentration higher than 0.6 M was used, the homogenate became viscous resulting in poor extract recovery. Zero point six molar is not commonly used in extract preparations as they are significantly less transcriptionally active than those prepared at 0.4 M (Dignam et al., 1983b). However, based on the present observations, such diminished transcriptional activity does not necessarily preclude the effective recovery of transcription factors and binding to DNA.

Multiple retarded bands were observed when sciatic extracts obtained with the optimized protocol described in this report were incubated with Krox-20 oligonucleotide probes (Fig. 3.1). One of these supershifted with Krox-20 antibody (lane 3), demonstrating that Krox-20 is present in sciatic nerve extracts. Further, 100-fold excess of an oligonucleotide bearing a mutation that abolishes Krox-20 binding (GCGGGGGCGG $\rightarrow$ GCGGTTTCGG; (Chavrier et al., 1990)) did not compete the band supershifted with the Krox-20 antibody (data not shown). It is noteworthy that the Krox-20 shift is very sensitive to binding buffer components and the temperature of incubation; if the binding reaction is carried out on ice, a predominant non-specific complex forms. Lastly, using EMSAs, we evaluated the distribution of Krox-20 in the subcellular

fractions prepared with this protocol. Little or no signal was observed when the cytoplasmic fraction was used even at concentrations twice that of the nuclear extract (data not shown).

DISCUSSION

Since the original nuclear extraction protocol was described (Dignam et al., 1983a; Dignam et al., 1983b), microextraction protocols optimized for a wide variety of tissues have been developed (Hoppe-Seyler et al., 1991; Roy et al., 1991). Relative to these established procedures, the modifications that led to success with sciatic nerves, while subtle, were numerous. A systematic evaluation was conducted of three major variables: the maturity of the donor animals; the method of sample accumulation and the salt concentration used for extraction. Using nerves from young mice, we overcame homogenization problems related to the high collagen content of this tissue in mature animals. One further difference of unknown significance is that the exceptionally dense intermediate filament cytoskeleton typical of mature axons assembles largely after the stage of maturation we investigated (Eyer and Peterson, 1994). The collection and homogenization technique that we adopted proved efficient for samples consisting of multiple small nerves. Finally, we determined the optimal salt concentration for effective extraction of nuclear proteins from peripheral nerves.

Since optimizing the protocol, we have seen consistent binding activity in EMSAs with 10 additional extract preparations using both Krox-20 and additional consensus

sequences as probes. Therefore, this method has been demonstrated to be both effective and reliable for extraction of multiple nuclear proteins from the sciatic nerves of mice. While the small amounts of nerve tissue obtainable from pre-weaning mice necessitate sacrificing multiple donor animals, the technique we describe may have application to nerves from larger animals provided that, as typical of immature animals and spinal roots of mature animals, such nerves have minimal collagen investment. Regardless of the breadth of species to which this method proves to be useful, its applicability to the mouse permits access to an experimentally important source of myelinating Schwann cells including developing, regenerating and mutant models. Consequently, we believe that it overcomes a major experimental obstacle that previously interfered with the investigation of DNA-protein interactions underlying myelin gene expression and the further investigation of axon-Schwann cell signaling.

ACKNOWLEDGEMENTS

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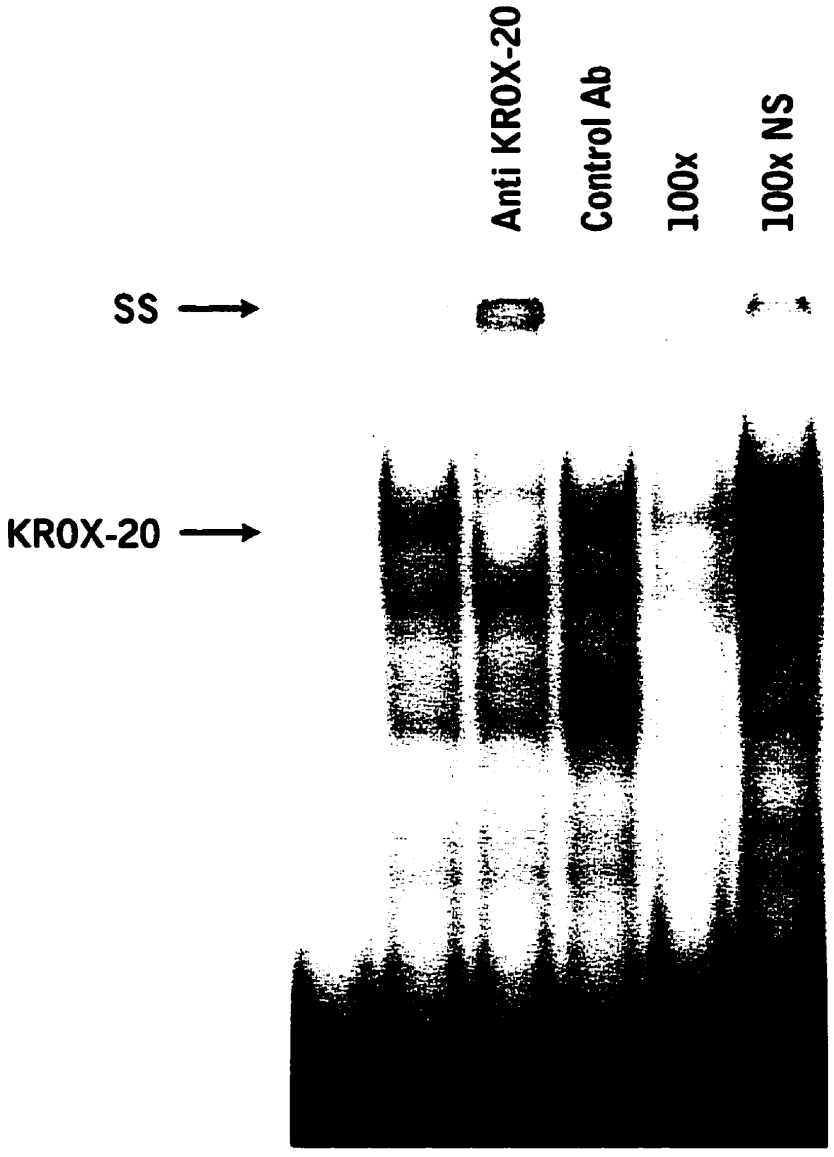
Figure 3.1. Sciatic nerve extracts contain the transcription factor Krox-20: EMSAs were performed with oligonucleotides containing the Krox-20 binding site as probe. Reactions contain probe alone (lane 1) or probe in the presence of 20 μg of nuclear extract (lanes 2-6) in binding buffer. Competitions were done with 100x excess of cold Krox-20 oligos (lane 5, 100x) or control (consensus AP-1 site) oligos (lane 6, 100x NS). For supershifts, 2 μg affinity purified rabbit polyclonal Krox-20 antibody (BABCO) (lane3) or control (rabbit Met antiserum) (lane 4) was added. Reactions were run in a 5% polyacrylamide gel (0.5 x Tris - Boric Acid - EDTA) at room temperature. Gels were dried and exposed overnight with an intensifying screen.


Anti KROX-20
Control Ab
100x
100x NS
SS
KROX-20

CHAPTER 4

CHAPTER 4

MYELIN BASIC PROTEIN EXPRESSION IN SCHWANN CELLS IS CONTROLLED THROUGH THE INTEGRATED OUTPUT OF MULTIPLE REGULATORY SUB-DOMAINS

R. Forghani, H. F. Farhadi, W. Orfali, G.J. Snipes, and A. Peterson

(*In preparation*)

PREFACE

In previous chapters, the discovery and characterization of Schwann cell enhancers (SCE1 and SCE2) of the Myelin Basic Protein (MBP) gene was described and a protocol for the extraction of nuclear factors from myelinating Schwann cells in peripheral nerves was established. The aim of this study was to use a multi-disciplinary approach combining sequence analysis, in *vitro* DNA-protein interaction assays, and functional promoter assays in *vivo* in order to locate and characterize the regulatory components underlying SCE1-mediated expression in Schwann cells, providing insight into the transcriptional regulation of MBP in these cells.

ABSTRACT

In the rodent peripheral nervous system, innervating axons instruct Schwann cells to myelinate in part through the coordinate up-regulation of genes encoding myelin structural proteins. In the mouse, expression of the Myelin Basic Protein (MBP) gene appears to be controlled by at least 2 far upstream Schwann cell enhancers, SCE1 and SCE2, that correspond to a highly conserved sequence in the human locus. Previously, we demonstrated that the 0.6 kb SCE1 contains all of the regulatory sequences necessary to confer high-level expression of reporter constructs to myelinating Schwann cells in transgenic mice. To gain further insight into the regulatory elements underlying SCE1-mediated expression, we used a combination of sequence analysis, in *vitro* DNA-protein interaction assays, and in *vivo* functional assays. We have identified 2 targeting sub-domains and a third functional domain that enhances transgene expression level, providing the basis for a model in which the expression of MBP in Schwann cells is regulated by multiple regulatory sub-domains. In addition, using electrophoretic mobility shift assays with peripheral nerve extracts, we identified multiple regulatory elements that may function in myelinating Schwann cells to regulate MBP expression. Lastly, to perform quantitative studies of MBP regulation, we have adopted a transgenic method in which a single copy of the reporter construct is inserted into a common and predetermined site in the HPRT locus. Initial investigations using this experimental strategy support a complex model of regulation and provide the basis for future high-resolution studies of the mechanism regulating MBP expression.

INTRODUCTION

Myelination is an essential step in the maturation of fast-conducting vertebrate axons, allowing nerve fibers to convey the electrical signals generated in the cell body rapidly and in an energy efficient manner (Bray et al., 1981). In the rodent peripheral nervous system (PNS), myelinogenesis begins in the peri-natal period and is tightly regulated by the innervating axon (Aguayo et al., 1976a; Aguayo et al., 1976b; Aguayo et al., 1977a; Gupta et al., 1988; Lemke and Chao, 1988; Trapp et al., 1988; LeBlanc and Poduslo, 1990; Snipes et al., 1992). Coincident with the initiation of myelin formation, Schwann cells coordinately up-regulate myelin specific genes such as myelin basic protein (MBP), myelin protein zero (P0), and peripheral myelin protein 22 (PMP-22), which reach maximal levels of expression by approximately 3 weeks postnatally. Thereafter, lower steady state levels are maintained throughout maturity.

Multiple lines of evidence demonstrate that myelin induction, its maintenance, and the associated pattern of gene expression requires that Schwann cells are in contact with a large caliber axon. In experimental models of axonal injury, Schwann cells in the distal stump rapidly down-regulate myelin gene expression and pre-existing myelin sheaths are shed. In such "denervated" Schwann cells, up-regulation of myelin genes and elaboration of new myelin sheaths occurs only when Schwann cells re-establish contact with regenerating axons (Gupta et al., 1988; Trapp et al., 1988; LeBlanc and Poduslo, 1990; Snipes et al., 1992). Consistent with these observations, when Schwann cells are isolated in vitro, myelin gene expression generally down-regulates to barely detectable levels

under most circumstances (Lemke and Chao, 1988). Combined, both in *vivo* and in *vitro* investigations suggest that axons signal Schwann cells to myelinate, tightly regulating myelin gene expression.

Despite the significant advances made in the understanding of the molecular regulation of myelination over the past decade, our knowledge of the transcriptional regulation of myelin genes remains rudimentary. One approach has been to study the promoters of myelin genes in *vitro* in order to identify *cis*-acting elements that in turn could be used to identify transcription factors regulating myelination (Monuki et al., 1989; Monuki et al., 1990; He et al., 1991; Monuki et al., 1993; Li et al., 1994; Shy et al., 1996; Brown and Lemke, 1997; Desarnaud et al., 1998; Peirano et al., 2000). However, since no in vitro model completely recapitulates the phenotype of myelinating Schwann cells in vivo, and given the apparent importance of axon-Schwann cell interactions in regulating myelin gene expression, the relevance of such in vitro findings is somewhat controversial. Consequently, the promoters of myelin genes have also been analyzed in vivo in transgenic preparations. Despite caveats such as position variegation and different copy numbers, this is a traditionally powerful approach for identifying and characterizing the expression programming conferred by DNA regulatory sequences. Using this approach, 5' flanking sequence from the myelin genes P0 (Messing et al., 1992), MBP (Forghani et al., in press), and CNPase (Gravel et al., 1998) that can target reporter gene expression to Schwann cells has been identified.

A further approach has involved the disruption or mutation of specific transcription factors. In mice homozygous for null Krox-20 alleles, Schwann cells associate with axons but do not myelinate them (Topilko et al., 1994), demonstrating the essential role of this factor in the advancement of Schwann cells into myelinating cells. However, it remains to be shown whether Krox-20 is required for the maturation of Schwann cells to a myelinating competent state or whether it directs the myelination program per se. Because expression of Krox-20 significantly precedes myelin gene up-regulation, any role in the direct regulation of myelin genes would require interaction with co-regulators or through downstream effectors.

Another transcription factor of importance for peripheral nerve maturation is SCIP. There is some evidence suggesting than SCIP represses myelination, however myelination is delayed in SCIP null mutants and the precise role of SCIP is still not clear (Monuki et al., 1989; Monuki et al., 1990; He et al., 1991; Monuki et al., 1993; Weinstein et al., 1995; Bermingham et al., 1996; Jaegle et al., 1996). Other molecules implicated in myelination include progestins, which have been shown to potentiate myelination (Koenig et al., 1995), and the intracellular second messenger cAMP (Lemke and Chao, 1988; Morgan et al., 1991; Poduslo et al., 1995). The role of the latter is based on the observation that agents that increase intracellular levels of cAMP, such as forskolin, partly restore myelin gene expression in isolated Schwann cells in culture. Interestingly, it also has been shown that under some experimental conditions (notably in the absence of serum and glial growth factor) isolated Schwann cells can express myelin genes (Cheng

and Mudge, 1996). The relevance of these observations to processes occurring in vivo remains to be established.

Previously, we demonstrated that two far upstream enhancers in the MBP locus confer reporter construct expression to myelinating Schwann cells in transgenic animals and these were designated SCE1, 0.6 kb, and SCE2, 3 kb (Schwann Cell Enhancer 1 and 2; Forghani et al., in press). The 0.6 kb SCE1 was shown to contain elements sufficient for conferring Schwann cell expression and here, we have searched for the functional sequences that confer SCE1 activity. We used a combination of sub-sequence and comparative sequence analysis, in *vitro* DNA-protein interaction assays, and in *vivo* functional promoter analysis. We demonstrate that SCE1 is composed of multiple interacting sub-domains and corroborate the evidence for this complex and sophisticated regulatory system by direct comparison between reporter constructs inserted into a common HPRT docking site.

MATERIALS AND METHODS

Sequence analysis

Subsequence analysis was performed for Krox-20 (Chavrier et al., 1990; Sham et al., 1993; Nonchev et al., 1996; Vesque et al., 1996), SCIP (He et al., 1991), and over 2000 additional sites (TFDSITES.SUBSEQ.7.0.aa database, MacVector®, no mismatch allowed) using MacVector® software. Sequence analysis for known transcription factor

sites was also performed with the online TFSEARCH program (Yutaka Akiyama: "TFSEARCH: Searching Transcription Factor Binding Sites", http://www.rwcp.or.jp/papia/) supported by TRANSFAC databases (Heinemeyer et al., 1998). SCE1 (0.6 kb; GenBank accession number AF277397; Forghani et al., in press) and P0 (979 bp; (Lemke et al., 1988)) sequences were compared using PIP analysis (PipMaker; http://bio.cse.psu.edu/pipmaker).

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSAs) were performed with a plasmid purified fragment (Probe 1; Pr1) or overlapping oligonucleotides spanning the -8.791 to -8.682 kb sequence (Probes 2 to 7), part of the highly conserved region within SCE1. To generate Pr1 (114 bp bearing 104 bp of SCE1 sequence), the -8.788 to -8685 kb (104 bp) SCE1 sub-fragment was released from a plasmid containing the 192 bp (-8.788 kb to -8.597 kb) sequence in pKS by restriction digestion. Following dephosphorylation (Calf Intestinal Phosphatase), Pr1 was isolated after electrophoresis in a 5% non-denaturing polyacrylamide gel. To generate radioactive probe, gel purifies Pr1 was end-labeled with γ^{32} ATP with T4 polynucleotide kinase. Probes 2 – 7 were generated using synthetic oligonucleotides encoding wild type sequence with added BamHI and XbaI flanks (custom made by Sheldon Biotechnology Centre, McGill University). Complementary strands were annealed, leaving BamHI and XbaI flanks, and radioactive probes were generated by end filling with αP^{32} dCTP using Klenow. To generate Probe 2, complementary oligonucleotides bearing the -8.787 to -8.735 kb (53 bp; Pr2) sequence

with BamHI (5') and XbaI (3') flanks were annealed (gatccGGTGACCCCAAGCCCAGGCTGCCAGCGGCAGAAGTATTCCA TGAACAAGGAAGt and ctagaCTTCCTTGTTCATGGAATACTTCTGCCGCTGGC AGCCTGGGCTTGGGGTCACCg). Probes 3 to 7 were generated in a similar manner (with BamHI and XbaI flanks) using synthetic oligonucleotides encoding the –8.791 kb to -8.762 kb (30 bp; Pr3), -8.771 kb to -8.742 kb (30 bp; Pr4), -8.752 kb to -8.723 kb (30 bp; Pr5), -8.732 kb to -8.703 (30 bp; Pr6), and –8.712 kb to -8.682 kb (31 bp; Pr7) overlapping sequences. To identify nucleotides essential for DNA-protein interactions, oligonucleotides were generated and annealed to form mutant variants of Pr3 [δ1-5(Pr3)]. The core sequences for Pr3 and Pr3 mutants are shown in Fig.2 (only one strand is shown and similar to Pr3, these probes bear BamHI and XbaI flanks, which are <u>not</u> shown in the figure; mutations are underlined). Oligonucleotides encoding transcription factor recognition elements were also custom made and annealed as above bearing recognition sequences for Krox-20 (Chavrier et al., 1990; Nardelli et al., 1991; Forghani et al., 1999), AP-1 (Lee et al., 1987; Xanthopoulos et al., 1991), NGFI-B/Nur77/NAK1/TR3/N10 (NBRE (NGFI-B response element) and δNBRE (NBRE mutant); Wilson et al., 1991), estrogen receptor (ERE (estrogen receptor response element); Wilson et al., 1991), H-2RII (Wilson et al., 1991), COUP (Wilson et al., 1991), CF1 (Wilson et al., 1991), and ERRE (Sladek et al., 1997). The sequence for one strand of each oligonucleotide is shown in Fig.3, with the core recognition element underlined. Radionucleotides and all enzymes except Klenow were from Amersham Pharmacia, Baie d'Urfé, Quebec. Klenow was from Boehringer Mannheim, Laval, Quebec.

Electrophoretic mobility shift assay (EMSA) reactions (40 µl) were carried out at room temperature with probe alone or by incubating with 20 µg of either peripheral nerve extract (Forghani et al., 1999) or extract from a Schwann cell line for control (Haynes et al., 1994; courtesy of P. Richardson) with 2 µg poly(d*I*-d*C*)•poly(d*I*-d*C*) (Amersham Pharmacia, Baie d'Urfé, Quebec) in 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.25 µg/µl BSA, 5% glycerol for 5 min. Then, 1 ng of probe (± 100 fold cold competitor) was added and incubation continued for 15 min at room temperature. Competitions assays were done by addition of 100x molar excess of cold oligonucleotide to the reaction mixture. Reactions were run in a 5% polyacrylamide gel (0.5 x Tris-Boric Acid-EDTA) at 4 °C. Gels were dried and exposed overnight with an intensifying screen.

Generation of reporter constructs for transgenesis

Nine constructs were generated in order to locate MBP regulatory sequences within SCE1 by zygote pronuclear injection (Table 4.1). In addition, 4 additional constructs were incorporated into a HPRT targeting vector (Bronson et al., 1996; more detail below; courtesy of S. Bronson) for ES cells (Table 4.2). All constructs used for pronuclear zygote injection were derived using the same basic strategy. First, plasmids containing SCE1 sub-domains or SCE1 sequences bearing specific mutations were derived using routine cloning techniques (SCE1 (0.6 kb) was isolated from clone 5 or clone 8 by SacII/SacI digestion (Forghani et al., in press)), and cloned in various configurations in Bluescript (pKS- or pSK+). Some were simply derived by restriction

enzyme digestion and cloning of SCE1 sub-fragments, and the restriction sites flanking these sub-fragments are included in Table 4.1. Others were derived using oligonucleotides and/or PCR. Once plasmids bearing SCE1 sub-domains or specifically mutated SCE1 were generated, the 6 kb proximal MBP promoter-lacZ was isolated from clone 5 (Forghani et al., in press) by BamHI/KpnI digestion and cloned into plasmids bearing mutated SCE1 or sub-domains of SCE1 by a common strategy in order to generate final constructs in which SCE1 sub-domains or mutated SCE1 are ligated to the 3' end of lacZ in the 5' → 3' orientation (Table 4.1; Figs.4.4 and 4.5). The SCE1 portion of all clones was sequenced to confirm that the appropriate deletions or mutations were present. All constructs were then released by restriction enzyme digestion and isolated by agarose gel electrophoresis for pronuclear injection. On the other hand, HPRT targeting vectors were used to introduce constructs into ES cells by homologous recombination (below).

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) or by insertion into a common HPRT docking site in ES cells (Bronson et al., 1996). The former were derived by injection of reporter construct into the zygote pronucleus, followed by transplantation of zygotes into the oviducts of B6C3F1 females rendered pseudopregnant by mating with vasectomized males. Litters were delivered either spontaneously or by cesarean section 18 days later. In most instances, postnatal day 3-15 primary transgenic animals were

less than one hour to overnight in stain consisting of 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ and 0.4 mg/ml Bluo-gal (Gibco/BRL, Burlington, Ontario). In some preparations, detergents sodium deoxycholate and NP-40 were added to the stain at 0.01% and 0.03% respectively to permeabilize the tissue thus assisting the penetration of ß-galactosidase substrate. Following the whole mount histochemical reaction, some samples were processed for plastic embedding. Typically, tissue was osmicated prior to dehydration and embedding in Epon. Following polymerization, the blocks were sectioned at 1µm, and the sections were mounted on slides and viewed either directly or after staining with toluidine blue. Additional tissue was cryoprotected by immersion in 30% sucrose prior to freezing and 12 µm cryostat sections were made and subsequently incubated in stain containing 0.8 mg/ml X-gal (Gibco/BRL, Burlington, Ontario).

Immunocytochemistry was performed as previously described with some modifications (Eyer and Peterson, 1994). After anesthesia with avertin, mice were perfused transcardially with 2% parafomaldehyde-lysine-periodate at 4 °C. Tissues were placed in the same fixative for an additional 2-5 hr and transferred to 30% sucrose in 0.1 M phosphate buffer at 4 °C, where they remained for 1-3 days. Tissues were then briefly rinsed in 0.1 M phosphate and embedded in OCT (VWR Canlab, Montreal, Quebec). Tissue blocks were frozen in isopentane at –40 °C and stored under isopentane at –80 °C under sectioning. Cryostat sections (12 µm thick) were placed on coated slides and postfixed in ice-cold formol-sucrose for 30 min. The sections were then blocked with 10% normal goat serum plus 0.3% Triton X-100 for 1 hr at room temperature and incubated in 100 µl of primary antibody overnight at 4 °C. Biotinylated secondary

antibodies (goat anti-rabbit IgG, 1:200; DAKO Diagnostics Canada Inc., Mississauga, Ontario) allowed the visualization of bound primary antibody using the ABC kit (DAKO Diagnostics Canada Inc., Mississauga, Ontario), according to the supplier's instructions. Antibodies and dilutions employed were as follows: polyclonal mouse anti-β-galactosidase (1:1000, 1:1500, 1:2000, 1:2500, 1:3000; 5' → 3' Inc.®, Mandel Scientific Company Ltd., Guelph, Ontario). To visualize the reaction, the DAB (Zymed® Laboratories Inc., Inter Medico, Markham, Ontario) kit containing the diaminobenzidine substrate was used as per supplier's instructions.

RESULTS

To identify the regulatory elements controlling Schwann cell expression, we searched for known regulatory sequences (search strategy is described in Materials & Methods), with emphasis on those recognized by transcription factors associated with myelination, such as Krox-20/Egr-2 (Krox-20), SCIP/Oct-6/Tst-1 (SCIP), CRE (cAMP response element), and progesterone (Lemke and Chao, 1988; Monuki et al., 1989; Monuki et al., 1990; He et al., 1991; Morgan et al., 1991; Monuki et al., 1993; Topilko et al., 1994; Koenig et al., 1995; Weinstein et al., 1995; Bermingham et al., 1996; Jaegle et al., 1996; Jung-Testas et al., 1996). As reported previously, there is a Krox-20 site [(-) GCGTGGGTG] near the 3' end of SCE1 which is neither essential nor sufficient for conferring Schwann cell expression (Forghani et al., in press). Among the additional 150 or more potential regulatory sequences revealed by this strategy, none were obvious candidates for myelin gene regulation. We extended our sequence analysis efforts to a

comparison between the SCE1 sequence and the 1 kb P0 promoter (Lemke et al., 1988) which contains sufficient regulatory sequences for targeting expression to myelinating Schwann cells (Messing et al., 1992). Comparative sequence alignment using PIP analysis did not identify significant homology.

In an alternate strategy to highlight candidate regulatory elements within SCE1, we performed electrophoretic mobility shift assays (EMSAs) using peripheral nerve extracts demonstrated previously to contain Schwann cell transcription factors (Forghani et al., 1999). We focused this assay on part of the most highly conserved sequence within SCE1. Using small sequences (30 – 102 bp) spanning the –8.791 to –8.682 kb sequence as probes (Fig. 4.1), we identified prominent bands in assays employing probes that encoded the 30 bp sequence contained within the -8.791 kb to –8.762 kb region. Notably, we observed two prominent bands using Probe 3 (Pr3; -8.791 kb to –8.762 kb), designated complex I and II, that can be competed with a 100x excess of unlabelled probe (Fig. 4.2).

To identify the DNA bases mediating complex I and II binding activities, competition assays were performed using 100x excess of unlabelled wild type Pr3 and oligonucleotides bearing stretches of mutations 3 to 7 bp long (Fig. 4.2). Based on the inability of some mutant oligonucleotides to compete complex I and II, a 15 bp contiguous sequence (5'CCAGGTGACCCCAAG3') mediates both complex I and II formation. Furthermore, an oligonucleotide bearing mutations in the first 5 of those bases (δ5-Pr3; CCAGG → AATTC) relatively spares complex I in competition assays,

suggesting that these bases are only essential for complex II binding activity. Neither complex was seen when extracts from a Schwann cell line (Haynes et al., 1994) were used in control experiments. The 1 kb P0 promoter sequence (Lemke et al., 1988) was also analyzed to determine whether it contains the 15 bp sequence or its shorter version lacking the five 5' bases (TGACCCCAAG) but there was no perfect match to either one.

Within the 15 bp sequence mediating binding activity lies an ER half site, 5'GGTCA3' (*minus* strand; corresponding sequence on the *plus* strand is underlined: 5'CCAGGTGACCCCAAG3'). To assess whether a member of the nuclear receptor family of transcription factors (Mangelsdorf and Evans, 1995; Mangelsdorf et al., 1995; Giguere, 1999) may mediate complex I or II formation, excess of unlabelled oligonucleotides encoding recognition sites for known members of this family of transcription factors were used in similar competition assays (Fig. 4.3). Notably, the transcription factor NGFI-B (nerve growth factor-inducible B; Milbrandt, 1988; Watson and Milbrandt, 1989; Fahrner et al., 1990) that is present in sciatic nerves during early postnatal development in the developing rat (Watson and Milbrandt, 1990) was of interest. Therefore, some of the recognition sequence encoding oligonucleotides employed in competition assays were similar to those used previously by Wilson et al. in determining the binding properties of NGFI-B (Wilson et al., 1991), such as NBRE and δNBRE (NGFI-B response element and a NBRE mutant), ERE (estrogen receptor response element), H-2RII site (H-2 region II binding protein), COUP site, and CF-1 site (Wilson et al., 1991). As shown in figure 4.3, all of these oligonucleotides effectively compete complex II with relative sparing of complex I, suggesting that complex II is

mediated by a transcription factor member of the nuclear receptor superfamily. However, this profile does not perfectly match that established for in vitro produced NGFI-B protein, especially given the effectiveness of δNBRE in competing complex II. Interestingly, the ERRE encoding oligonucleotide (Sladek et al., 1997; Giguere, 1999) did not compete either complex effectively. Excess of an oligonucleotide encoding the Krox-20 recognition site (control) did not compete complex I or II.

To further investigate complex II, a mutant Pr3 oligonucleotide bearing a 3 bp mutation at the core of the ER half site [δ4-Pr3: TGACC → ATTCC (minus strand: GGTCA → GGAAT)] was used in competition assays (Fig. 4.2). As expected, excess unlabelled δ4-Pr3 effectively competes complex II formation. Interestingly, this mutant oligonucleotide also competes complex I, suggesting that the factors giving rise to complex I and II interact closely through adjacent DNA recognition elements. In further competition assays, 100x excess of an oligonucleotide bearing the recognition sequence for the transcription factor AP-1 effectively competed complex I but not II, suggesting that the former band may represent AP-1 site mediated binding activity.

As complexes I and II represented compelling candidate regulatory elements within a highly conserved portion of SCE1, we used site-directed mutagenesis to determine whether they are essential for the expression conferred by SCE1. We introduced in the 0.6 kb SCE1 the 3 bp mutation [GGTCA → GGAAT (minus strand)] that abolishes both binding activities and derived a construct bearing this mutant SCE1 at the 3' end of 6 kb MBP-lacZ (Fig. 4.4). As the 6 kb MBP promoter confers expression to

oligodendrocytes and not Schwann cells in transgenic mice (Forghani et al., in press), it was used to drive lacZ and provide a positive internal control. In 3/3 independently derived δER#1-SCE1 primary transgenic pups, there was high-level transgene expression in both oligodendrocytes and Schwann cells (Fig. 4.4; Table 4.1). Surprisingly, this investigation demonstrates that these compelling regulatory candidates are not essential for SCE1 activity.

As an alternative approach in elucidating SCE1-directed expression, SCE1 reporter lines were crossed to mice bearing mutations in candidate transcription factors. Since the minor PNS abnormalities that result from the absence of MBP can be overlooked in routine morphologic studies (Kirschner and Ganser, 1980; Rosenbluth, 1980; Peterson and Bray, 1984), SCE1-regulated transgene expression was used to monitor any contribution to MBP expression. Members of the nuclear receptor family of transcription factors were candidates for multiple reasons. There are multiple ER half sites within SCE1, and evidence of DNA-protein interactions was obtained for these sites. Moreover, they have a well-established role in lipogenesis in other cell types (Giguere, 1999).

Among available null mutants, mice bearing a mutation in the transcription factor RORα (Hamilton et al., 1996; Matysiak-Scholze and Nehls, 1997; Dussault et al., 1998) seemed particularly appealing given that one of the three ER half sites within SCE1 (AAGTAGGTCAAGTG; designated ER#2 in Fig. 4.1) satisfies the additional base criteria for specific recognition by this member of the nuclear receptor superfamily

Using this approach, a systematic functional analysis of SCE1 sub-domains was performed (key constructs are illustrated in Fig. 4.5; Table 4.1 provides a list of all constructs that were used for pronuclear injection in this investigation).

This investigation demonstrated that a contiguous 192 bp sequence (-8.802 kb to -8.611 kb), flanked by BstE II (5') and Bgl II (3'), can confer expression to Schwann cells, albeit only in a small percentage of mice (1/2; Fig. 4.5; Table 4.1). Analysis of 13 additional independently derived transgenic mice [5 constructs; (Δ2, 5, 6, 7, 8) - SCE1] bearing shorter sub-sequences from this 192 bp sequence did not reveal any Schwann cell expression. In addition, a downstream 232 bp sub-domain (Δ3-SCE1; -8.600 kb to –8.369 kb) also confers Schwann cell expression in a fraction of transgenic animals (1/2) if isolated, but apparently not if the approximately 0.1 kb sequence immediately upstream is also present (Δ2-SCE1; -8.684 to –8.369 kb; 0/3 express; although this may reflect the inefficiency of the 232 bp sequence and not the effect of the upstream 0.1 kb). Neither of the expressing mice demonstrated the apparently high-level expression characteristic of SCE1 lines (compare Fig. 4.5 with Figs. 2.2 and 2.3, chapter 2) and unlike the larger SCE1 configurations tested, only a small proportion of the mice bearing these constructs expressed.

In a previous study, among mice bearing transgenes expressed in oligodendrocytes all (9/9) constructs that included the full SCE1 also expressed in Schwann cells (Forghani et al., in press). Consequently, the decreased faithfulness of expression observed with SCE1 sub-domains was striking. Although this might be

attributed to non-specific effects on shorter enhancer sequences, it was also a possibility that sequences within SCE1 facilitate expression and in their absence, the effectiveness of the enhancer to confer expression is significantly decreased. As shown in Table 4.1 and Fig. 4.5, the decreased expression efficiency was not limited to the 192 and 232 bp sub-domains, but also was encountered with the 510 bp sub-domain (Δ1-SCE1; -8.878 kb to – 8.369 kb; Table 4.1). Since all of the constructs discussed lack the 73 bp sequence normally present at the 3' end of SCE1, these results suggested that elements within this sequence facilitate Schwann cell expression by modulating the activity of the upstream targeting sequences.

Our observations suggesting that SCE1-mediated expression occurs through the activity of multiple targeting and activity modulating sub-domains had significant implications for future experiments. The apparent presence of multiple targeting and modulating sub-domains made further investigation using a strategy based on a qualitative analysis for expression versus no expression impractical or outright uninformative. Likewise, in light of these results, certain of our previous observations cannot be interpreted with certainty. For example, it is possible that mutated putative elements function in concert with others to contribute to SCE1 function, but their individual contributions are not essential. Overall, given this apparent level of complexity, further investigations using traditional transgenic mice were not likely to provide significant additional insight and a new approach was needed.

A strategy of transgenesis devised by Bronson et al. (Bronson et al., 1996) in which constructs are inserted into the HPRT locus, keeping the site of integration and transgene copy number constant among independent lines, appeared capable of providing the necessary high-resolution quantitative comparisons. To evaluate this method, we used constructs bearing the 0.6 kb SCE1 or the 510 bp sub-domain in the context of MBP proximal 5' flanking sequences (Table 4.2). Constructs were inserted into the HPRT docking site in ES cells by homologous recombination, chimeras were derived from ES cells and their nervous system assessed for β-galactosidase expression. Both constructs conferred expression to Schwann cells and oligodendrocytes demonstrating that the HPRT locus can provide a premium tool for future high-resolution studies of the MBP promoter (Fig. 4.6).

Previous observations on the function of SCE1 sub-domains were confirmed using this preparation. First, both constructs targeted expression to Schwann cells, consistent with our previous conclusion that the 3' 73 bp at the end of SCE1 (containing the Krox-20 site) is not essential for expression. Also predicted from the decreased efficiency of SCE1 sub-domains lacking the 73 bp 3' sequence, a lower level of β-galactosidase accumulation was observed in Schwann cells in mice bearing the 510 bp sub-domain of SCE1 (Fig. 4.6).

Within the 3' 73 bp sequence, the most intriguing putative regulatory element is Krox-20. Previous results derived from a transgenic line derived by pronuclear injection demonstrated that the Krox-20 site within SCE1 is not essential for conferring Schwann

cell expression (Forghani et al., in press). The targeting capability of SCE1 sub-domains described here also support this conclusion. However, the likelihood that the 73 bp sequence was involved in a quantitative modulation required a re-evaluation of the role of the Krox-20 site. We generated chimeras bearing a reporter construct (in the HPRT docking site) in which the Krox-20 site within the 0.6 kb SCE1 was mutated [(-) GCGTGGGTG → GCGGTTTCG; Chavrier et al., 1990; Forghani et al., 1999; Forghani et al., in press). The level of Schwann cell expression in this chimera chimera (estimated to be composed of 80 % ES derived cells by coat color chimerization) was compared with transgenic lines bearing reporter constructs that contained the 510 bp and 0.6 kb versions of SCE1 also in the HPRT locus. β-galactosidase histochemistry as well as immunohistochemistry for the β-galactosidase enzyme was used. As shown in Table 4.3, those cells containing constructs regulated by the 0.6 kb SCE1 bearing an intact Krox-20 site were more intensely labeled. While very promising, these observations are considered to be preliminary due to both the non-linear signals generated by the technique applied and by the visual assessment of labeling intensity. Investigations designed to overcome these technical limitations are ongoing (see discussion) and will be included in this manuscript prior to submission for publication.

DISCUSSION

Myelination of peripheral fibers requires close association and signaling between Schwann cells and axons (Aguayo et al., 1976a; Aguayo et al., 1976b; Aguayo et al., 1977b; Gupta et al., 1988; Lemke and Chao, 1988; Trapp et al., 1988; LeBlanc and

Poduslo, 1990; Snipes et al., 1992; Bolin and Shooter, 1993). During myelination, genes encoding the structural proteins of myelin are up-regulated through poorly defined signaling pathways and unknown transcription factors. In this report, we have used a multi-disciplinary approach to characterize the regulatory sequences of a Schwann cell enhancer of the MBP gene (SCE1). Our observations suggest that the regulation of MBP expression in Schwann cells is achieved through the complex output of multiple regulatory sequences analogous to better characterized loci in lower and higher eukaryotes (Yun and Wold, 1996; Yuh et al., 1998). The complex and quantitative model that our data suggest for the regulation of this locus would be refractory to further analysis using traditional transgenic techniques but we demonstrate that a chosen site, single copy strategy of transgenesis (Bronson et al., 1996) is applicable to MBP regulated reporter constructs thus providing a basis for future high-resolution studies.

Transcriptional regulation of eukaryotic genes is complex involving multiple DNA cis elements and transcription factors that bind the promoter region, co-regulators that interact with transcription factors but do not bind DNA directly, as well as regulation through alterations in chromatin structure (Yun and Wold, 1996; Glass et al., 1997; Kadonaga, 1998; Wray, 1998; Freedman, 1999; Engel and Tanimoto, 2000). Such complexity is reflected in the functional organization of SCE1 described here and is consistent with our previous observations that multiple enhancers and repressors regulate MBP expression in Schwann cells (Forghani et al., in press). In that report, the two adjacent sequences capable of targeting reporter gene expression to Schwann cells were arbitrarily assigned the designations SCE1 and SCE2. However, SCE2 appears to have

similar function to the two targeting sub-domains identified within SCE1 suggesting that it may be an additional component of a single sophisticated regulatory module that integrates the activity of multiple elements. This notion is supported by the fact that a highly conserved mouse-human sequence spanning much of SCE1 extends upstream to include part of SCE2 (Lepage et al., in preparation).

The striking conservation observed between the human sequence and mouse SCE1 suggests an important role for this sequence (Lepage et al., submitted). However, from the functional results presented here, it is evident that not all SCE1 sub-domains regulating Schwann cell expression in the mouse are completely conserved. For example, while most of the192 bp targeting sub-domain lies within a highly conserved region, the 232 bp and 73 bp sub-domains are located outside the region of highest conservation. This may reflect evolutionary changes in the individual components of the regulatory network controlling MBP expression in mouse and human Schwann cells. Alternatively, the regulatory element(s) within these sub-domains may be present within the human MBP promoter but not readily identifiable at the resolution provided by the comparative sequencing algorithm and parameters used. One possibility given the well-established degeneracy in the recognition of regulatory elements by many transcription factors is that individual elements may have altered sequence but still interact with the same transcription factors. These issues will be clarified once the individual regulatory element(s) within various sub-domains have been identified.

In transgenic mice, 1.1 kb of P0 5' flanking sequence confers a program of expression to reporter constructs with similarities to that observed for MBP SCE1 (Messing et al., 1992). Consequently, it is plausible that the seemingly coordinate expression programs displayed by these reporter constructs involves enhancers or repressors in each promoter that bind identical transcription factors that are themselves regulated through shared signaling pathways, similar to the regulatory model described in myogenesis (Yun and Wold, 1996). However, PIP analysis did not reveal significant sequence homology between SCE1 and this region of the P0 promoter. The interpretation to be placed on this negative result is not simple. Most sequence comparative algorithms set a lower limit for significance at greater than 50% identities over 70 bp. Consequently, multiple elements would have to be aligned and spaced identically in different myelin genes. While this could occur through direct descent it seems inconceivably remote that multiple genes would have independently evolved the same configuration of regulatory elements. Furthermore, P0 regulatory elements that contribute to Schwann cell expression also appear to reside outside the 1.1 kb promoter sequence (Feltri et al., 1999). Whether the same elements are shared with MBP and/or other myelin genes awaits identification of the elements regulating such genes.

Our results define a complex of elements that function to regulate MBP and underline the experimental challenges to be faced in elucidating myelin gene regulation. Despite numerous well-designed investigations (Monuki et al., 1989; Monuki et al., 1990; He et al., 1991; Monuki et al., 1993; Li et al., 1994; Shy et al., 1996; Brown and Lemke, 1997; Peirano et al., 2000), the molecular pathways regulating myelin gene expression

remain poorly defined. In part, this difficulty appears to arise from the existence of multiple interacting regulatory components with at times overlapping functions. Given the emergence of this complexity for SCE1, the previous site-directed mutagenesis experiments, which can be conclusive in simple "on-off" switch models, cannot rule out a role for the elements tested in this study. Furthermore, our original experimental paradigm using randomly integrated transgenes was of limited use in assigning a quantitative phenotype. Although these experiments provided evidence consistent with a complex model, they were not conclusive regarding many key features.

During the course of these investigations a controlled method for inserting constructs into the genome was introduced leading to the insertion of constructs into the 5' flank of the HPRT gene. By eliminating both the variable construct copy number at each transgene locus and the effects of different insertion sites that traditionally have made quantitative comparisons in transgenic animals impossible, Bronson et al. (Bronson et al., 1996) have demonstrated that quantitative comparisons of transgene expression level among independently derived transgenic lines are possible. Here, we demonstrate that MBP regulated constructs behave in a predictable manner when inserted into the HPRT docking site, expressing in oligodendorcytes and Schwann cells. Furthermore, this approach has enabled us to detect a quantitative contribution of the 73 bp sub-domain.

The observation that a regulatory sequence (the 73 bp 3' sequence within SCE1) modulates the expression conferred by targeting sub-domains quantitatively and improves the efficiency of transgene expression is consistent with the complex transcriptional

regulation described at other loci involving multiple elements that interact to give rise to an integrated transcriptional output (Yun and Wold, 1996; Yuh et al., 1998) and is somewhat analogous to the recent demonstration that P0 regulatory sequences outside the 1.1 kb targeting sequence significantly enhance Schwann cell expression (Feltri et al., 1999). Our initial design using oligodendrocyte expression as an internal control was based on the fact that even reporter constructs driven by known functional enhancers fail to express in a significant percentage of random integration events (Palmiter and Brinster, 1986). Given that this could confound the interpretation of a negative result (i.e. deleted or mutated forms of SCE1 that do not express), we assumed that a transcriptionally "unfavorable" chromatin domain would similarly effect transgene expression in both oligodendorcytes and Schwann cells. Thus, expression in oligodendrocytes would indicate that a given SCE1 sub-domain should express in Schwann cells if it bears sufficient Schwann cell specific regulatory elements. While this may be an oversimplified view, of the lines bearing proximal MBP sequences and SCE1, all that expressed in oligodendrocytes also expressed in Schwann cells. Based on this observation, the reduced efficiency of reporter gene expression in the absence of the 73 bp sub-domain following random integration was striking. Furthermore, as this sequence confers a quantitative effect on the expression of reporter constructs inserted into the HPRT locus, it seems highly probable that it is required for high-level Schwann cell expression. Thus, it seems probable that this sequence would also facilitate Schwann cell expression from the endogenous MBP locus.

Within Schwann cells, candidate molecules regulating myelination include the transcriptions factors Krox-20 (Topilko et al., 1994) and SCIP (Monuki et al., 1989; Monuki et al., 1990; He et al., 1991; Monuki et al., 1993; Scherer et al., 1994; Weinstein et al., 1995; Bermingham et al., 1996; Jaegle et al., 1996), progesterone (Koenig et al., 1995; Desarnaud et al., 1998), and the second messenger cAMP (Lemke and Chao, 1988; Morgan et al., 1991; Poduslo et al., 1995). While SCE1 sequence analysis did not reveal sites for SCIP, the progesterone receptor, or CRE (cAMP response element), a Krox-20 element as well as three ER half sites were identified. Previously, we demonstrated the Krox-20 site is neither sufficient nor essential for SCE1-mediated expression (Forghani et al., in press) and our description here of two targeting sub-domains outside the Krox-20 bearing region are consistent with that conclusion. However, preliminary observations suggest that the Krox-20 site enhances SCE1 activity and therefore functions in conjunction with additional elements within SCE1. Although a quantitative role for Krox-20 has not been described previously, Krox-20 is known to function in conjunction with an additional element designated Box1 in conferring expression via the Hoxb-2 enhancer (Vesque et al., 1996). There is no Box1 site within SCE1.

To rigorously confirm our current conclusion based upon β-galactosidase histochemistry and immunocytochemistry, we are deriving transgenic lines to support biochemical analysis of the β-galactosidase expression phenotype. In addition, the proximal promoter of the line bearing the wild type 0.6 kb SCE1 is different (it bears the –6 to –3 kb MBP sequence plus the 0.3 kb proximal MBP promoter) from the line bearing the 510 bp sub-domain and the chimera bearing the mutated 0.6 kb SCE1 (both have the

same 3.1 kb MBP proximal promoter), and it is conceivable that this difference in the proximal promoter could exert an unanticipated influence on expression levels. A targeting construct bearing the wild type 0.6 kb SCE1 with the same 3.1 kb MBP promoter has been generated and no difference in the expression conferred has been observed between Schwann cells in the single chimera analyzed so far and the SCE1 bearing line driven by the –6 to –3 kb plus 0.3 kb MBP promoter.

The results presented here define a framework for understanding the regulation of MBP expression and identify one effective experimental strategy for future in vivo studies of myelin gene regulation. The Schwann cell enhancer described here along with its various sub-domains should provide experimental tools yielding unique opportunities to introduce controlled expression of transgenes into culture, transplant and regeneration preparations. As the elements become defined, elucidation of their transcription factors and the related signaling pathways may expose strategies through which the expression levels of endogenous myelin genes may be modulated experimentally and therapeutically.

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Table 4.1. Functional analysis of mutated SCE1 or SCE1 sub-domain driven reporter constructs in transgenic mice derived by pronuclear zygote injection: the designations + (indicates reporter gene expression) and – (no expression) were made based on the β-galactosidase histochemical assay in whole mount preparations and epon embedded tissue sections during the period of maximal myelin gene expression in early postnatal development (P3-P21). With two exceptions (below***), all mice analyzed were *primary* transgenic animals. In cases of mosaic animals where there was expression in the CNS but not PNS, mice were considered informative only if approximately >50% of cells in the CNS were labeled in whole mount preparations. Otherwise, they were considered uninformative and not included in the results.

* For technical reasons, δER#1-SCE1 component is slightly shorter (591 bp) than the 600 bp SCE1 used in some previous constructs.

** As all constructs contain the 6 kb proximal MBP promoter, expression in the CNS was used as a positive control and is used to indicate the total number of *expressing* mice (hence the denominator). As expected, all transgenic animals expressing lacZ in the PNS also expressed lacZ in the CNS.

*** Of transgenic mice bearing the Δ1-SCE1 and Δ3-SCE1 constructs, one of each constitutes results from an established line rather than primary transgenic animals. For Δ1-SCE1, mice from the established line did not express in the PNS (whereas a single primary transgenic mouse expressed in the PNS). Meanwhile, for Δ3-SCE1, mice from the established line expressed in the PNS, while the independently derived primary transgenic animal did not.

Construct identifier	Construct	SCE1 component	Number of independently derived mice expressing in the PNS/total expressing (total based on CNS expression)**
δER#1-SCE1	-6 kb MBP-lacZ-δER#1-SCE1 (0.6 kb; 5' → 3')	-8.891 to –8.301 kb (591 bp*) bearing a mutation in –8.785 to –8.783 kb: (-) TCA → ATT	3/3
Δ1-SCE1 (sub-domain #1)	-6 kb MBP-lacZ-Δ1-SCE1 (5' → 3')	-8.878 to –8.369 kb (510 bp) (5')NaeI/ (3')NaeI	1/2***
Δ2-SCE1 (sub-domain #2)	-6 kb MBP-lacZ-Δ2-SCE1 (5' → 3')	-8.684 to –8.369 kb (316 bp) (5')StuI/ (3')Nae I	0/3
Δ3-SCE1 (sub-domain #3)	-6 kb MBP-lacZ-Δ3-SCE1 (5' → 3')	-8.600 to –8.369 kb (232 bp) (5')BglII/ (3')NaeI	1/2***
Δ4-SCE1 (sub-domain #4)	-6 kb MBP-lacZ-Δ4-SCE1 (5' → 3')	-8.802 to -8.611 kb (192 bp) (5')BstEII/ (3')BglII	1/2
Δ5-SCE1 (sub-domain #5)	-6 kb MBP-lacZ-Δ5-SCE1 (5' → 3')	-8.802 to –8.699 kb (104 bp) (5')BstEII/ (3')StuI	0/5
Δ6-SCE1 (sub-domain #6)	-6 kb MBP-lacZ-Δ6-SCE1 (5' → 3')	-8.750 to –8.658 kb (93 bp) (5')Eco0109I/ (3')Eco0109I	0/1
Δ7-SCE1 (sub-domain #7)	-6 kb MBP-lacZ-Δ7-SCE1 (5' → 3')	-8.801 to –8.747 kb (55 bp)	0/2
Δ8-SCE1 (sub-domain #8)	-6 kb MBP-lacZ-Δ8-SCE1 (5' → 3')	-8.766 to -8.737 kb (30 bp) (dimerized; head to tail)	0/2

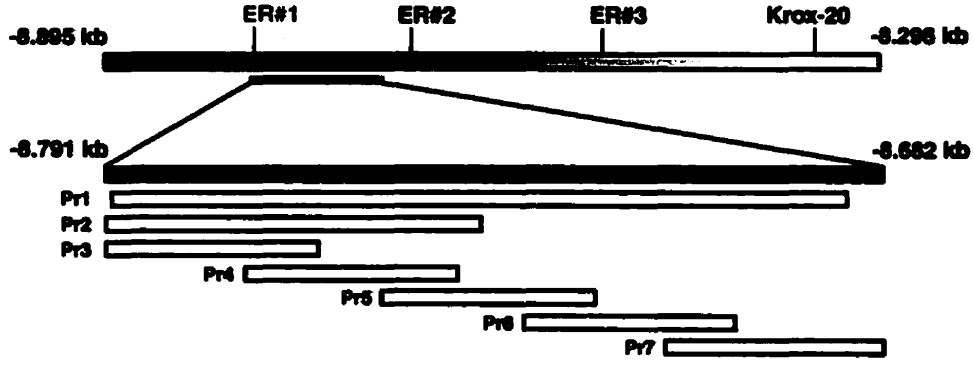
Table 4.2. Transgenic mice derived by insertion of reporter constructs in the pre-determined HPRT site and their analysis by β-galactosidase histochemistry: a single copy of the MBP promoted constructs listed was targeted to the HPRT locus in ES cells by homologous recombination and transgenic chimeras derived following blastocyst injection. Chimeras or mice from established lines were analyzed between P3 and P90 as whole mount preparations and sections stained for β-galactosidase histochemistry. Expression was designated as either low (+) or high (++) based on the visual assessment of labeling intensity in whole mount preparations and tissue sections with emphasis on the labeling intensity of individual cells. Designations of relative intensity were made based on the analysis of mice with a similar age (the chimeras described here, and their controls from established lines, were 2 mo old). Figure 6 provides an example of the difference in labeling intensity between mice from SCE1-*HPRT* #1 and Δ1-SCE1-*HPRT* lines.

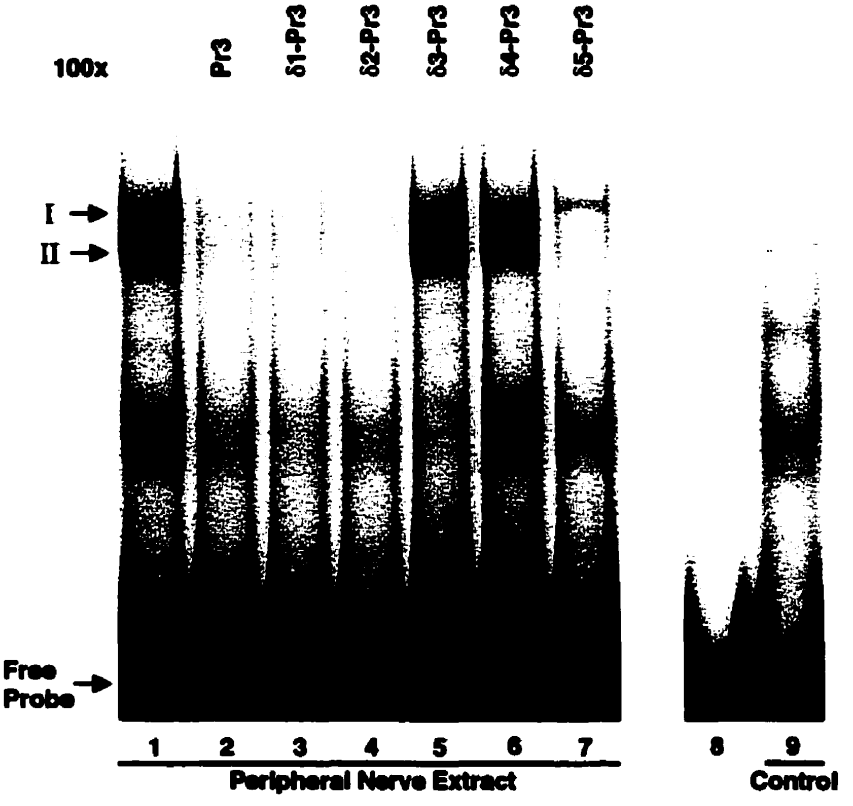
Construct identifier	MBP promoted construct (as part of HPRT targeting cassette)	SCE1 component	# of chimeras analyzed/ expression in the PNS (+) or (++)	# of lines analyzed/ expression in the PNS (+) or (++)
SCE1-*HPRT* #1	–6 to –3 kb MBP + –0.3 kb MBP + lacZ + SCE1 (0.6 kb)	–8.891 to –8.301 kb (591 bp) (5')SacII/ (3')SacI	1/++	1/++
SCE1-*HPRT* #2	–3.1 kb MBP + lacZ + SCE1 (0.6 kb)	–8.891 to –8.301 kb (591 bp) (5')SacII/ (3')SacI	1/++	
Δ1-SCE1-*HPRT*	–3.1 kb MBP + lacZ + Δ1-SCE1	–8.878 to –8.369 kb (510 bp) (5')NaeI/ (3')NaeI	1/+	1/+
δKrox-20-SCE1-*HPRT*	–3.1 kb MBP + lacZ + δKrox-20-SCE1	–8.891 to –8.301 kb (591 bp) bearing a mutation in –8.357 to –8.353 kb: ACCCA → GAAAC	1/+	

1° Antibody Dilution	1:1000	1:1500	1:2000	1:2500	1:3000
Relative staining in SCE1-*HPRT* #1 peripheral nerves compared to nerves from a δKrox-20-SCE1-*HPRT* chimera (+)/(+++)	+	+	++	+++	+++

Table 4.3. Comparison of β-galactosidase immunocytochemistry in mice bearing reporter constructs driven by SCE1 or a mutated version bearing an altered Krox-20 site: So far, we have been most successful in detecting β-galactosidase with a technique involving a secondary antibody and this table presents the results from the comparison of a 2 mo δKrox-20-SCE1-*HPRT* chimera (composed of approximately 80 % ES derived cells by coat color chimerization) and a 2 mo offspring from a SCE1-*HPRT* #1 line. Given the non-linear signal generated from such an approach, different dilutions of the primary antibody were used. At all dilutions, the apparent staining of individual Schwann cell profiles was greater in intensity in SCE1-*HPRT* #1 peripheral nerves than in nerves from the δKrox-20-SCE1-*HPRT* chimera. However, in samples incubated with 1:1000, 1:1500, and 1:2000 diluted antibody, this difference was very subtle (designated +) and sometimes not convincing. On the other hand, when 1:2500 or 1:3000 dilutions of the 1° antibody were used, the difference was clear and striking (+++). As discussed in the text of the manuscript, these results are preliminary and additional investigations are ongoing.

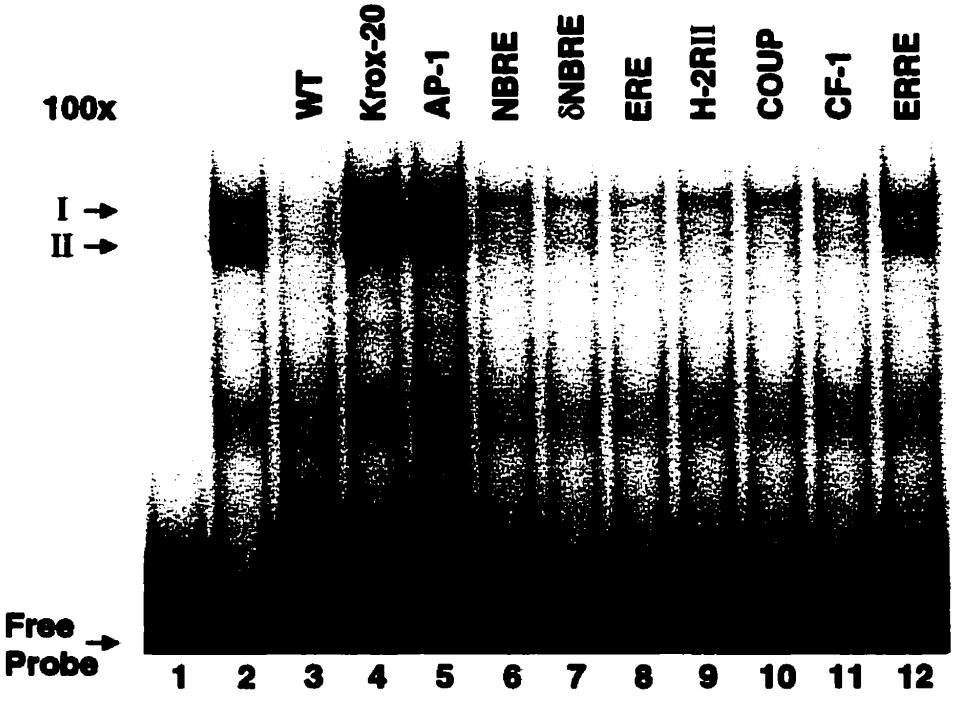
Figure 4.1. SCE1 sequence analysis and the use of partially overlapping oligonucleotides to detect binding activity in EMSAs: in order to identify potential elements underlying SCE1 function, EMSAs were performed using extracts from peripheral nerves that have previously been shown to contain transcription factors present in myelinating Schwann cells. This schematic diagram illustrates the relation of DNA probes used in EMSAs to SCE1. The top rectangle represents the 0.6 kb mouse SCE1 sequence [mouse SCE1 has high homology with a regulatory module in the human locus (Lepage et al., submitted) that is represented by the shade of gray - decreasing shade of gray represents decreasing homology with the human regulatory module]. Also shown are a few among the over 150 potential regulatory elements revealed by subsequence analysis of SCE1. Notably, only Krox-20 has a known association to myelination. Below the rectangle representing SCE1, the region tested in EMSAs for binding activity is shown. In an initial set of EMSAs, investigations were carried out using probes 1 to 7 which span part of this highly conserved SCE1 sequence. These investigations identified prominent binding activity in the region corresponding to Pr3, as shown in Figure 2.


ER#1
ER#2
ER#3
Krox-20
-8.895 kb
-8.296 kb
-8.791 kb
-8.682 kb
Pr1
Pr2
Pr3
Pr4
Pr5
Pr6
Pr7



Pr3	CCCAGGTGACCCCAAGCCCAGGCTGCCAGC
δ1-Pr3	CCCAGGTGACCCCAAGCCCAGGCAGAGTTG
δ2-Pr3	CCCAGGTGACCCCAAGGATCTTATGCCAGC
δ3-Pr3	CCCAGGTGAATAGCTTCCCAGGCTGCCAGC
δ4-Pr3	CCCAGGATTCCCCAAGCCCAGGCTGCCAGC
δ5-Pr3	CAATTCTGACCCCAAGCCCAGGCTGCCAGC

Figure 4.3. Recognition sequences for nuclear receptor superfamily of transcription factors and AP-1 specifically compete binding activity represented by Complex II and I, respectively: in additional EMSAs using Pr3 as probe, oligonucleotides bearing recognition sites for candidate transcription factors were used in competition assays. Labeled Pr3 was incubated with peripheral nerve extract (lanes 2 – 12) or without (lane 1). Competition assays were performed using 100x excess cold Pr3 (WT; lane 3), control (Krox-20; lane 4), or oligonucleotides bearing recognition sequences for candidate transcription factors, as indicated at the top of the lanes 5 – 12 (sequences appear at the bottom of the panel; the sequence for Pr3 is provided in Fig.2). As before, two complexes form when Pr3 is incubated with peripheral nerve extracts (I and II) and oligonucleotides bearing the AP-1 recognition sequence specifically compete complex I (lane 5). Consistent with the presence of an ER half site in Pr3, many characterized recognition sequences for members of the nuclear receptor family of transcription factors specifically compete complex II (lanes 6 – 11), with the exception of ERRE that does not compete very effectively (lane 12). The oligonucleotides encoding some of these sites also display a limited ability to compete complex I.



Krox-20	GATCCTTGTACGCGGGGGCGGTTAGT
AP-1	GATCCAGGGGCCATGTGACTCATTACACCAGT
NBRE	GATCCGAGTTTTAAAAGGTCATGCTCAATTT
δNBRE	GATCCGAGTTTT[redacted]AGGTCATGCTCAATTT
ERE	GTCCAAAGTCAGGTCACAGTGACCTGATCAAAGTT
H-2RII	GGTGAGGTCAGGGGGTGGGGACCATGG
COUP	ATGGTGTCAAAGGTCAAA
CF-1	TAGTGTATATAGGTCACGTAAATGTC
ERRE	TTATCATCGTTTCTCAAGGTCAGAATGA

Figure 4.4. Site-directed mutagenesis did not reveal an essential role for complexes I and II in mediating SCE1 activity in vivo: to test whether the binding activities represented by complexes I and II are essential for SCE1 activity, the 3 bp mutation that abolishes both binding activities in EMSAs (Fig. 2) was introduced into the 0.6 kb SCE1. The mutated SCE1 (δER#1-SCE1) was then ligated to the 3' end of ~6 kb MBP-lacZ and this construct was used to derive transgenic mice by zygote pronuclear injection. Primary transgenic animals were analyzed at P3-P10 and three pups expressed both in the CNS and PNS, demonstrating that this site is not essential for SCE1 activity.


5'→3'
- 6.0 kb
lacZ
δER#1-
SCE1

Figure 4.5. SCE1 sub-domains used for the functional breakdown of SCE1 activity: in order to locate sequence(s) underlying SCE1 activity, SCE1 sub-domains were generated and ligated to the 3' end of –6 kb MBP-lacZ and constructs were used to derive transgenic mice by pronuclear injection. (a) This diagram shows the basic design of all constructs used for this functional breakdown and sub-domains contained in key constructs (a full list of constructs is available in Table 1). On the left side of the panel, the targeting ability of sub-domains, based on β-galacotosidase histochemistry in whole mount and tissue sections, is designated (+, if any of the transgenic animals derived from a construct expressed in Schwann cells, and –, if none did). Beside the +/– designations, the number that expressed in the PNS/total number of expressing transgenic animals is indicated (total is based on expression in oligodendrocytes). The –6 kb MBP proximal promoter was used to drive expression in the CNS, providing a positive internal control for insertion into a location in the genome that is transcriptionally permissible. The result indicated for the 0.6 kb SCE1 (9/9) is from a previous study (Forghani et al., in press). This investigation shows that SCE1 contains at least two independent targeting sub-domains. In addition, all targeting sub-domains fail to express consistently in the absence of the 3' 73 bp sequence . (b) Whole mount preparations of a P3 Δ4-SCE1 (192 bp) bearing *primary* transgenic animal and a P3 Δ3-SCE1 (232 bp) bearing pup from an established line demonstrate expression in PNS and CNS. Neither of these mice display the apparent robust staining seen in transgenic mice bearing the full 0.6 kb SCE1 (Forghani et al., in press).

Figure 4.6. The 3' 73 bp sub-domain enhances the expression level conferred by SCE1: to enable a quantitative comparison of expression level conferred by reporter constructs, a single copy of constructs bearing the 0.6 kb SCE1 (SCE1-*HPRT* #1) and the 510 bp sub-domain that lacks the 3' 73 bp sequence (Δ1-SCE1-*HPRT*) was inserted in the pre-determined HPRT site. Then, transgenic lines were established from chimeras and expression assessed by β-galactosidase histochemistry after overnight incubation in stain. (a) A 2 mo SCE1-*HPRT* #1 transgenic animal displays high-level expression in peripheral nerves. (b) A 2 mo old Δ1-SCE1-*HPRT* mouse lacking the 73 bp sub-domain (and therefore the Krox-20 site) continues to express in the PNS, consistent with the investigation showing targeting sub-domains within SCE1 outside of the 3' 73 bp region. However, consistent with the observation that SCE1 expression is less consistent and robust in mice lacking the 73 bp sub-domain, this mouse displays reduced labeling of its peripheral nerves compared to the one bearing the full 0.6 kb SCE1, suggesting that the 73 bp sequence quantitatively modulates the expression level conferred by targeting sub-domains. A developmental study has also revealed expression in oligodendrocytes in mice bearing this construct, but at this age such expression is not robust. This is consistent with previous observations using the 3.1 kb MBP proximal promoter (Foran and Peterson, 1992).

CHAPTER 5

CHAPTER 5

GENERAL DISCUSSION

PREFACE

This chapter provides a general discussion of the investigations described in chapters two, three, and four and a discussion of future directions and implications for axon-glia biology and beyond. Regretfully but by necessity, there is some redundancy between the content of this chapter and the individual discussions in previous chapters.

The development of mature peripheral nerve fibers requires multiple levels of interaction between axons and ensheathing Schwann cells (Peters and Muir, 1959; Webster, 1971; Webster et al., 1973; Aguayo et al., 1976a; Aguayo et al., 1976b; Aguayo et al., 1977; Peters et al., 1991; Mirsky and Jessen, 1996). An important developmental milestone in the maturation of the vertebrate nervous system is myelination of large caliber axons and this is achieved in part through the coordinate up-regulation of genes encoding myelin structural proteins (Gupta et al., 1988; Lemke and Chao, 1988; Trapp et al., 1988; Lamperth et al., 1990; LeBlanc and Poduslo, 1990; Stahl et al., 1990; Snipes et al., 1992). This thesis examines the transcriptional regulation of one such gene, the Myelin Basic Protein (MBP). Using in vivo studies of reporter constructs driven by MBP 5' flanking sequence, including a high-resolution transgenic preparation in which a single copy of a reporter construct is inserted in the HPRT docking site, multiple positive and negative Schwann cell regulatory sequences were located across 12 kb of MBP 5' flanking sequence. These investigations demonstrated that Schwann cell expression is controlled through three regulatory "sub-domains" with independent targeting capability. A further sub-domain appears to modulate the quantitative level of expression. In parallel, a method was devised for obtaining nuclear extracts containing transcription factors from myelinating Schwann cells in peripheral nerves. Using sequence analysis and in vitro DNA-protein interaction assays with such extracts, multiple candidate regulatory elements were revealed. Combined, our observations provide the basis for a complex model in which the expression of MBP in Schwann cells is regulated through the

integrated output of multiple regulatory sequences, analogous to better characterized loci in lower and higher eukaryotes (Yun and Wold, 1996; Yuh et al., 1998).

Identification of MBP Schwann cell enhancers: a complex network with multiple regulatory components

Transcriptional regulation of eukaryotic genes is complex involving multiple DNA cis elements and transcription factors that bind the promoter region, co-regulators that interact with transcription factors but do not bind DNA directly, as well as regulation through alterations in chromatin structure (Yun and Wold, 1996; Glass et al., 1997; Kadonaga, 1998; Wray, 1998; Freedman, 1999; Engel and Tanimoto, 2000). Such complexity is reflected in the functional organization of MBP Schwann cell regulatory sequences described here. In our search for MBP Schwann cell enhancers, we initially identified two sequences capable of targeting reporter gene expression to Schwann cells independently and arbitrarily designated them SCE1 (0.6 kb) and SCE2 (3kb). While SCE1 was characterized most thoroughly, both sequences target Schwann cell expression when ligated to the 3' of lacZ in reporter constructs and hence appear to function as classic enhancers. However, the previously designated SCE2 appears to have function similar to the two targeting sub-domains identified within SCE1 and may well be an additional component of a single sophisticated regulatory module.

Reporter constructs promoted by relatively short P0 and CNPase 5' flanking sequences have been shown to express in Schwann cells of transgenic mice (Messing et

al., 1992; Gravel et al., 1998). In contrast, constructs regulated by similar lengths of 5' sequence from the MBP gene express in oligodendrocytes, the myelin forming cell type in the central nervous system, but not in Schwann cells (Foran and Peterson, 1992; Gow et al., 1992; Miskimins et al., 1992; Goujet-Zalc et al., 1993; Stankoff et al., 1996). Our investigations demonstrate that at the MBP locus, Schwann cell enhancer(s) reside further upstream. As SCE1 has sufficient regulatory elements to confer appropriate Schwann cell expression from the heterologous hsp68 promoter, MBP expression in Schwann cells and oligodendrocytes appears to be controlled through different and, in large part, independent sets of regulatory elements. Therefore, despite the common role of MBP as a structural protein in both CNS and PNS myelin (Privat et al., 1979; Kirschner and Ganser, 1980; Roach et al., 1983; Roach et al., 1985; Lemke, 1988; Martini et al., 1995a), the promoter may function through different transcription factors and regulatory elements in these two cell types.

The robust and independent expression conferred by the 0.6 kb SCE1 made it an ideal candidate for locating and identifying positive regulatory elements controlling MBP expression. In the most simplistic model, one or a few essential elements with distinct activities would underlie SCE1 function. The functional breakdown assays were based upon this assumption and were designed to locate such essential element(s) within a short sequence in order to facilitate their further characterization through in vitro DNA-protein interaction assays and in vivo functional assays. However, instead of highlighting a single target sequence, our investigations revealed a much richer and more complex regulatory

network with multiple targeting components and a sub-domain that modulates expression level.

Perhaps the most intriguing observation emanating from the SCE1 breakdown experiments was the reduced probability of expression in transgenic mice bearing any sub-component of SCE1 that lacked the 3' 73 bp sequence. Our initial design using oligodendrocyte expression as an internal control was based on the fact that even reporter constructs driven by known functional enhancers fail to express in a significant percentage of random integration events (Palmiter and Brinster, 1986). Given that this could confound the interpretation of a negative result (i.e. deleted or mutated forms of SCE1 that do not express), we assumed that a transcriptionally "unfavorable" chromatin domain would similarly effect transgene expression in both oligodendorcytes and Schwann cells. Thus, expression in oligodendrocytes would indicate that a given SCE1 sub-domain should express in Schwann cells if it bears sufficient Schwann cell specific regulatory elements. While this may be an oversimplified view, of the lines bearing proximal MBP sequences and SCE1, all that expressed in oligodendrocytes also expressed in Schwann cells. Based on this observation, the reduced efficiency of reporter gene expression in the absence of the 73 bp sub-domain following random integration was striking. Furthermore, as this sequence confers a quantitative effect on the expression of reporter constructs inserted into the HPRT locus, it seems highly probable that it is required for high-level Schwann cell expression from the endogenous MBP locus. This result is somewhat analogous to the recent demonstration that P0 regulatory sequences

reflects normal MBP transcriptional output. One example of this deviation is the unanticipated down-regulation of transgene expression in ventral roots of some mature SCE1 bearing lines. Furthermore, some transgenes that were expressed robustly during myelination were shut-off in mature animals entirely. Consequently, either additional regulatory domains are yet to be identified and/or more normalized function will be observed when the elements contained within SCE1 and SCE2 are combined.

Unanticipated differential transgene expression in motor and sensory fibers of SCE1 lines

An entirely unanticipated observation was the differential regulation of transgenes in motor and sensory fibers of mature animals in some SCE1 bearing transgenic 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). As 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 diverges from the normal expression pattern of the endogenous MBP locus. Such differential regulation, presumably arising from a subset of MBP regulatory elements (i.e. differential utilization of elements within SCE1), suggests a novel level of Schwann cell heterogeneity directed by the innervating axon. It remains an intriguing possibility that the unequal susceptibility recognized for motor and sensory fiber types in

multiple human neuropathies and experimental animal models (Martini et al., 1995b; Wrabetz et al., 2000) may arise from differences within the Schwann cells populating such fibers.

Although the differential expression in motor and sensory fibers is striking, it was not observed in all SCE1 lines. Notably, while this was a prominent feature of several moderately expressing lines, it was not detected in mice from lines in which histochemical labeling was particularly rapid and intense. One possible explanation for this discrepancy is that significant differences exist in all lines but are obscured in high-expressing lines. However, additional investigations are needed before conclusions are drawn from these preliminary observations with certainty. In that regard, a re-assessment of this phenotype in transgenic animals bearing reporter construct in the pre-determined HPRT site may lead to more consistent and conclusive observations.

Regulation of SCE1 output by proximal repressor(s) during embryogenesis

Despite the close relationship between the expression in peripheral nerves of SCE1-bearing transgenes and the endogenous MBP locus, an apparent discrepancy was observed during mid-fetal development. Of the three SCE1-hsp68 lines examined (17, 18, and 42), all three consistently revealed transient ß-galactosidase expression in a small sub-population of cells in the neural tube, brain and retina. Because constructs bearing SCE1 with the 6 kb MBP promoter are not similarly expressed, putative negative regulatory elements serving to repress MBP expression in these cell populations appear to

be located within the first 6kb of MBP 5' flanking sequence. These putative repressors appear to contribute to the cell-specificity of MBP expression during embryogenesis, similar to the utilization of such elements in directing tissue-specific expression at other loci (Yun and Wold, 1996). Further investigations are required to locate and define the role of these putative MBP repressor elements more precisely. Consistent with the identification of multiple positive regulatory sequences, these observations provide further support for a model of MBP expression involving multiple interacting regulatory sequences.

SCE1 as a target sequence for the identification of DNA regulatory elements controlling MBP expression: knowledge of myelin gene regulation and the role of Krox-20

The 0.6 kb SCE1 which contained sufficient regulatory element(s) for conferring Schwann cell expression provided an ideal target sequence to support a search for the precise DNA regulatory elements controlling MBP expression and ultimately the transcription factors that interact with them. The most obvious next step, having characterized SCE1, was to use sequence analysis algorithms to identify known regulatory elements. While a powerful approach, sequence analysis is limited by the current state of knowledge and 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.

20 site is not essential for SCE1-mediated expression. However, preliminary observations suggest that the Krox-20 site enhances SCE1 activity and therefore functions in conjunction with additional elements within SCE1. Although a quantitative role for Krox-20 has not been described previously, Krox-20 is known to function in conjunction with an additional element designated Box1 in conferring expression via the Hoxb-2 enhancer (Vesque et al., 1996).

To rigorously confirm our current conclusion based upon β-galactosidase histochemistry and immunocytochemistry, it will be necessary to derive transgenic lines and apply biochemical analysis of the β-galactosidase expression phenotype. In addition, the proximal promoter of the line bearing the wild type 0.6 kb SCE1 is different (it bears the –6 to –3 kb MBP sequence plus the 0.3 kb proximal MBP promoter) from the line bearing the 510 bp sub-domain and the chimera bearing the mutated 0.6 kb SCE1 (both have the same 3.1 kb MBP proximal promoter). It is conceivable that this difference in the proximal promoter could exert an unanticipated influence on expression levels. However, a targeting construct bearing the wild type 0.6 kb SCE1 with the same 3.1 kb MBP promoter has been generated and in the single chimera analyzed so far, expression similar to that observed in the SCE1 bearing line driven by the –6 to –3 kb plus 0.3 kb MBP promoter was observed. While not yet conclusive, these observations suggest that Krox-20 may directly enhance MBP expression. As sequence(s) outside of a targeting domain have also been shown to enhance the expression of transgenes regulated by the P0 promoter (Feltri et al., 1999), it remains an intriguing possibility that a Krox-20 site with a similar role is present in other myelin gene promoters and regulates their expression.

Another transcription factor implicated in the control of myelin gene expression is the POU domain factor Tst-1/SCIP/Oct-6 (SCIP) (He et al., 1989; Monuki et al., 1989). It is down-regulated immediately prior to 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; Monuki et al., 1990; He et al., 1991; Weinstein et al., 1995). However, myelination in the PNS of homozygous null SCIP mutants is delayed (Bermingham 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.

Despite numerous well-designed investigations (Monuki et al., 1989; Monuki et al., 1990; He et al., 1991; Monuki et al., 1993; Li et al., 1994b; Shy et al., 1996; Brown and Lemke, 1997; Peirano et al., 2000), the molecular pathways and DNA cis regulatory elements controlling myelin gene expression remain poorly defined. In part, this difficulty appears to arise from the existence of multiple interacting regulatory components with potentially overlapping functions. Furthermore, given the importance of axon-Schwann cell interactions in controlling myelin gene regulation and the lack of an in vitro model that completely recapitulates the phenotype of a myelinated fiber, experimental design and interpretation has been significantly limited. Given the apparent complexity emerging for SCE1, the single copy chosen site of insertion strategy of transgenesis may provide a sufficiently high-resolution tool for investigating myelin gene regulation.

Subsequence and comparative analysis fails to narrow down target regulatory sequence

While analysis of SCE1 sequence only revealed one site with a known association to myelination (i.e. Krox-20), numerous additional putative elements were identified, as would be expected for a sequence this size. However, in the absence of a known link to myelination, these could not be used to narrow down regulatory region(s) within SCE1. Another approach was to compare the SCE1 sequence with the published 1.1 kb P0 promoter that confers an expression program with similarities to that described here for MBP SCE1 (Messing et al., 1992) in order to identify potentially shared elements. It is plausible that the seemingly coordinate expression programs displayed by these reporter constructs involves enhancers or repressors in each promoter that bind identical transcription factors that are themselves regulated through shared signaling pathways, similar to the regulatory model described in myogenesis (Yun and Wold, 1996). However, PIP analysis did not reveal significant sequence homology between SCE1 and this region of the P0 promoter. The interpretation to be placed on this negative result is not simple. Most sequence comparative algorithms set a lower limit for significance at greater than 50% identities over 70 bp. Consequently, multiple elements would have to be similarly aligned and spaced in different myelin genes to be recognized. While this could occur through direct descent it seems inconceivably remote that multiple genes would have independently evolved the same configuration of regulatory elements. Furthermore, P0 regulatory elements that contribute to Schwann cell expression also appear to reside outside the 1.1 kb promoter sequence (Feltri et al., 1999). Whether the same elements are

shared with MBP and/or other myelin genes awaits identification of the elements regulating such genes.

Elements regulating MBP expression: other experimental obstacles and the development of a method for preparing peripheral nerve extracts

The effort to characterize the cis element(s) within SCE1 could be significantly facilitated by the application of DNA-protein binding techniques such as electrophoretic mobility shift assays (EMSAs). However, precluding this approach was the lack of an effective method for obtaining nuclear extracts from myelinating Schwann cells. Since Dignam et al.'s original nuclear extraction protocol (Dignam et al., 1983a; Dignam et al., 1983b), microextraction protocols applicable to a wide variety of tissues have been described (Hoppe-Seyler et al., 1991; Roy et al., 1991). However, despite attempts by various investigators in the field, there has been no effective method for obtaining Schwann cell nuclear extracts from peripheral nerves prior to the method described in this thesis. This difficulty arises from anatomic features unique to peripheral nerves. Each Schwann cell elaborates a basal lamina and the nerve is heavily invested with collagen and three separate connective tissue sheaths which limit the flow of macromolecules (Peters et al., 1991). These features make simple nuclear preparation strategies ineffective and interfere with traditional methods of extracting nuclear proteins. While methods were available for obtaining nuclear proteins from cultured cells, our preference was to use a source that is most likely to contain myelin gene relevant transcription factors, i.e. peripheral nerves containing myelinating Schwann cells.

Regulation of MBP expression through multiple cis elements

The EMSAs reported in this thesis identified multiple and prominent DNA-protein interactions within SCE1. While the site-directed mutagenesis experiments performed in the context of the 0.6 kb SCE1 in transgenic animals derived by pronuclear injection have not revealed an essential role for any individual element, this does not rule out important roles for these elements. Indeed, this would be anticipated in light of our new understanding that there are at least two independent targeting sequences within SCE1. As the DNA-protein interactions described here are prominent and highly specific, as well as from a source that contains transcription factors regulating myelinogenesis, they are likely to play an important role in the overall regulation of MBP expression. Therefore, these, along with element(s) yet to be identified by investigation of other parts of SCE1 using a similar approach, will provide excellent candidates for future mutagenesis experiments leading to constructs that will be assayed functionally after insertion into the HPRT docking site.

A complex regulatory model for MBP expression: a platform for future investigations using high resolution transgenic models

Our investigations support a model in which the transcriptional regulation of MBP is achieved through the integrated output of multiple regulatory sequences sharing fundamental features with better characterized loci in lower and higher eukaryotes (Yun and Wold, 1996; Yuh et al., 1998). Given the complexity of this system, including

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). Lastly, the robust expression observed in numerous lines bearing SCE1 regulated constructs and the conservation observed between the mouse SCE1 and human MBP regulatory module (Lepage et al., in preparation) suggest that SCE1 will provide a powerful tool for promoting gene expression in myelinating Schwann cells for both biological investigations and potential therapeutic interventions. As the elements regulating myelin gene expression and their transcription factors partners are identified, these also will provide novel opportunities to modulate myelin gene levels experimentally and therapeutically.

Future prospects for the post genome world

In the last twenty years, the fields of genomics and genetic engineering have witnessed an unforeseen advancement (Wilmut et al., 1997; Collins et al., 1998; Hacia et al., 1998; Karanjawala and Collins, 1998; Collins, 1999a, b; Zajtchuk, 1999). With the completion of the human genome project, there is wide opportunity for the investigation of the function of both DNA regulatory sequences and proteins, providing insight into basic biology that has the potential to significantly improve our understanding of the genetic susceptibility and basis of disease and widen the arsenal of therapeutic modalities for disease prevention and treatment. Among the many technological advances witnessed in the past decade, high throughput sequencing will enable more effective basic and clinical investigation. Such technology may eventually be applicable to routine clinical

investigations for disease prevention and diagnosis, as well as to provide information that will help determine the best treatment course for an individual based on her genetic profile. Although the recent technological advances raise serious ethical questions that should not be overlooked, controlled and judicious use of this technology provides great potential for improving the field of clinical medicine and ultimately the lives of all of us. Perhaps the most challenging endeavor, given the potential to derive any organ from most of the genetically identical cells in our bodies, is to genetically engineer and derive organs in vitro for transplantation from a source as simple as a skin biopsy. As the mechanisms governing transcriptional regulation of tissue differentiation are elucidated, the basis for such tissue engineering will be established. While still a remote possibility, given the changes witnessed over the past century, these endeavors may be realizable in a not so distant future.

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