Doctoral Thesis

Promoter analysis and transcriptional regulation of the CMT1A-disease gene peripheral myelin protein PMP22

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Promoter Analysis and Transcriptional Regulation of the CMT1A-Disease Gene
Peripheral Myelin Protein PMP22

A dissertation submitted to the
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2003
for Carina and my parents
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SUMMARY</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>ZUSAMMENFASSUNG</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>INTRODUCTION</td>
<td>9</td>
</tr>
<tr>
<td>3.1</td>
<td>Origin and differentiation of Schwann cells</td>
<td>9</td>
</tr>
<tr>
<td>3.2</td>
<td>Genetic defects in myelin proteins are associated with hereditary peripheral neuropathies</td>
<td>11</td>
</tr>
<tr>
<td>3.3</td>
<td>Transcriptional control of myelin genes</td>
<td>13</td>
</tr>
<tr>
<td>3.3.1</td>
<td>Glial transcription factors and their function</td>
<td>15</td>
</tr>
<tr>
<td>3.3.2</td>
<td>Transcriptional control elements in myelin genes</td>
<td>16</td>
</tr>
<tr>
<td>3.4</td>
<td>Aim and motivation of the work</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>MYELIN GENE REGULATION IN VITRO</td>
<td>27</td>
</tr>
<tr>
<td>4.1</td>
<td>Aims and Experimental strategies</td>
<td>27</td>
</tr>
<tr>
<td>4.2</td>
<td>Promoterdeletion study in MSC80 and NIH 3T3 cells</td>
<td>29</td>
</tr>
<tr>
<td>4.3</td>
<td>Is PMP22 regulated by Sox10 in N2a cells?</td>
<td>32</td>
</tr>
<tr>
<td>4.4</td>
<td>Screening for transcription factors upregulated upon Krox20 induced myelin gene expression</td>
<td>33</td>
</tr>
<tr>
<td>5</td>
<td>REGULATORY ELEMENTS ON THE -10/0 KB PMP22 LACZ TRANSGENE</td>
<td>37</td>
</tr>
<tr>
<td>5.1</td>
<td>Aims and Experimental strategies</td>
<td>37</td>
</tr>
<tr>
<td>5.2</td>
<td>Generation of transgenic mice expressing the reporter lacZ gene under the control of the PMP22 promoters (-10/0kb PMP22 LacZ)</td>
<td>37</td>
</tr>
<tr>
<td>5.3</td>
<td>Analysis of the first wave of -10/0kb PMP22 lacZ expression during embryonic development</td>
<td>39</td>
</tr>
<tr>
<td>5.4</td>
<td>-10/0kb PMP22 lacZ expression is strongly upregulated in postnatal neurons and Schwann cells of peripheral nerves</td>
<td>41</td>
</tr>
<tr>
<td>5.5</td>
<td>Transgenic β-gal expression in sensory and motor neurons of cranial nerves</td>
<td>45</td>
</tr>
<tr>
<td>5.6</td>
<td>Tissue specificity of the -10/0kb PMP22 lacZ transgene</td>
<td>46</td>
</tr>
<tr>
<td>5.7</td>
<td>-10/0kb PMP22 lacZ transgene regulation in Schwann cells after loss of axonal contact and in regeneration</td>
<td>48</td>
</tr>
<tr>
<td>5.8</td>
<td>Sciatic nerve of PMP22 mutant animals show reduced β-gal levels</td>
<td>50</td>
</tr>
</tbody>
</table>
6 **PMP22 PROMOTER DELETION ANALYSIS IN VIVO** ....53  
   6.1 Late myelination Schwann cell specific elements (LMSE) reside in the 6 kb DNA fragment upstream of promoter ....... 55  
   6.2 The LMSE confer Schwann cell specificity to the non-cell type-specific *hsp68* promoter and are functionally independent of core promoter 1 and exon 1A ..................................................... 59  
   6.3 The 4 kb sequence upstream of exon 2, including promoter 2, contains elements directing expression in sensory neurons ...... 62  

7 **COMPUTATIONAL ANALYSIS OF CONSERVED PROMOTER ELEMENTS AND REPETITIVE GENOMIC REGIONS** 65  

8 **DISCUSSION AND OUTLOOK** ................................. 69  
   8.1 PART I: Myelin Gene Regulation *in vitro* ......................... 69  
   8.2 PART II: Regulatory Elements on the -10/0kb PMP22 lacZ Transgene ................................................................................... 73  
   8.3 PART III & IV: PMP22 Promoter Deletion Analysis *in vivo* .... 77  

9 **EXPERIMENTAL METHODS** ................................. 87  
   9.1 Generation of reporter constructs ............................................... 87  
   9.2 Generation of transgenic animals ............................................... 88  
   9.3 ß-gal histochemical analysis ....................................................... 89  
   9.4 ß-gal solution assay .................................................................... 90  
   9.5 Quantitative analysis of PMP22 mRNA levels ...................... 91  
   9.6 PMP22-lacZ x PMP22-/- and PMP22-lacZ x Tr mice ............... 92  
   9.7 Sciatic nerve transsection and crush ........................................... 93  
   9.8 Immunocytochemistry of dissociated DRG ............................... 93  
   9.9 Cell culture, transfection and reporter assays ............................. 94  
   9.10 Sequence analysis and determination of potential binding sites 96  

10 **REFERENCES** ............................................................... 97  
11 **ACKNOWLEDGEMENTS** .............................................111  
12 **CURRICULUM VITAE** ................................................112
1 SUMMARY

Minor changes in Peripheral Myelin Protein 22 (PMP22) gene dosage have profound effects on the development and maintenance of peripheral nerves. This is evident from the genetic disease mechanisms in the hereditary peripheral neuropathy Charcot-Marie-Tooth disease type 1A (CMT1A) and Hereditary Neuropathy with liability to Pressure Palsies (HNPP) as well as in transgenic animals with altered PMP22 gene dosage. Thus, regulation of PMP22 is a crucial aspect in understanding the function of this protein in health and disease.

As a first approach to study PMP22 transcriptional regulation, I have generated transgenic mice containing ten kilobases of 5'-flanking region of the PMP22 gene, including the two previously identified alternative promoters, fused to a lacZ reporter gene. I showed that this part of the PMP22 gene contains the necessary information to reflect the endogenous expression pattern in peripheral nerves during development, regeneration, and in mouse models of demyelination due to genetic lesions. Transgene expression is strongly regulated during myelination, demyelination and remyelination in Schwann cells, demonstrating the important influence of neuron-Schwann cell interactions in the regulation of PMP22. In addition, the region of the PMP22 gene present in this transgene also directs expression in sensory and motor neurons.

These results provided the basis for further analysis of the elements that direct these specific aspects of temporal and spatial regulation of the PMP22 gene. To this end, I subdivided the ten kilobase 5’-flanking region of the PMP22 gene and analyzed different cis-acting elements as a fusion with either the corresponding PMP22 promoter or a heterologous hsp68 promoter, both together with a lacZ reporter gene in vivo. This revealed the existence of two separate elements. The first is a late myelinating Schwann cell specific element (LMSE) located 5’ to promoter 1 of PMP22. The LMSE is strongly regulated during myelination and is responsible for specific expression of PMP22 during the later phase of myelination in Schwann cells. The second element, 2kb 5’ of and including promoter 2 of PMP22, was active postnatally specifically in sensory neurons. The activities of these two elements contribute to distinct parts of the endogenous PMP22 expression.
These in vivo studies were complemented by cell culture experiments analysing PMP22 regulatory elements in a promoter deletion study with transient transfection assays of PMP22 promoter-driven lacZ reporter constructs. Furthermore, I established a system with which to further analyse the initiation of myelin gene expression in cell culture. Combined with a bioinformatics-based determination of conserved regulatory elements and potential binding sites for transcription factors, these different approaches contributed to a better understanding of the temporal and spatial regulation of PMP22. Furthermore, these results provide the basis for further dissection of the molecular basis responsible for late postnatal expression of PMP22 and the corresponding pathways converging on the LMSE. These pathways may be important in myelin maintenance or in demyelinating peripheral neuropathies.
Aenderungen in der Kopienzahl für das Periphere Myelin Protein 22 (PMP22) haben erhebliche Auswirkungen auf die Entwicklung und Erhaltung des peripheren Nervensystems (PNS). Dies wurde offenkundig durch die Erforschung der genetischen Mechanismen der hereditären Motorischen und Sensiblen Neuropathie Charcot-Marie-Tooth Typ 1A (CMT1A), der Hereditären Neuropathie mit Neigung zu Druckparesen (HNPP-Hereditary Neuropathy with liability to Pressure Pulsies) sowie dem Studium von Tiermodellen mit veränderter PMP22 Kopienzahl. Diese Arbeiten zeigten, dass die Regulation des PMP22 Genes einen essentiellen Aspekt darstellt, um die Rolle dieses Proteins in der normalen Entwicklung sowie in der Entstehung von Neuropathien zu verstehen.


3 INTRODUCTION

3.1 Origin and differentiation of Schwann cells

The trunk neural crest gives rise to melanocytes, neurones and peripheral glia. Among the glia are the Schwann cell precursors, found in rat peripheral nerves at E14 to E15, and in mouse nerves at E12 to E13. In a relatively abrupt transition between E16 and E17 (rat) or E14 and E15 (mouse), immature Schwann cells are formed while migrating into the periphery along the axonal tracts. These then diverge around the time of birth to give rise to myelinating and non-myelinating Schwann cells (Fig. 3-1). Here, their fate is tightly controlled by axonal signals, e.g. by trophic factors such as neuregulin that promote the survival of the Schwann cells and their precursors (for review see Jessen and Mirsky, 1999; Lobsiger et al., 2002). Prospectively myelinating Schwann cells form a 1:1 relationship with axons prior to myelination. Schwann cells with this morphology are often referred to as pro-myelinating Schwann cells. Larger calibre axons (1µm and more) will be myelinated with each myelinating Schwann cell providing one myelin internode (Fig. 3-2). The transition from the promyelinating to the myelinating stage is then accompanied by a number of significant changes in the pattern of gene expression, including the activation of a set of genes encoding myelin structural proteins and lipid biosynthetic enzymes, and the inactivation of a set of genes expressed only in immature or nonmyelinating Schwann cells. Smaller calibre axons remain unmyelinated and are ensheathed in bundles of 5-30 axons by the non-myelinating Schwann cells (Friede and Samorajski, 1968). Both types of Schwann cells are surrounded by a basement membrane, which separates them from other components of the endoneurium in the peripheral nerve and anchors them in the extracellular matrix.

The myelin sheath formed by Schwann cells can be of extremely large diameter, consisting of up to 100 wrappings. Schwann cell myelin contains incisure (Schmidt-Lantermann incisures) which traverse the compact myelin. These funnel-shaped domains of uncompacted myelin contain remnants of cytoplasm and, similar to the paranodal loops, contain different proteins than are found in compact myelin (for review see Scherer, 1999; Scherer and Arroyo, 2002).
Figure 3-1: Diagrammatic representation of the development of Schwann cells in rat and mouse with their molecular markers (adapted with modifications from Jessen and Mirsky, 1999).

Upon nerve transection, or when dissociated and placed in tissue culture, myelinating and non-myelinating Schwann cells undergo a reversion in molecular phenotype comparable to that seen in immature nerves. In regenerating nerves, these Schwann cells again differentiate into myelinating and non-myelinating Schwann cells. Thus the Schwann cell molecular and morphological phenotype is reversible from the immature Schwann cell state onwards.
3.2 Genetic defects in myelin proteins are associated with hereditary peripheral neuropathies

The hereditary peripheral neuropathy Charcot-Marie-Tooth disease (CMT) comprises a heterogeneous group of genetic human disorders that affect the peripheral nervous system (PNS) with an estimated prevalence of 1:2500 (Skre, 1974). In recent years mutations in up to ten genes have now been identified in humans as culprits in different forms of CMT (reviewed by Berger et al., 2002; Maier et al., 2002b; Young and Suter, 2001), suggesting that there are different important players in the interplay between Schwann cells and neurons. Three of the PNS myelin proteins, PMP22, Cx32 and MPZ, have been linked to one of the most common forms of hereditary motor and sensory neuropathies (HMSN), the Charcot-Marie-Tooth disease type 1 (CMT1; reviewed by Suter et al., 1995).

In my thesis project, I focused on the gene encoding the peripheral myelin protein 22 (PMP22) which is the gene involved in the vast majority of patients affected by the
hereditary peripheral neuropathy Charcot-Marie-Tooth disease (CMT; subtype CMT1A; reviewed in Maier et al., 2002b; Young and Suter, 2001; Suter and Snipes, 1995). Patients suffering from CMT1A, the most common form, show slowed nerve conduction velocities, reduced compound motor and sensory nerve action potentials, progressive distal limb weakness, sensory loss, and decreased reflexes (Dyck et al., 1993). Histologically, characteristic findings in these patients are progressive demyelination of motor and sensory nerves associated with incomplete remyelination. In the majority of the patients the autosomal-dominant CMT1A is due to an intrachromosomal duplication on chromosome 17p11.2, leading to the presence of an extra copy of the gene for peripheral myelin protein 22. The reciprocal deletion of the same DNA fragment has also been identified in patients and is associated with hereditary neuropathy with liability to pressure palsies (HNPP; Chance et al., 1993), which is usually not progressive but rather characterized by temporary palsies after pressure trauma (Windebank, 1993; Amato et al., 1996). In addition, PMP22 missense mutations have been found in some rare cases of familial CMT1A without the usual chromosomal duplication (Roa et al., 1993; Naef and Suter, 1999).

The findings derived from the genetics of CMT1A and HNPP, and the fact that the PMP22 gene is neither disrupted by the CMT1A duplication nor mutated in these patients, led to the suggestion that altered PMP22 gene dosage may be responsible for these two frequent neuropathies. The observation that homozygous CMT1A duplication patients are often more affected than heterozygous relatives supports this hypothesis (Lupski et al., 1991; Kaku et al., 1993). Additional evidence that PMP22 is a dosage-sensitive gene has been provided by accurate animal models in which mice and rats carry variable numbers of the PMP22 gene (Adlkofer et al., 1995; Huxley et al., 1996; Magyar et al., 1996; Sereda et al., 1996). In particular, a twofold increased PMP22 gene dosage in transgenic rats led to a morphological phenotype comparable to CMT1A. Transgenic mice retaining only one functional PMP22 allele develop, as expected from the analogous human disease, a pathology comparable to HNPP, whereas mice lacking PMP22 completely develop a demyelinating peripheral neuropathy reminiscent of severe CMT1A (Adlkofer et al., 1995). Two different missense mutations in the PMP22 gene have also been found in the allelic mouse mutants Trembler (Tr) and Trembler-J (Tr-J), which initially implicated PMP22 as the critical gene within the CMT1A duplication.
Since these animals show a PNS-specific dismyelination, they have been proposed to be suitable models for CMT1A (Suter et al., 1992a, b). Taken together, these findings suggest that the \textit{PMP22} gene must be very tightly regulated since already slightly higher or lower gene dosage (150% in CMT1A; 50% in HNPP) lead to a disease phenotype.

Interestingly, a comparable phenomenon is observed for the proteolipid protein (\textit{Plp}) gene. Similar to the situation in CMT1A for \textit{PMP22} increased gene dosage resulting from a duplication of the proteolipid protein (\textit{Plp}) gene is one of the causes of the Pelizaeus-Merzbacher disease (PMD) and leads to myelination abnormalities in the CNS (reviewed by Anderson et al., 1999; Yool et al., 2000). Transgenic mice carrying extra copies of the wild-type \textit{Plp} gene provide a valid model of PMD. Variations in gene dosage can cause a wide range of phenotypes from severe, lethal dysmyelination through late-onset demyelination.

### 3.3 Transcriptional control of myelin genes

The extremely high and transient demand for newly synthesized myelin proteins during nerve development and regeneration is accompanied by the coordinated expression of genes which encode myelin components (reviewed by Toews et al., 1997). Deciphering the molecular basis of this regulation is crucial for our understanding of the interplay between neurons and glia cells in myelin formation in health and in diseases such as multiple sclerosis and neuropathies.

In the PNS, two main strategies have been employed to address this issue. First, efforts have been made to identify potential master regulators of myelin gene expression in analogy to the transcription factor MyoD in muscle development (reviewed by Borycki and Emerson, 1997). This search has proven to be quite difficult, although during the last years, some candidates have been identified. These will be introduced in chapter 2.3.1 below.

The second approach involves the search for regulatory regions within myelin genes, since cell-type specific transcriptional control mechanisms can be unravelled by the
identification and characterization of cis-acting control elements in genes preferentially expressed in a given cell-type or tissue. A number of myelin genes have been studied with regard to their transcriptional regulation. Sometimes this was done in transgenic mice, more frequently in tissue culture systems by transient transfection and in vitro by DNA binding studies. Each of these methods used in gene regulation studies has its advantages and its limitations. Transgenic approaches, for instance, unequivocally determine the capacity of a regulatory region to drive temporal and cell type-specific expression. Therefore transgenic mice provide an excellent assay system to examine gene regulation, in particular under conditions which require numerous developmental and physiological signals for correct interactions (reviewed by Duchala et al., 1996). This situation is obvious in the intensive axon-glia interactions in PNS development and thus, regulation of myelin genes and their regulators are most accurately characterized in vivo. Such studies have the disadvantage that they are time-consuming and labor-intensive, and hardly suited for an in-depth fine-mapping analysis. By contrast, transient transfections in tissue culture combined with in vitro DNA-binding experiments provide an efficient tool for a rapid molecular dissection of a regulatory region (for example, see Brown and Lemke, 1997). However, they do not always give a clear answer as to whether the region under study is by itself sufficient to elicit the cell type-specific expression of the gene to which it belongs. Furthermore such studies require a system in which the gene of interest is expressed at reasonable levels compared to the expression in vivo (compare also chapter 4.1 of this thesis). Especially when studying myelin genes there are very often limitations due to the fact that Schwann cells do not myelinate in vitro without co-culturing with neurons. Taking these limitations into consideration, a combination of in vivo, tissue culture, and in vitro methods is likely to give the most meaningful results. The region responsible for temporal and tissue-specific expression can first be localized to a larger genomic fragment by transgenic techniques. Well-chosen tissue culture systems and DNA-binding studies can then be used to map the potentially important cis-acting elements within this region. Finally, the importance of these elements should again be confirmed in vivo, for example by deleting the regulatory element by homologous recombination in embryonic stem cells, which may result in the generation of a cell-type specific allele or a cell type-specific null mutant in case of a cell type- specific enhancer (for example see Ghazvini et al., 2002). In the past, some myelin
genes and their regulatory elements have been analyzed in separate studies both in transgenic mice and in tissue culture. Some aspects of the transcription regulation of myelin genes with the focus on those which are expressed during myelination in the PNS are introduced below in chapter 3.3.2. They will give an idea about the variety of potential regulatory mechanisms which might be involved in the transcriptional regulation for \textit{PMP22}.

### 3.3.1 Glial transcription factors and their function

Several transcription factors that exert a pivotal role in Schwann cells have been identified as components of the molecular mechanisms initiating myelination. These include the zinc finger protein Krox20/Egr-2 and the POU protein Oct-6/SCIP/Tst-1/Pou3f1 (referred to Oct-6 in the thesis; reviewed by Zorick and Lemke, 1996; Zorick \textit{et al.}, 1999). In support of this hypothesis, the generation of null mutant mice has revealed important roles for these factors in the process of myelination. In both Krox20 and Oct-6 mutants, Schwann cells appear morphologically arrested at the promyelinating stage (Bermingham \textit{et al.}, 1996; Jaegle \textit{et al.}, 1996; Topilko \textit{et al.}, 1994). Detailed analysis of the Krox20 mutant showed severe defects in Schwann cell development resulting in an hyperproliferation and presumed differentiation arrest at the promyelinating stage (Topilko \textit{et al.}, 1994). Although Oct-6 mutant Schwann cells exhibit a phenotype similar to that of Krox20 mutants during the first week after birth, myelination subsequently resumes (Jaegle \textit{et al.}, 1996). This has led to the suggestion that Oct-6 is involved in the timing of myelination, whereas Krox20 would be integral to the myelination program (Jaegle and Meijer, 1998). Consistent with the latter hypothesis, genome expression profiling studies in Schwann cells revealed that Krox20 regulates multiple genes involved in myelin formation (Nagarajan \textit{et al.}, 2001). These results are reinforced by clinical studies on patients with hypomyelinating neuropathies including congenital neuropathies, Charcot-Marie-Tooth type 1, and Dejerine-Sottas syndrome (compare chapter 3.2). Some of these patients carry dominant or recessive point mutations affecting different domains of the Krox20 protein (Boerkoel \textit{et al.}, 2001; Timmerman \textit{et al.}, 1999; Warner \textit{et al.}, 1998).
Additional transcription factors that may affect late steps in the differentiation of Schwann cells and may be involved in myelin gene regulation include Pax-3, c-Jun, Krox24/Egr-1 and Sox10 (reviewed by Scherer, 1997; Wegner, 2000a). Sox10 has been identified as a common transcriptional modulator of Oct-6, Krox20 and Pax3 potentially conferring cell specificity to interacting transcription factors in developing and mature glia (Kuhlbrodt et al., 1998). Indeed, recent results suggest a crucial role of Sox10 in peripheral glia development (Britsch et al., 2001; Paratore et al., 2001) and regulation of the major PNS myelin protein MPZ by Sox10 has been demonstrated (Peirano et al., 2000) (compare chapter 4.3).

Recently, it was shown that the POU domain transcription factor Brn-5/Pou6f1 has a developmental expression pattern inverse to that of Oct-6, with Brn-5 stably expressed in the adult myelinating Schwann cell, but virtually absent during promyelination (Wu et al., 2001). Beside Brn-5, the discovery that another POU domain transcription factor Brn-2/Pou3f2 is not restricted to CNS remyelination, but is expressed in a pattern similar to that exhibited by Oct-6 during Schwann cell myelination of neonatal nerves and during regeneration of crushed adult nerve, made this transcription factor to an additional candidate for the regulation of myelin genes (Sim et al., 2002).

Besides the Schwann cell-specific transcription factors mentioned above binding sites have been also identified for various groups of transcription factors such as AP-1, CREB, STAT, NF-κB or several members of the nuclear receptor family, which are known to translate extracellular signals used by many different cell-types into changes of gene expression. These are well-described components of trans-acting factors in the transcription machinery of myelinating glia and may also take part in the regulation of myelin gene expression in the PNS (reviewed by Wegner, 2000a).

### 3.3.2 Transcriptional control elements in myelin genes

Much biological regulation takes place at the level of transcription initiation, where an assortment of varied ‘enhancer’ and ‘silencer’ sequences serve as docking sites for transcriptional activators and repressors, the precise combination of which controls gene
expression. Although inspection of myelin gene sequences has suggested the presence of many transcription factor binding sites, so far only a few transcription factors have been documented to bind to the promoter regions of myelin genes *in vitro* or *in vivo*. Nevertheless, the focus of the following chapter is on myelin gene transcription in the PNS, in particular on the structure and regulation of myelin gene promoters. An overview of regulation of glial transcription with the corresponding references can be found in Tab 1. The topic is reviewed in more detail in (Wegner, 2000a, b).

**Myelin Protein Zero (MPZ/P0)**

One of the best-analyzed myelin genes is the myelin protein zero gene (MPZ/P0), which encodes a tetramer-forming 31-kilodalton (kDa) transmembrane glycoprotein of the immunoglobulin superfamily with specific expression in the Schwann cell lineage. MPZ expression starts early in these cells, possibly in neural crest cells already committed to a glial fate (Hagedorn *et al.*, 1999; Lee *et al.*, 1997). It is massively upregulated in Schwann cells during the onset of myelination. MPZ is a major myelin component accounting for more than 50% of the protein in PNS myelin. It is actively involved in stabilizing the myelin sheath by binding to other MPZ molecules in opposing membranes via homophilic interactions (for review see Mirsky and Jessen, 1999; Scherer, 1997).

Experiments on the 5’ flanking region of the rat MPZ gene have shown, both in culture and in transgenic mice, that 1.1 kilobases (1kb) upstream of the transcription start site are sufficient to drive expression in Schwann cells (Lemke *et al.*, 1988; Messing *et al.*, 1992). However, transgene expression with this construct in mice was variable between single Schwann cells and there was evidence of ectopic expression. Consistently, another group could not express lacZ under control of a similar small pieces of the MPZ promoter specifically in Schwann cells. A more consistent expression was obtained using the complete mouse MPZ gene, including 6kb of 5’ flanking sequences plus all exons and introns (Feltri *et al.*, 1999). Nevertheless, the 1.1kb part of the MPZ promoter is capable of recapitulating some essential features of MPZ expression, including low-level expression during early phases of PNS development and increased expression in myelinating Schwann cells. Mapping studies in Schwann cell cultures have been used to dissect this 5’ flanking region into functional domains (Brown and Lemke, 1997). These
include (1) a core promoter, which has little activity on its own and which spans positions -100 to +45 relative to the transcription start site (defined as +1); (2) a potently activating proximal promoter region from position -350 to -100, which exhibits high sequence conservation among rat, mouse and humans, and which is responsible for greater than 50% of the activity of the MPZ promoter in Schwann cell cultures; and (3) an accessory distal region from positions -910 to -315 which makes up for the rest of the activity (Table 1).

Similarly structured promoters are also found in other myelin genes, for instances the P2 gene (Bharucha et al., 1993) and the myelin-associated glycoprotein gene (MAG) (Grubinska et al., 1994; Laszkiewicz et al., 1997; Ye et al., 1994) (Table 1).

DNase footprinting of the MPZ promoter revealed the presence of numerous binding sites in all three parts (Brown and Lemke, 1997). Some binding sites were occupied by proteins from glial and nonglial cells, while others showed differential protection in one cell type only. Most of the identified cis-acting elements in these experiments do not correspond to known transcription factor binding sites, with the exception of a GC rich binding site and CAAT boxes in the core promoter region. The GC-rich binding site is recognized by Sp1 family proteins, and the CAAT boxes are recognized by NF-Y (Brown and Lemke, 1997). Both CAAT boxes and GC-rich binding sites are found in a number of other glial promoters (Table 1), including that of Oct-6 (Kuhn et al., 1991) and Promoter 1 (see section below) of PMP22 (Suter et al., 1994).

*Myelin Basic Protein (MBP)*

Myelin basic proteins (MBP) occur both in myelinating Schwann cells and in oligodendrocytes as multiple isoforms transcribed from the same gene. MBP are highly charged proteins with high affinity for membranes and mediate the close apposition of myelin membranes on their cytoplasmic sides. MBP makes up 3% of total myelin in the PNS and 30% of total myelin protein in the CNS. The MBP gene is part of the larger Golli-mbp gene, which is conserved between humans and rodents (Campagnoni et al., 1993; Pribyl et al., 1993). Numerous transgene experiments have been carried out with 5’flanking regions, employing varying length ranging from 256bp to 6.5kb taken from mouse or human MBP gene (reviewed by Ikenaka and Kagawa, 1995). Interestingly, all the transgenes exhibited expression in cells of the oligodendrocyte lineage, although the
penetrance of transgene expression was lower for example in the transgenes containing only 256bp (Goujet-Zalc et al., 1993). None showed expression in Schwann cells. Nevertheless, this short proximal promoter region seems to contain the necessary information for oligodendrocyte-specific expression with further upstream sequences amplifying the effect. Expression of the transgenes comes on in the oligodendrocyte lineage with the developmental profile typical of MBP. Most transgenes, however, exhibit a decline in expression after peaking during the active myelination period, and do not show the strong maintained level of expression seen for MBP during adulthood.

Comparable observations were also made with a transgene under the control of the immediate 5’ flanking region of the CNP gene where the correct temporal expression of the transgene was obtained only in oligodendrocytes (Gravel et al., 1998) (Table 1). Together with the MBP transgenes, it was proposed that differential regulatory elements may exist for oligodendrocyte-specific versus Schwann cell-specific expression and for early versus late expression of myelin genes.

Indeed, a recent in vivo promoter study identified DNA elements responsible for MBP expression in the PNS (Forghani et al., 2001). Evidence was provided for the participation of multiple, widely distributed, positive and negative elements in the overall control of MBP expression. Especially, all constructs bearing a 0.6 kb far-upstream sequence, designated Schwann cell enhancer 1 (SCE1), expressed at high levels in myelin-forming Schwann cells. In addition the 0.6 kb SCE1 alone shows robust targeting activity independent of other MBP 5’ flanking sequence.

The MBP promoter has also been extensively characterized in transient transfections and in vitro using transcriptionally competent brain extracts. Taken together these studies agreed with the in vivo analysis in that a comparatively short region preceding the transcription start site was found to contain the information necessary for oligodendrocyte-specific expression (Table 1). Using various methods to detect protein-DNA interactions in vitro, this region was shown to contain numerous sites capable of binding both ubiquitous and cell type-specific nuclear proteins (e.g. Devine-Beach et al., 1990). Most of these binding activities are still poorly characterized at the molecular level. However, it has been shown that this proximal promoter region contains response elements for the thyroid hormone receptor (TR) (Farsetti et al., 1992; 1991) and for members of the NF-I and Sp1 family of transcription factors (Aoyama et al., 1990;
Tamura et al., 1988) (Table 1). The latter two transcription factors are fairly ubiquitous proteins and are generally believed to contribute to basal rather than to cell type-dependent expression. Nevertheless, also an ubiquitous transcription factor like NF-I may contribute to oligodendrocyte specificity by additional protein-protein and DNA-protein interactions around the binding site of NF-I. Indeed, there are indications that the NF-I site in the MBP promoter is part of a larger composite regulatory element, which is flanked in the case of the human promoter on its 5’ site by a site for a not further characterized activity called MEBA (Taveggia et al., 1998). In the mouse promoter the 3’ flanking region is assumed to mediate binding of an activity called M1 (Aoyama et al., 1990). Thus, one might speculate that such an NF-I centered nucleoprotein complex could be an important determinant in creating the oligodendrocyte specificity of MBP gene expression, without NF-I itself being oligodendrocyte specific.

**TABLE 1: Regulatory regions of genes expressed in myelinating glia**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression</th>
<th>Regulatory regions</th>
<th>Binding sites</th>
<th>Transgene (expression)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>PNS</td>
<td>Core: - 100 to + 45 Proc: - 350 to - 100 Dist: - 915 to - 350</td>
<td>TATA, CAAT, GC, Sox10, other fp</td>
<td>1.1 kb 5’ flank (SC)</td>
<td>Lemke et al., 1988; Messing et al., 1992; Brown et al., 1997</td>
</tr>
<tr>
<td>P2</td>
<td>PNS</td>
<td>Core: - 293 to + 125 Proc: - 435 to - 293 Dist: - 870 to - 435</td>
<td>TATA, CAAT</td>
<td>–</td>
<td>Bharucha et al., 1993</td>
</tr>
<tr>
<td>PMP-22</td>
<td>ubiq.</td>
<td>P1: - 298 to + 172 P2: - 352 to + 144</td>
<td>NFI, CAAT, TATA GC</td>
<td>10 kb 5’ flanking (myelinating SC)</td>
<td>Suter et al., 1994; Maier et al., 2002</td>
</tr>
<tr>
<td>L1</td>
<td>PNS, CNS</td>
<td>Core: - 150 to + 118 Enhancer: int 1 Silence: int 2</td>
<td>NFI, GC, K-Pax homeoproteins NRSF</td>
<td>2.9 kb 5’ flank ex 1a, int 1,3 (CNS, PNS)</td>
<td>Kallunki et al., 1997, 1998; Meech et al., 1999</td>
</tr>
<tr>
<td>MBP</td>
<td>CNS, PNS</td>
<td>Core: - 36 to + 12 Proc: - 256 to - 36 FynRE: - 675 to - 647</td>
<td>TATA, GC, NFI, MEBA, M1, TR, other FP STAT, C/EBP</td>
<td>6.5±0.3 kb 59 flank (OL, not SC)</td>
<td>e.g., Goujet-Zalc et al., 1993; Tamura et al., 1990; Unemori et al., 1999</td>
</tr>
<tr>
<td>PLP</td>
<td>CNS, (PNS)</td>
<td>Core: - 186 to 1 87 Promoter: - 1038 to - 87 Enhancer: int 1</td>
<td>TATA, CAAT, MyT1, other fp</td>
<td>human: 4.2 kb 59 and 1.5 kb 3’ flank; mouse: 2.4 kb 5’ flank, ex. 1, 12, int 1 (OL)</td>
<td>Nave and Lemke, 1991; Berndt et al., 1992; Nadon et al., 1994; Wight et al., 1993, 1997</td>
</tr>
<tr>
<td>CNP</td>
<td>CNS, PNS</td>
<td>P1 P2</td>
<td>TATA TATA</td>
<td>4·kb 5’ flank (OL, early SC)</td>
<td>Gravel et al., 1998</td>
</tr>
<tr>
<td>MAG</td>
<td>CNS, PNS</td>
<td>Core: - 138 to 1 21 Proc: - 583 to - 138 Dist: - 861 to - 583</td>
<td>GC, AP2</td>
<td>–</td>
<td>Ye et al., 1994; Grubinska et al., 1994</td>
</tr>
<tr>
<td>Krox-20</td>
<td>PNS</td>
<td></td>
<td>SRE, CARG-1, CRE, AP-1, SP-1</td>
<td>Immature SC element (immature SC, 5’ flank) Myelinating SC element (myelinating SC, 3’ flank)</td>
<td>review: Beckmann et al., 1997; Ghislain et al., 2002</td>
</tr>
<tr>
<td>Oct-6/Tst-1</td>
<td>PNS, CNS</td>
<td>Core: - 93 to + 49 Enhancer: - 5kb</td>
<td>TATA, CAAT, GC AP-1, ER</td>
<td>SC Enhancer, about 5kb 3’ flank</td>
<td>Kuhn et al., 1991; Renner et al., 1996a Mandemakers et al., 2000 Ghazvini et al., 2002</td>
</tr>
</tbody>
</table>

ubiq., ubiquitous expression; core, core promoter; prox, proximal promoter; dist, distal promoter; fp, footprinted region; SC, Schwann cell; OL, oligodendrocyte; ex, exon; int, intron.

(adapted with modifications from Wegner, 2000a)
**Proteolipid Protein (PLP)**

The promoter topology of the gene encoding the proteolipid protein (PLP) is quite different from those described so far. PLP is a highly conserved myelin protein with four transmembrane domains, and functions in oligodendrocyte development, myelin compaction, and axonal integrity. It is expressed as the two splice-variants, PLP and DM-20, which exhibit different developmental expression profiles and display non-redundant functions (Nadon *et al.*, 1994, and references therein). The major splice variant PLP makes up 40% of total myelin protein in the CNS. PLP is also expressed in Schwann cells, albeit at much lower levels (e.g. Garbern *et al.*, 1997).

For the rat, mouse and human PLP promoters the proximal 186, 145, and 204 base pairs (bp), respectively, have been described as sufficient to generate basal promoter activity in transiently transfected cells (Berndt *et al.*, 1992; Nave and Lemke, 1991; Wight *et al.*, 1993). This region appears to contain multiple start sites that are differentially used in oligodendrocytes versus Schwann cells (Kamholz *et al.*, 1992; Scherer *et al.*, 1992). This region as well as the sequences immediately upstream contain a number of protein binding sites, as judged by DNase footprinting experiments and electrophoretic mobility shift assays (EMSA) (Berndt *et al.*, 1992; Nave and Lemke, 1991). However, even 1kb immediately upstream of the transcription start site is not sufficient to direct oligodendrocyte-specific expression of a transgene (Nadon *et al.*, 1994). Instead, 4.2kb of 5’ flanking sequence from the human PLP gene were needed in combination with 1.5kb of 3’ flanking sequence to obtain oligodendrocyte-specific expression in transgenic animals. Several additional transgenes with oligodendrocyte-specific expression were generated using different regions from the mouse PLP gene, leading also to PNS specific expression (reviewed by Ikenaka and Kagawa, 1995; Kagawa *et al.*, 1994; Readhead *et al.*, 1994; Wight *et al.*, 1993). One conclusion to draw from the sum of transgenic experiments is to postulate the presence of multiple oligodendrocyte-specific regulatory elements in the PLP gene, one in the 5’ flanking region and the other in intron 1 - at least in the mouse sequence.

To assume the presence of regulatory element necessary for oligodendrocyte-specific expression within intron 1 is not unreasonable, as important regulatory elements have been found in intronic sequences of many genes. In the case of L1, for instance, regulatory elements have been found both in intron 1 and in intron 2, each responsible
for a specific expression pattern (Kallunki et al., 1998; 1997; Meech et al., 1999). The L1 gene codes for an integral membrane protein of the immunoglobulin superfamily, modulates neuron-neuron and neuron-glia interactions, and exhibits strong expression in the PNS during embryonic development and later in non-myelinating Schwann cells.

Oct-6/SCIP

In the case of the Oct-6 gene regulatory elements were mapped as DNase I-hypersensitive sites (HSS). A combination of two of the HSS was shown to act as Oct-6 Schwann cell-specific enhancer (SCE) and were located very distally to the gene (Mandemakers et al., 2000). The SCE is sufficient to drive spatially and temporally correct expression of Oct-6, during both normal peripheral nerve development and regeneration. Because Oct-6 expression is under the control of axonal signals during nerve development and regeneration, this SCE provides a cis-acting genetic element that responds to converging signalling pathways to drive myelination in the PNS. Consequently in a recent study an Oct-6 allele with reduced expression in Schwann cells was generated through deletion of the SCE in the Oct-6 locus (Ghazvini et al., 2002). Indeed, the analysis of mice homozygous for this allele reveals that rate-limiting levels of Oct-6 in Schwann cells are dependent on the SCE and that this element does not contribute detectably to Oct-6 regulation in other cell types.

Krox20/Egr-2

Analysis of cis-acting elements governing Krox20 expression with transgenes in Schwann cells revealed the existence of two separate elements (Ghislain et al., 2002). The first, designated immature Schwann cell element (ISE), was active in immature but not myelinating SC, whereas the second, designated myelinating Schwann cell element (MSE), was active from the onset of myelination through adulthood in myelinating SC. In vivo sciatic nerve regeneration experiments demonstrated that both elements were activated during this process, in an axon-dependent manner. Together the activity of these elements reproduced the profile of Krox20 expression during development and regeneration. The MSE was localised to a 1.3 kb fragment 35 kb downstream of Krox20. The identification of multiple Oct-6 binding sites within this fragment suggested that Oct-6 directly controls Krox20 transcription. The ISE was located in the -31/-4.5kb
region of the Krox20 gene. A series of four different overlapping fragments from this region were tested in mouse transgenesis by fusion to a β-globin minimal promoter/lacZ reporter. Although regulatory sequences controlling Krox20 in the nerve roots were identified, none of the constructs showed expression distally in the peripheral nerves. Taken together, these data indicate that, although Krox20 is expressed continuously from 15.5 dpc in Schwann cells, the regulation of its expression is a biphasic, axon-dependent process involving two cis-acting elements that act in succession during development. In addition, they provide insight into the complexity of the transcription factor regulatory network controlling myelination.

In vitro studies showed that the Krox-20 promoter contains two sequences similar to serum response elements, with an CArG-box as an inner core element (CArG-1 and CArG-2). CArG-1 is responsible for the serum and AP-1 responsiveness of Krox-20 via PKC-dependent and -independent pathways, respectively (reviewed by Beckmann and Wilce, 1997).

Peripheral Myelin Protein 22 (PMP22)
The 22 kDa hydrophobic glycoprotein PMP22 is predominantly expressed by myelinating Schwann cells in peripheral nerves, where the PMP22 protein is incorporated into compact myelin (Haney et al., 1996; Snipes et al., 1992; Welcher et al., 1991). It accounts for 2-5% of the total protein found in isolated myelin preparations (Pareek et al., 1993). The PMP22 molecule consists of an 18kDa polypeptide core, as predicted from its primary amino acid sequence, and of carbohydrates, which are linked to an asparagine residue. Some of the PMP22-bound glycosyl moieties carry the L2/HNK-1 carbohydrate epitope, which has been implicated in intercellular recognition and adhesion processes (Snipes et al., 1993).

Although PMP22 is found mainly in Schwann cells and seems to be required for correct development of peripheral nerves, the maintenance of axons and the determination of myelin thickness and stability (Adlkofer et al., 1997a; 1995), it is not myelin-specific. Hence a more general role of PMP22 in cell biology has been proposed. Indeed, the cDNA encoding PMP22 was first isolated from NIH3T3 fibroblasts as a growth arrest-specific gene (gas-3) (Manfioletti et al., 1990; Schneider et al., 1988). The function of PMP22 is unknown, but results from in vitro studies suggest that exogenously altered
PMP22 expression affects proliferation, cell shape, and spreading, as well as apoptotic cell death of Schwann cells (Brancolini et al., 1999; 2000; Fabbretti et al., 1995; Zoidl et al., 1995). On the other hand, studies of certain PMP22 mutations with an aberrant intracellular trafficking (Naef and Suter, 1999; 1998) and association of PMP22 with Calnexin (Dickson et al., 2002) propose a role of PMP22 in membrane organization in the mechanism of myelin formation.

In the rat, two different PMP22 cDNAs, SR13 and CD25, have been isolated (Spreyer et al., 1991; Welcher et al., 1991), and meanwhile analogous transcripts been described in humans and mice (Suter et al., 1994; van de Wetering et al., 1999). These transcripts consist of different 5’ untranslated regions, resulting from two alternatively transcribed exons (Suter et al., 1994). The mapping of separate transcription start sites to each of these exons indicated that PMP22 expression is regulated by two alternatively used promoters. The relative expression of the alternative PMP22-transcripts is tissue specific, and high levels of the exon 1A-containing transcript (CD25, 1A-PMP22) are coupled to myelin formation. In contrast, transcripts containing exon 1B (SR13, 1B-PMP22) are also present in non-neural tissues such as the lung, intestine, heart and skeletal muscle (Suter et al., 1994). In addition, PMP22 transcripts were localized by in situ hybridization in motor nuclei of certain cranial nerves in the brainstem and in the motoneurons of spinal nerves localized in the ventral horn of the spinal cord (Parmantier et al., 1995). During embryonic development PMP22 mRNA was also detected in a variety of murine tissues (Baechner et al., 1995; Parmantier et al., 1997). Beside the strong expression found in Schwann cells especially during myelination, immunoreactivity for PMP22 has been described in dorsal root ganglia (DRG) neurons, satellite cells, and in the spinal cord predominantly in the dorsal horn (De Leon et al., 1994).

Recent studies characterized the transcriptional startpoints and methylation pattern in the PMP22 promoters in tumour cell lines and in the peripheral nerve. They proposed multiple transcriptional start points for exon 1B and proposed a third promoter in front of exon 2, which seems to be active in the osteosarcoma and glioblastoma cell lines RH30 and SF763 (Huehne and Rautenstrauss, 2001; Huhne et al., 1999).

For PMP22, so far only initial in vitro studies have been performed on the human 5’ regulatory region, resulting in identification of a cAMP sensitive silencer element.
between -0.3kb and -3.5kb relative to the transcription start site of promoter 1 (Saberan-Djoneidi et al., 2000) in the mouse Schwann cell line MSC80 (Boutry et al., 1992). In the RT4-D6P2T schwannoma cell line a positive regulatory element (-105 to -43bp) was described immediately upstream of the minimal promoter 1. This element forms a DNA-protein complex in vitro (Hai et al., 2001).

3.4 Aim and motivation of the work

To improve our understanding of the mechanisms involved in CMT1A and HNPP and to determine the molecular and cellular function of the dosage-sensitive gene PMP22, it is important to determine the role of PMP22 transcriptional regulation during development and in pathogenesis of the peripheral nervous system. Deciphering the molecular basis of this regulation is crucial for our understanding of the interplay between neurons and glial cells in myelin formation in health and in diseases like multiple sclerosis and neuropathies. Furthermore, such studies may provide the rational basis for potential gene therapeutic approaches to normalize PMP22 expression in CMT1A (Hai et al., 2001) and will provide additional insights into the coordinate regulation of myelin genes.

To this end, I started with an additional characterisation of the PMP22 promoter in vitro (see chapter 4 of the thesis). Second, I performed an extensive and systematic dissection of the regulatory regions of PMP22 in transgenic mice as presented in chapter 5 and 6 of the thesis. Finally, the results will be compared with a computer analysis of conserved sequences and potential binding sites as presented in chapter 7.
4 MYELIN GENE REGULATION IN VITRO

4.1 Aims and Experimental strategies

The following chapter describes the validation of different cell culture systems to study PMP22 transcriptional regulation in cell culture. As discussed earlier, a cell culture system does focus more on the cell intrinsic mechanism of a certain cell type, and therefore neglects the fact that developing or myelinating Schwann cells integrate a complex pattern of extracellular signals in addition to their cell intrinsic mechanisms in the in vivo situation, which then finally results in their coordinate regulation of genes. On the other hand a well-chosen cell culture system may allow, for example, a rapid molecular dissection of a regulatory region, the determination of DNA-binding factors, or the study of certain intracellular pathways by specific activation or inhibition. It is possible to study certain mechanisms in ways that are either not possible with the available techniques so far in vivo or are very time consuming. However, the results obtained in a cell culture system should be confirmed in vivo whenever possible. Consequently, our aim was to find a system in which some aspects of PMP22 gene regulation could be studied and modulated in a fast and reliable way.

A classical and frequently used way to characterize and identify negative and positive regulatory elements in promoter regions is to transiently transfecct cell lines with reporter constructs harbouring different fragments of the regulatory region of the gene. For PMP22, initial experiments, performed with constructs specific for promoter 1 or promoter 2 driven expression of PMP22, already indicated that promoter 2 of PMP22 shows stronger transcriptional activity in cultured Schwann cells than does the myelin associated promoter 1 (Suter et al., 1994). To gain more insight into PMP22 transcriptional regulation in cultured Schwann cells, which correspond, based on their molecular markers, to non-myelinating Schwann cells, we performed a more extensive promoter deletion study, similar in design to previous studies of other myelin gene promoters such as the Myelin basic protein (MBP) (Li et al., 1994; Miura et al., 1989; Tamura et al., 1989), myelin protein zero (MPZ) (Brown and Lemke, 1997; Lemke et al., 1988) or proteolipid protein (PLP) promoters (Berndt et al., 1992; Nave and Lemke,
The results obtained from such studies depend – among other things – on the cell type in which the transfection studies are performed. In principle two different strategies can be followed to select a cell type. Either one uses a non-specific cell line where only basal promoter activity can be expected for the gene of interest, and subsequently tries to modulate this basal activity by the cotransfection of additional regulatory factors. The second approach is to choose a cell line which resembles the cell type in which the gene of interest is as highly expressed as possible. With this choice one anticipates that additional endogenous factors from the cell line contribute to the complex transcriptional regulation of the gene. We decided to use the second approach, using the mouse Schwann cell line MSC80 (Boutry et al., 1992) which does express PMP22. As a second cell line we selected NIH3T3 fibroblasts, since previous studies characterized PMP22 regulation in this cell type (Fabbretti et al., 1995; Manfioletti et al., 1990; Zoidl et al., 1997). The results of this study are presented below in section 4.2.

In the case of MPZ a successful identification of regulatory factors was possible in cell culture either in cotransfection assays (Monuki et al., 1990; Zorick et al., 1999) or with doxycycline-inducible expression of transcription factors in N2A cells (Peirano et al., 2000). The latter study revealed a clear induction of MPZ expression specifically by Sox10. We wondered whether we could find coregulation of PMP22 in this system. The Sox10 induction can be performed in cell culture media with very low level of fetal calf serum (FCS), an agent known from earlier studies to prevent upregulation of PMP22 when present at high concentrations (Suter et al., 1994). Similarly PMP22/gas3 is upregulated upon growth arrest of the cells which can be induced by serum withdrawal (Manfioletti et al., 1990; Schneider et al., 1988). In a first attempt we tried classical cotransfection experiments with Krox20, Sox10, pax3, Oct-6/SCIP, Ski, and combinations of these, together with a PM22-lacZ reporter construct (-10/0kb PMP22 lacZ, see Fig. 4-1). In the presence of 10% FCS, reporter gene expression was either not influenced or inhibited by these factors. This corresponds to observations that others have made in cell culture (e.g. Bharucha et al., 1994; Monuki et al., 1993). In the absence of serum we faced the problem that Schwann cells are not efficiently transfectable, as they stop proliferating after serum withdrawal. Therefore this system
was not reliable, and the positive results obtained in certain experiments were not reproducible (data not shown).

As a next system we analysed the regulation of \textit{PMP22} in N2A cells stably transfected with a construct allowing induction of Sox 10 expression by doxycycline, as established by Peirano and coworkers (2000). The results obtained in collaboration with this group are presented in part 4.3 below.

While the \textit{in vivo} experiments presented in chapters 5 and 6 were in progress, Nagarajan and coworkers found a way to circumvent the low transfection efficiency in non-proliferating Schwann cells in the absence of serum. They used an adenoviral system to overexpress Krox20 in rat and mouse Schwann cells and could indeed observe upregulation of different myelin genes including PMP22. Nagarajan \textit{et al.}, also presented a list of Krox20-regulated genes identified in a whole genome expression study (Nagarajan \textit{et al.}, 2001). We established this \textit{in vitro} system in which \textit{PMP22} can be upregulated directly by a transcription factor, and could reproduce the upregulation of myelin genes by Krox20. In addition we confirmed our assumption that upregulation of \textit{PMP22} is reduced in the presence of serum. With this system, we started to test Krox20-regulated transcription factors, based in part on the published gene chip data (Nagarajan \textit{et al.}, 2001) including other candidates from other gene profiling experiments (Araki \textit{et al.}, 2001). The results, which still have preliminary character but for some candidates are very promising for future studies, are presented in section 4.4 below.

4.2 Promoter Deletion study in MSC80 and NIH 3T3 cells

As a first step, we generated the basic PMP22 promoter construct (-10/0kb \textit{PMP22 lacZ}) using the \textit{sh ble}-\textit{lacZ} fusion gene driven by promoter 1, promoter 2 and upstream sequences of the mouse \textit{PMP22} gene. Ten kb of 5’-flanking \textit{PMP22} DNA were used, comprising 5.8 kb upstream of exon 1A including promoter 1, 2.3 kb between exon1A and exon 1B including promoter 2, and 1.5 kb downstream of exon 1B with the first 43 bp of exon 2 (see Fig. 4-1). In a next step we generated additional reporter constructs harbouring a 5’ deletion of the first 6kb region (-4/0 kb Pro2 \textit{lacZ}), or the first 7kb region (-3/0 kb Pro2 \textit{lacZ}) leading to a reporter construct containing only promoter 2 with 1kb
or 2kb sequences upstream of exon 1B, respectively (see Fig. 6-1). The deletion of exon 1A or the exon 1B with the associated promoter resulted in the constructs Del1AlacZ and Del1BlacZ (see Fig. 4-1). In the Del1AlacZ construct we deleted the -230 bp to +330 bp and in the Del1BlacZ construct the -170 bp to +410 bp region in relation to the transcription start site as mapped by Suter, et al. (1994). All these construct were transfected in the mouse Schwann cell line MSC80 (Boutry et al., 1992) and in NIH 3T3 cells. We used equimolar amount of each reporter construct and corrected for different transfection efficiencies by cotransfection of a constant amount of SV40 luciferase plasmid. The measured β-gal activities are shown in relation to the SV40-lacZ expression (set to a 100% in both cell lines; Fig. 4-1).

The mouse Schwann cell line (MSC80) is one of the few Schwann cell lines derived from mice. It was established from purified mouse Schwann cell cultures using high levels of fetal calf serum. Most of the MSC80 cells are bipolar or stellate (3-5 processes) in shape, and express antigens of Schwann cells such as S-100 and laminin but also the non-myelinating Schwann cell antigen GFAP. In vivo after transplantation in or at a distance from a lysolecithin-induced lesion, MSC80 cells form myelin around the demyelinated host axons (Boutry et al., 1992).

As a general finding, higher expression levels were seen in the MSC80 cell line compared to the NIH3T3 fibroblasts. Highest relative reporter gene activity, with levels about two fold above the SV40 promoter, can be found with the -10/0kb PMP22 lacZ construct in MSC80 cells in the presence of forskolin. Forskolin increases the intracellular levels of cAMP and is thought to mimic some aspects of axonal contact of Schwann cells. In our experiment, forskolin increased PMP22 promoted reporter gene expression about twofold in MSC80 cells, whereas in NIH 3T3 cells no consistent effect of forskolin was observed.

The observation that deletion of promoter 2 and exon1B (construct Del1BlacZ, Fig. 4-1) led to a complete loss of expression and that the deletion of exon 1A and its associated promoter 1 (construct Del1AlacZ, Fig. 4-1) did not change expression levels substantially, shows that mainly promoter 2 is active in the MSC80 cell line. The same was observed in NIH 3T3 cells. Indeed, quantitative RT-PCR with RNA from MSC80 cells transfected with the -10/0kb PMP22 lacZ construct, using primers specific for exons 1A or 1B and either the reporter or the endogenous gene, confirmed that promoter
Figure 4-1: Activity of different PMP22-promoted reporter constructs in the mouse Schwann cell line MSC80 (grey bars) and in NIH3T3 fibroblast cells (black bars) (a), and mRNA expression levels of transfected lacZ constructs and endogenous PMP22 (b). (a) The relative expression of the lacZ reporter gene obtained 40 hours after transfection is shown for each construct in the presence (+) or absence (-) of 20µM forskolin. The expression levels of the SV40 promoter in the absence of forskolin were defined as 100%. (b) mRNA expression levels of 1A-lacZ and 1B-lacZ transcripts derived from the transfected -10/0kb PMP22 lacZ construct and the 1A-PMP22 and 1B-PMP22 transcripts of the endogenous PMP22 promoters in MSC80 cells, determined by quantitative RT-PCR (see Fig. 5-1). The cells were maintained in DMEM containing 10%FCS. Error bars represent the SD of values obtained from three independent transfections.
2 is about 100 times more active than promoter 1, both for the transfected and the endogenous gene (Fig. 4-1b, for details of the quantitative RT-PCR see Fig. 5-1 below). The differential promoter activities also did not change considerably in MSC80 cells upon addition of 10µM forskolin to the cell culture medium (data not shown). This is in contrast to the situation in the sciatic nerve, where about four times more 1A-PMP22 than 1B-PMP22 mRNA was detected (compare Fig. 5-6). This supports the hypothesis that promoter 1 is the myelin-associated promoter and therefore only basal levels of 1A-PMP22 mRNA are detected in non-myelinating Schwann cells. In other words: the myelin specific promoter 1 is active only at very basal levels in cultured Schwann cells.

4.3 Is PMP22 regulated by Sox10 in N2a cells?

Peirano et al., (2000) showed that MPZ can be upregulated by Sox10 in N2A Neuroblastoma cells. We therefore asked whether PMP22 might be similarly regulated. In collaboration with the group of M. Wegner (Erlangen), N2A neuroblastoma cells expressing the reverse tetracycline-controlled transactivator (rtTA) and the cDNA of Sox10 under control of a tetracycline-regulatable promoter were induced with doxycycline or vehicle alone in DMEM Medium containing 0.5% FCS for 48 hours before harvesting the RNA. A high induction of MPZ upon doxycycline induced expression of Sox10 (+ Dox) compared to non-induced control (- Dox) can be observed (Fig. 4-2a,b; Peirano et al., 2000; for technical review see Mansuy and Suter, 2000). In contrast to MPZ neither a substantial upregulation of the very low levels of the 1A-PMP22 mRNA derived from promoter 1 (Fig. 4-2b), nor of the 1B-PMP22 mRNA derived from promoter 2 (Fig. 4-2c) can be detected by quantitative RT-PCR. As a control, the total amount of PMP22 mRNA was determined with a different set of TaqMan Primers specific for the translated region and detecting all PMP22 mRNA species. Also in this case total levels of PMP22 mRNA did not show any upregulation, as expected since the total PMP22 mRNA consists again mainly of 1B-PMP22 transcripts. In fact, the opposite was the case: a weak inhibition of PMP22 expression was observed.
(Fig. 4-2e). As a conclusion, PMP22 seems not to be co-regulated with the myelin protein zero under these conditions.

Figure 4-2: mRNA expression levels of Sox10 (a), Myelin Protein Zero (b, MPZ) and PMP22 (c, d, e) with (+Dox) or without (-Dox) doxycycline induction of Sox10 in N2a cells, detected with semiquantitative (a,b) or quantitative (c-e) RT-PCR.

4.4 Screening for transcription factors upregulated upon Krox20 induced myelin gene expression

Schwann cells are well infectable with relatively low titres of adenovirus. In contrast to retrovirus, adenovirus can also infect non-proliferating cells (Fig. 4-3a-c). Therefore infection with adenoviral vectors offers a very efficient and reliable way to express, for example, a transcription factor in nonproliferating Schwann cells. Nagarajan and coworkers (2001) exploited this fact and studied changes in gene expression after infection of rat Schwann cell with an adenovirus expressing Krox20 and eGFP under control of a CMV promoter (Ehrengruber et al., 2000). They used microarrays in a global analysis of gene expression in rat Schwann cells infected with an adenovirus expressing Krox20, as compared to control-virus infected Schwann cells (Nagarajan et al., 2001). Our hypothesis was that transcription factors regulated by Krox20 are directly or indirectly involved in the regulatory network of myelin gene regulation. Based on the published gene chip data, educated guesses and other gene profiling studies (Araki et al., 2001), we started to confirm the Krox-20 regulation of our candidates. These included the KRAB-zinc finger protein KZF-1 and KZF-1 like ((Bellefroid et al., 1998), accession
Figure 4-3: Quantitative RT-PCR screening for Krox-20 regulated genes in rat Schwann cells upon infection with an adenovirus expressing Krox-20. (a-c) High expression levels of GFP encoded by the viral vector (taken as a surrogate for measuring Krox20 directly) can be detected in nearly all rat Schwann cells infected in DMEM medium in the presence of 10% FCS with a dilution of 1:100 (a) or 1:10 (b) of the Adegr2GFP virus (Ehrengruber et al., 2000) after 48 hours. When the infection was performed in defined N2 medium (c) with the same dilution of the virus (1:10), weaker expression levels were observed, which nevertheless induced the upregulation of PMP22 and periaxin mRNA (d). (d) Differences in mRNA expression levels (fold difference) of Krox20 versus control-virus infected Schwann cells, 30 hours (experiment 1), 24 or 48 hours (experiment 2) after infection, assayed with triplicate measurement of the indicated TaqMan or SYBRGreen PCR (corresponding dissociation curve shown in the last column). Abbreviation: n.a. (ME): not applicated; dissociation curve done by M. Ehrengruber.
number AF175222 and BC004747 for the mouse cDNA), the broadly expressed POU domain transcription factor Brn-2 (Donahue and Reinhart, 1998; Schreiber et al., 1997), the Iron responsive element binding protein (IREBP; accession number X61147), Egr1 and Egr3 to confirm the effect of specific induction of Egr2/Krox20 and the Schwann cell marker S100 as a control which should show unchanged levels (Fig. 4-3d).

In the absence of serum and within 48 hours after infection, we could confirm the upregulation of total PMP22 levels, and an upregulation of the 1B-PMP22 message. The 1A-PMP22 levels were strongly increased (20-80 fold), but it must be emphasized that even the induced levels were still low. We did not observe a robust induction of PMP22 in the presence of serum. At least in experiment 2 we detected a seven-fold induction of Periaxin expression (Fig. 4-3d).

In regard to the screening we could only confirm the two-fold up-regulation of Brn-2, but not of KZF-1, which in the published gene chip analysis was upregulated about five-fold (Nagarajan et al., 2001). No regulation was observed for IREBP. The negative first derivative of the fluorescence versus temperature curve of the PCR products (Fig. 4-3d) shows only a single peak for IREBP, indicating that only one single product was amplified. In the case of KZF-1 a more sensitive PCR might show different results since, the gene was expressed at very low levels in cultured Schwann cells. In contrast to the gene chip data where a five-fold upregulation of Egr1 was detected, we determined in our experiment either unchanged or decreased levels of Egr1 upon Krox20 expression. Expression levels of egr3 seem not to be influenced by Krox20 expression.

In principle, the system can be used to study the regulatory network of PMP22 gene regulation in cell culture, since we are able to induce myelin gene expression in this in vitro system. In addition, in the case of Brn-2 our screening approach revealed a valuable candidate for further studies, as Brn-2 regulation in mice was confirmed in the meantime by (Sim et al., 2002).
5 REGULATORY ELEMENTS ON THE -10/0KB PMP22 LACZ TRANSGENE

5.1 Aims and Experimental strategies

Cultured Schwann cells without coculturing with neurons do not myelinate *in vitro*, so that the Schwann cells do not integrate axonal signals into their transcriptional regulation. Therefore a systematic dissection of the regulatory regions in transgenic mice provides an excellent assay to study gene regulation involving numerous developmental and physiological signals. Consequently, we examine the role of the PMP22 promoters and their 5’ regulatory sequences on the transcriptional regulation of PMP22 *in vivo* which is described in the following section of my thesis. To this end, we produced transgenic mice in which promoters 1 and 2 drive expression of a lacZ reporter gene, either in wildtype mice or in animal models for CMT1A.

Part of the following results have been published in:

5.2 Generation of transgenic mice expressing the reporter lacZ gene under the control of the PMP22 gene promoters (-10/0 kb PMP22 LacZ)

Transgenic mouse lines were generated using the sh ble-lacZ fusion gene driven by promoter 1, promoter 2 and regulatory sequences of the mouse PMP22 gene (Suter et al., 1994). Ten kb of 5'-flanking PMP22 DNA were used including promoter 1 with exon1A, promoter 2 with the corresponding exon 1B and 1.5 kb downstream of exon 1B with the first 43 bp of exon 2 (Fig. 5-1) (Suter et al., 1994; van de Wetering et al., 1999). The sh ble gene confers resistance to the antibiotics of the zeomycin group (Drocourt et al.,

![Diagram of PMP22 Promoter-sh ble-lacZ (-10/0kb PMP22 lacZ) transgenic mice. Diagram of the endogenous mPMP22 genomic locus and the -10/0kb PMP22 lacZ transgene. (a) Promoter 1 is located in front of the non-translated exon 1A, Promoter 2 in front of the alternatively used exon 1B. All numbers refer to the nucleotide +1 which was defined at the translation start codon on exon 2 (ATG). The specific forward and backward primers for the detection of the two endogenous mRNAs, 1A-PMP22 (Pr1A and Pr2PMP22) and 1B-PMP22 are indicated (Pr1B and Pr2PMP22). (b) To generate a reporter transgene under the control of the PMP22 promoters, the endogenous PMP22 sequence was replaced at the translation start site with the coding sequence of a sh ble-lacZ fusion reporter gene. The specific primers for the two transgenic mRNA species 1A-lacZ (Pr1A, Pr2lacZ), 1B-lacZ (Pr1B, Pr2lacZ) are indicated. The PMP22 TaqMan probe (black rectangle with 5' reporter and 3' quencher dye modifications) used for quantification is located in the first half of exon 2 in a sequence common to all four different RNA transcripts.]

Figure 5-1: Generation of PMP22 Promoter-sh ble-lacZ (-10/0kb PMP22 lacZ) transgenic mice. Diagram of the endogenous mPMP22 genomic locus and the -10/0kb PMP22 lacZ transgene. (a) Promoter 1 is located in front of the non-translated exon 1A, Promoter 2 in front of the alternatively used exon 1B. All numbers refer to the nucleotide +1 which was defined at the translation start codon on exon 2 (ATG). The specific forward and backward primers for the detection of the two endogenous mRNAs, 1A-PMP22 (Pr1A and Pr2PMP22) and 1B-PMP22 are indicated (Pr1B and Pr2PMP22). (b) To generate a reporter transgene under the control of the PMP22 promoters, the endogenous PMP22 sequence was replaced at the translation start site with the coding sequence of a sh ble-lacZ fusion reporter gene. The specific primers for the two transgenic mRNA species 1A-lacZ (Pr1A, Pr2lacZ), 1B-lacZ (Pr1B, Pr2lacZ) are indicated. The PMP22 TaqMan probe (black rectangle with 5' reporter and 3' quencher dye modifications) used for quantification is located in the first half of exon 2 in a sequence common to all four different RNA transcripts.
1990; Gatignol et al., 1988) and the addition of the *Escherichia coli lacZ* gene in frame to the *sh ble* sequence allows monitoring of *PMP22* gene expression. DNA extracted from the tails of 85 offspring mice was analyzed by PCR for the presence of the transgene which was detected in 25 animals. Expression of the transgene in innervating peripheral nerves of the tail was analyzed on cross-sections derived from biopsies of the founder animals. Three founders showed high lacZ expression levels and seventeen showed a moderate or weak expression. Five founders were mated for further analysis with B6D2F1 hybrid mice and stable lines with high (lines 48.4, 44.2, 49.3), moderate (line 37.1) or low (line 45.2) expression levels were established. The expression levels were confirmed on whole mount X-gal stainings of sciatic nerve in F1 animals (data not shown) which were then bred again with hybrid mice. Lines 48.4 and 44.2 were analyzed in detail and showed identical patterns of expression of the transgene both in the embryo and adult. With all five lines, transgenic animals were fertile and appeared normal throughout live.

### 5.3 Analysis of the first wave of -10/0kb PMP22 lacZ expression during embryonic development

β-gal expressing cells were first detected at E10.5 in the limb buds of the developing fore- (Fig. 5-2a, arrowhead) and hindlimbs as well as at the midbrain-hindbrain border (Fig. 5-2a, arrow). The expression pattern at E11.5 (data not shown) was similar to that seen at E12.5 (Fig. 5-2b). At E12.5 a prominent staining was detected in the lateral and medial regions of the limbs (Fig. 5-2b, arrowhead). This expression of the -10/0kb PMP22 lacZ transgene is consistent with the finding of endogenous PMP22 RNA by in situ hybridization in the developing limbs (Baechner et al., 1995). Furthermore, we detected β-gal expression in the ventricular epithelium of the rhombencephalon, but neither more rostrally in the myencephalon or prosencephalon, nor more caudally in the alar plate of the spinal cord, either at E12.5 (Fig. 5-2b) or at E14.5 (Fig. 5-2c, d, f). These results are in agreement with the endogenous expression pattern of the *PMP22* gene in the developing CNS as determined by (Parmantier et al., 1997). This is also true for some
weak X-gal staining that we detected in the neuroepithelium of the olfactory bulb and in some tongue muscles (data not shown). The spatial expression pattern at E14.5 (Fig. 5-2d,e) is similar to the pattern at E12.5. On whole-mount X-Gal stainings (Fig. 5-2c) or sections of E14.5 embryos, we detected additional prominent staining in the outer ear and scattered blue cells were found in the skin and in the retina of the eye (data not shown). This finding contrasts to the data of (Baechner et al., 1995) who localized endogenous PMP22 mRNA predominantly to the inner ear and the lens of the eye. How-

Figure 5-2: β-gal expression during embryonic development of -10/0kb PMP22 lacZ transgenic mice. Whole mount X-gal stainings from embryonic day E10.5 (a), E12.5 (b) and E14.5 (c). Histological X-Gal stainings of longitudinal (d) and transverse (e, f) cryosections at E14.5 at the levels of the embryo as indicated in (c). At E10.5 (a) β-gal activity was detected in the outer and inner region of the limbs (arrowhead) and in the region of the midbrain-hindbrain boundary (arrow). At E12.5 (b) and E14.5 (c, d, e, f) β-gal activity could be detected most prominently in the limb muscles (arrowhead in b, d, e), in the roof of the midbrain (f), in the external ear (arrow in c) and in some scattered cells of the olfactory epithelia and epidermis. mv: mesencephalic vesicle. Scale bars: 500µm (a-e), 100µm (f).
ever, the pattern of transgene expression was consistent in our transgenic lines (data not shown). Thus, it is likely that negative regulatory elements that inhibit the expression of the endogenous \textit{PMP22} gene in these areas are missing on our transgene. In accordance with \textit{PMP22} mRNA \textit{in situ} studies (Baechner \textit{et al}., 1995; Parmantier \textit{et al}., 1997) a reduced expression of the transgene was observed in late embryonic development (data not shown). At E16.5 and E18.5 remaining ß-gal expression could be detected in the outer ear and in the limb muscles of -10/0kb \textit{PMP22 lacZ} transgenic animals.

5.4 \textbf{-10/0kb \textit{PMP22 lacZ} expression is strongly upregulated in postnatal neurons and Schwann cells of peripheral nerves}

The second wave of -10/0kb \textit{PMP22 lacZ} transgene expression starts around birth. First ß-gal positive cells in the DRG can be detected at E19.5 on whole mount stainings (data not shown). An increasing number of ß-gal positive DRG neurons can be observed on sections at postnatal day 1 (P1, Fig. 5-3a) and P5 (Fig. 5-3b, inset). At this age, X-gal staining was also observed in the cartilage regions of the bones (Fig. 5-3b, asterisk), as had been observed for \textit{PMP22} mRNA during late embryogenesis (Baechner \textit{et al}., 1995). Transgene expression in the muscles persisted throughout postnatal development, but in contrast to the embryonic expression at E14.5, staining was detectable additionally in muscles outside of the limbs and was more localized around single muscle fibers (Figs. 5-3a, b, arrowhead).

Postnatal day 10 (P10) was the first time at which ß-gal expressing cells were seen in the ventral horn of the spinal cord (data not shown). At P21, the ß-gal activity was more intense than at P10, and on sections through the thoracic and lumbar spinal cord (Fig. 5-3c, d) a diffuse X-gal staining was additionally observed in the dorsal horn of the spinal cord, mainly located in the area of lamina II. Also the dorsal column tracts, especially the gracile fasciculus as well as some large cell bodies in the ventral horn of the gray matter, were positive for ß-gal. These large, multipolar cells have been described to express
endogenous PMP22 mRNA and were identified as motor neurons by (Parmantier et al., 1995).

Figure 5-3: Developmental appearance of β-gal-positive cells in DRGs and spinal cords of -10/0kb PMP22 lacZ transgenic animals. X-Gal stainings of spinal cord cross-sections of P1 (a) and P5 (b) mice. Expression of lacZ starts around birth and is upregulated during the first postnatal week in the DRG (arrows in a, inset in b). During the first postnatal days, additional expression can be detected around muscle fibers (arrowhead in a, b) and in the cartilage regions of the bones (asterisk in b). (c, d) At P21, lacZ expression is seen in the ventral horns of the grey matter in some motor neurons (mn), in the sensory neurons of the lamina II, in the dorsal column neurons and in the ventral roots at thoracic level of the spinal cord (c) as well as at lumbar level (d). (e) Immunohistochemical stainings of dissociated DRGs from E19 embryos. Neuro-filament (NF, red) and β-galactosidase (β-gal, green) were detected in the same cells. (f) X-Gal histochemistry on teased nerve fibers of a ten week-old -10/0kb PMP22 lacZ transgenic mouse. Virtually all fibers with large (1, inset), medium (2, inset) and small (3, inset) caliber axons were enwrapped with a β-gal positive Schwann cell, albeit with different level of expression. mn: motoneurons, vr: ventral roots, sn: sensory neurons, gt: gracile tract. Scale bar: 200µm (a-d), 20µm (f)
DRG neurons give rise to a long peripheral axon and a shorter central axon. Depending on the sensory input, the central axons terminate either directly in the dorsolateral region of the spinal cord, or ascend ipsilaterally through the dorsal columns of the cord and terminate in the dorsal column nuclei located in the lower medulla. Thus, it appears likely that the dot-like X-gal staining found in lamina II and the staining in the dorsal column tracts derive from β-gal that has diffused into the projections of the sensory DRG neurons since the cell soma of these neurons are strongly β-gal positive. This interpretation is supported by the analysis of longitudinally cut axons at places where they are myelinated by β-gal-negative oligodendrocytes (Fig. 5-4d, asterisk), for example at the central-peripheral nervous system transition zone of the trigeminal nerve.

Additional evidence that the β-gal positive DRG cells are neurons was provided by analysis of the β-gal expression of the different cell types in vitro. DRG from E19.5 embryos were dissected, enzymatically dissociated, and plated on collagen-coated cell culture dishes. Staining of the mixed cell population after three days with antibodies against β-gal and neurofilament revealed localization of β-gal immunoreactivity in DRG neurons (Fig. 5-3e). All neurofilament positive cells on the cell culture plate also showed immunoreactivity for β-gal. In addition, X-gal staining of sister plates showed β-gal positive cells identified by morphology as neurons with variable staining intensities (data not shown).

Transgene expression in Schwann cells of the sciatic nerve was first detected around P8 with a few scattered blue cells visible in whole mount preparations. During peak period of myelination around P10, the number of stained Schwann cells as well as the staining intensity increased dramatically (data not shown). At P21, β-gal positive Schwann cells were found in the spinal nerves (for sciatic nerve, see Fig. 5-3f) as well as in some cranial nerves (for trigeminal nerve, see Fig. 5-4d). The staining intensity of the Schwann cells was considerably stronger than that of the motor and DRG neurons, and was maintained in old adult animals (up to 1 year of age). β-gal positive Schwann cells showed comparable staining intensity in the dorsal and ventral roots of the spinal cord (Fig. 5-3c, only ventral roots visible on this section).
X-Gal stainings on teased nerve fibers of adult -10/0kb PMP22 lacZ transgenic animals showed expression of the reporter gene in the majority of the fibers (Fig. 5-3f). Virtually all Schwann cells are positive for X-Gal whether they enwrap relatively small (inset Fig. 5-3f, fiber No. 3), medium (inset Fig. 5-3f, fiber No. 2) or large caliber axons (inset Fig. 5-3f, fiber No. 1). Accumulation of cytoplasmatic β-gal can be observed in regions with increased Schwann cell cytoplasm, the paranodes (arrow in inset, Fig. 5-3f) and Schmidt-Lanterman incisures (arrowheads in inset, Fig. 5-3f).

To confirm the onset of transgene expression and to quantitate the expression of the -10/0kb PMP22 lacZ transgene, the β-gal enzymatic activity was determined in homogenates of the sciatic nerve at different time points (Fig. 5-5a). Strong upregulation of the transgene was observed after P10. This confirms the rather late upregulation of the transgene seen in X-gal stainings compared to what has been described for the endogenous PMP22 mRNA in rat sciatic nerve (Suter et al., 1994). To compare the transcriptional regulation of the endogenous PMP22 with the transgene in the sciatic nerve of mice, transgene expression was determined directly on the mRNA level. For this purpose, the levels of the exon 1A-containing-lacZ (1A-lacZ) mRNA and the exon 1B-containing-lacZ (1B-lacZ) mRNA was determined by quantitative RT-PCR, and compared with the expression of the exon 1A-containing-PMP22 (1A-PMP22) mRNA and the exon 1B-containing-PMP22 (1B-PMP22) mRNA (Fig. 5-1). GAPDH mRNA was used as an internal standard. The lacZ mRNA species in transgenic animals (Fig. 5-5b) were compared to PMP22 mRNA from wildtype littermates (Fig. 5-5c) at different time point during postnatal development. The quantitative RT-PCR for the lacZ mRNA confirmed the delayed upregulation of the -10/0kb PMP22 lacZ transgene around P10 also at the transcriptional level. The expression profile of the endogenous PMP22 mRNA in wildtype mice was similar to that found in rats during myelination (Suter et al., 1994) with the main upregulation of endogenous PMP22 mRNA occurring between postnatal days P1 and P10 during the onset of myelination (Fig. 5-5c).

We also compared the relative contributions of the endogenous promoters 1 and 2 to the regulation by the exogenous promoters 1 and 2 of the transgene (in two different lines). No significant difference in the ratio of 1A to 1B transcripts was seen for the lacZ-
containing transcripts compared to wildtype PMP22 mRNA (Fig. 5-6b, c). Furthermore, the amount of endogenous PMP22 mRNA was similar in both wildtype and transgenic mice, indicating that the expression of the reporter gene does not alter the endogenous PMP22 mRNA levels (data not shown). This is consistent with the fact that no phenotype was observed in the transgenic animals and indicates that the presence of additional copies of the PMP22 promoter region in the genome does not have a major influence on the transcriptional regulation of the endogenous PMP22 gene. The absolute abundance of endogenous PMP22 and transgenic lacZ mRNA can therefore be compared directly revealing that, at P21, the lacZ mRNA is approximately two orders of magnitude less abundant than the PMP22 mRNA (Fig. 5-5b, c).

5.5 Transgenic β-gal expression in sensory and motor neurons of cranial nerves

Serial transverse cryosections through the brainstem of three week-old -10/0kb PMP22 lacZ transgenic mice revealed that β-gal positive cells can also be detected in several cranial nerve nuclei of the brainstem (Fig. 5-4a, b, c). Except for the nuclei of the oculomotor (nerve III), trochlear (nerve IV), abducens (nerve VI) and auditory nerve (nerve VIII), where no signal was detected, all other motor and sensory nuclei of cranial nerves showed a definitive X-gal staining. This is illustrated for the hypoglossal and dorsal vagus nuclei (nerve XII and X, respectively, Fig. 5-4a), the motor nuclei of the facial (VII) nerve (Fig. 5-4b), and the motor nuclei of the trigeminal (V) nerve (Fig. 5-4c). This expression pattern of the transgene is in accordance with the endogenous expression of PMP22 (Parmantier et al., 1995) which was found in the same subset of nuclei of cranial nerves.

In Figure 5-4d, the different staining intensities of neuronal and Schwann cell expression of the -10/0kb PMP22 lacZ reporter gene are illustrated for the trigeminal nerve at the border between the central and peripheral nervous system (arrowhead in Fig. 5-4d). As soon as the cranial nerve neurons are myelinated by Schwann cells the staining intensity
increased dramatically, and confirms that oligodendrocytes around these β-gal positive neurons do not express the transgene in detectable amounts.

Figure 5-4: β-gal expression in spinal nerve nuclei in the brainstem of -10/0kb PMP22 lacZ transgenic mice. At P21, X-Gal staining is found in the hypoglossal (a, arrows) and dorsal vagus nuclei (a, arrowhead), in the motor nuclei of the facial nerve (b, arrows) and in the trigeminal nerve nuclei of the brainstem (c, arrows). In addition, expression of the transgene was detected in the sensory fibers of the spinal trigeminal tract (a, asterisks) and in the region of the mesencephalic nuclei of the trigeminal nerve (c, arrowheads). (d) The neuronal expression (d, asterisk) is much weaker than the expression by myelinating Schwann cells as shown on longitudinal sections of the trigeminal nerve at the transition zone between the central and peripheral nervous system (arrowhead). Scale bar: 200µm
5.6 Tissue specificity of the -10/0kb PMP22 lacZ transgene

In a next step, we investigated whether transgene expression is also found in non-neural organs. In earlier studies, endogenous expression of PMP22 mRNA outside the nervous system was detected in small intestine, lung, to some extent in heart by RNase protection assays (Suter et al., 1994) and in a variety of other tissues by RT-PCR (Parmantier et al., 1995; van de Wetering et al., 1999). Since no specific staining was observed on sections of heart, lung, intestine and liver (data not shown), we decided to use the sensitive luminescence β-gal assay. We determined the β-gal activity in homogenates of brainstem, lung, intestine, muscle, heart, liver and sciatic nerve in -10/0kb PMP22 lacZ transgenic animals compared to background levels in wildtype animals (Fig. 5-6a). Beside the strong promoter activity in the sciatic nerve, we detected β-gal activity significantly over background of non-transgenic littermates only in brainstem and muscle tissue, although the expression levels were about four orders of magnitude lower than in the sciatic nerve. β-gal activity in lung, intestine and heart was not distinguishable from background and was not detectable at all in liver.

To confirm the expression of endogenous PMP22 mRNA in these organs, quantitative RT-PCR was performed. High expression of 1A- and 1B-PMP22 mRNA in the sciatic nerve and lower expression levels of 1B-PMP22 mRNA in lung and intestine (Fig. 5-6b) were observed, in accordance with data from the rat system (Suter et al., 1994). In contrast, no lacZ mRNA was detectable in lung or intestine. Whether this is due to the general low abundance of the lacZ mRNA at levels below the limit of reliable detection with RT-PCR, or due to missing promoter activity remains open.
Figure 5-5: Temporal expression profile of β-galactosidase in the sciatic nerve during postnatal development. (a) β-gal activity in homogenates from the sciatic nerve increases dramatically in parallel with myelination. Four to six sciatic nerves were analyzed. (b) Total RNA was isolated from sciatic nerve of -10/0kb PMP22 lacZ transgenic animals at postnatal days 0, 4, 8, 10, 21 and 60, and the amount of transgenic 1A-lacZ or 1B-lacZ mRNA per GAPDH mRNA was determined by quantitative RT-PCR. Total mRNA levels correlate well with the regulation of lacZ expression. Error bars represent the SD of six values obtained from two independent experiments. β-gal activities are shown in relative light unit (RLU)*10^6. (c) Endogenous levels of 1A-PMP22 and 1B-PMP22 transcripts as measured by quantitative RT-PCR.

Figure 5-6: Spatial distribution of PMP22 promoter activity. (a) β-gal expression levels in organs of three week-old -10/0kb PMP22 lacZ transgenic animals and wildtype littermates. The highest expression levels by far were found in homogenates of the sciatic nerve. About four orders of magnitude less but still significant levels of activity could be detected in brainstem and muscle tissue. Error bars represent the SD of the β-gal activities/µg protein of three different animals.
Asterisks indicate statistical significance between values of transgenic and wildtype animals (Mann-Whitney U test, P<0.05). (b, c) Comparison of the endogenous PMP22 promoter with the transgenic PMP22 promoter activities in different tissues. (b) About 3-fold more exon 1A than 1B-containing endogenous PMP22 mRNA was detected in the sciatic nerve of 3-week old wildtype mice. In other tissues with PMP22 expression, mainly the 1B mRNA was found. (b) A similar ratio of 1A- to 1B-lacZ messages derived from the transgenic promoters could be detected in the two -10/0kb PMP22 lacZ transgenic lines 44.2 and 48.4, although the abundance of LacZ mRNA was approximately two orders of magnitude lower than the endogenous PMP22 mRNA. Error bars represent the SD of all values (n=4-6) obtained from two independent experiments.

5.7 -10/0kb PMP22 lacZ transgene regulation in Schwann cells after loss of axonal contact and in regeneration.

After a crush lesion of peripheral nerves, the axons distal to the injury degenerate. During regeneration, they are replaced by regenerating axons growing out from the nerve stump. During this process called Wallerian degeneration, the Schwann cells that lost their axonal contact, initially dedifferentiate and proliferate, and finally remyelinate the regenerated axons. Using this experimental paradigm, we examined whether the -10/0kb PMP22 lacZ transgene also contains the necessary DNA elements for regulation of the reporter gene by axonal contact as described for the endogenous PMP22 gene. Strong downregulation of PMP22 had been seen within two weeks after a nerve crush followed by an increase of PMP22 mRNA and immunoreactivity during remyelination (Snipes et al., 1992; Suter et al., 1994). To this end, we performed nerve crush experiments in adult -10/0kb PMP22 lacZ transgenic mice and analyzed β-gal expression 9, 14 and 80 days after the nerve crush in the distal part of lesioned nerves. These data were compared to transgene expression levels of the corresponding nerves in the non-lesioned contralateral nerve (Fig. 5-7a). Significant down-regulation of the transgene was detected nine days after injury, even more pronounced after 14 days, followed by an upregulation to expression levels similar to the contralateral nerve after remyelination (80 days post crush).
Figure 5-7: Regulation of the -10/0kb PMP22 lacZ transgene during Wallerian degeneration and after regeneration. Temporal regulation of β-gal expression after a nerve crush (a) and after nerve transsection (b) of adult -10/0kb PMP22 lacZ animals. (a) Nine and 14 days after nerve crush, reduced β-gal levels were observed during Wallerian degeneration. After regeneration (80 days), β-gal levels similar to the contralateral nerve were detected. (b) 60 days after a nerve cut, the β-gal activity of the distal part of the lesioned nerve was dramatically reduced compared to the equivalent segment of the contralateral nerve, indicating that high β-gal levels depend on axonal contact and myelination of the Schwann cells. Error bars represent the SD of the values for 3-4 animals. Asterisks indicate statistical significance between values of lesioned and contralateral nerve (Mann-Whitney U test, P<0.05).

By cutting peripheral nerves, reinervation distal to the cut is prevented. Using this approach, we analyzed transgene expression in Schwann cells without axonal contact in the distal part of the remaining nerve. Sixty days after the nerve cut, a strong downregulation of the transgene was observed compared to the normal expression levels in the non-lesioned contralateral nerve (Fig. 5-7b), indicating that axonal contact and remyelination is necessary for full transgene activity.

To know whether there is a distal-proximal gradient of the transgene expression along a nerve, we prepared in parallel additional homogenates of the proximal part of the unlesioned nerve. Taken all measurement together, no significant difference could be detected between the expression of the transgene in distal versus the proximal part of unlesioned nerves (data not shown).
5.8 Sciatic nerves of PMP22 mutant animals show reduced β-gal levels

In a next step, we examined how the -10/0kb PMP22 lacZ transgene is regulated in animal models for inherited peripheral neuropathies such as the Trembler mutant (Tr), which has a point mutation in the coding region of the PMP22 gene (Suter et al., 1992a, b), or in animals deficient for PMP22 (Adlkofer et al., 1995). These animals show demyelination and incomplete remyelination without acute injury to the nerves (Sancho et al., 1999, 2001). We analyzed the β-gal activity in sciatic nerve homogenates of -10/0kb PMP22 lacZ transgenic animals heterozygous for the Tr mutation compared to the β-gal activity in -10/0kb PMP22 lacZ transgenic animals without the Tr mutation. The presence of the Tr allele led to a drastic reduction of the β-gal activity in the sciatic nerve at the age of three weeks (P21, Fig. 5-8a) and in adult animals (P60 and P90, Fig. 5-8a). X-gal staining on teased nerve fibers of -10/0kb PMP22 lacZ transgenic Tr showed a general reduced staining in all nerve fibers (data not shown).

In the same way, we examined the effects of PMP22 deficiency on -10/0kb PMP22 lacZ transgene expression. -10/0kb PMP22 lacZ transgenic and PMP22-deficient mice were analyzed at P21 and P60 and showed a significantly reduced β-gal activity of the transgene in the sciatic nerve compared to age-matched -10/0kb PMP22 lacZ transgenic animals without PMP22 deletion (Fig. 5-8b). To check whether the neuronal expression pattern of the transgene is changed in animals deficient for PMP22, whole mount stainings of spinal cord and brainstem slices were analyzed, but no obvious changes were observed (data not shown). X-gal staining on teased fiber preparations of the same animals showed an inhomogeneous staining. Along the same fiber, some internodes showed a strong X-gal staining whereas others stained only weakly (data not shown). It has been previously reported that focal hypermyelination and myelin degeneration occurs in the peripheral nervous system of adult PMP22 deficient mice (Adlkofer et al., 1995). Therefore, the intensity of X-gal staining is likely to reflect whether the corresponding Schwann cell is in a demyelinating or remyelinating phase. In addition, the stripe-like accumulation of β-gal at non-compacted myelin regions as seen in wildtype animals (Fig. 5-3f), was not visible in either of the PMP22 mutants, indicating an altered architecture of the myelin sheath as described by (Neuberg et al., 1999).
Figure 5-8: Activity of the -10/0kb PMP22 lacZ transgene in PMP22-mutant mice. β-gal activity in sciatic nerve homogenates of the -10/0kb PMP22 lacZ transgene on a Tr mutant (a) or PMP22-deficient (b) background. A strong reduction of β-gal expression in the sciatic nerve was observed in both PMP22 mutants at postnatal day 21 and in adult animals. β-gal activities are shown in RLU*10^6. Asterisks indicate statistical significance between values of PMP22 mutant and wildtype animals (Mann-Whitney U test, P<0.05).
6   PMP22 PROMOTER DELETION ANALYSIS IN VIVO

Part of this chapter will be the basis for a publication:

Marcel Maier, Francois Castagner, Philipp Berger and Ueli Suter (2003). Dissection of the Peripheral Myelin Protein 22 (PMP22) Promoter in vivo Reveals a Late Myelinating Schwann Cell Specific Element, manuscript in preparation

In a search for the cis-acting regulatory elements that control PMP22 expression in the peripheral nervous system (PNS), we analyzed the expression of PMP22-regulated reporter constructs in transgenic mice. In a previous study, we started our analysis with a ten kilobase fragment upstream of exon 2 of the PMP22 locus (Fig. 6-1a, cf. part II; Maier et al., 2002a; referred to as -10/0kb PMP22 lacZ (PMP22-lacZ) construct, Fig. 6-1b). In figure 1 and for the rest of the text, base +1 was defined at the A of the translation start codon on exon 2. The 10 kb fragment contained promoter 1 preceding the nontranslated exon 1A, promoter 2 preceding the alternatively used exon 1B and the first part of exon 2 to the translation start codon. The alternative use of the two promoters results in two mRNA that encode the same protein and differ only in their 5’ non-coding region (Suter et al., 1994). We have shown previously, using quantitative RT-PCR, that both promoters are active on this transgene since both transgenic transcripts could be detected in addition to the two endogenous mRNA species in the siatic nerve (Maier et al., 2002a). Our analysis showed that this 10 kb fragment contains regulatory elements that reflect endogenous PMP22 expression in Schwann Cells during late myelination, in PNS sensory and motor neurons, as well as during Wallerian degeneration and remyelination after nerve crush.

To further dissect the cis-acting regulatory elements of PMP22 in the PNS, several additional transgenic mouse lines with subfragments of this 10 kb element fused to the sh ble-lacZ fusion reporter gene or to a heterologous hsp68 promoter fused to lacZ were generated. The different reporter constructs tested as transgenes in vivo can be split roughly into two groups. The first group of constructs was used to dissect the regulatory elements responsible for the expression of the reporter gene in Schwann cells during
Figure 6-1: Reporter constructs used in transgenic mice to map PNS specific elements in the PMP22 promoter. (a) Diagram of the endogenous mPMP22 genomic locus with promoter 1 preceding the nontranslated exon 1A and promoter 2 in front of the alternatively used exon 1B. All numbers refer to the nucleotide +1, defined as the "A" of the translation start codon on exon 2. (b-h) Constructs containing different parts of the 10 kb region 5' to exon 2 (-10/0kb region) were used to derive transgenic mice. The -10/0kb PMP22-lacZ construct (Fig. 6-1b) was used in a previous study (Maier et al., 2002a; PMP22-lacZ) and its activity reflects endogenous PMP22 expression in Schwann cells during late myelination, in sensory and motor neurons of the PNS, and during remyelination after a nerve crush. For this study 5' sequences extending from -10 kb to -4 kb (c), from -6.5 kb to -4 kb (d), or from -10 kb to -6.5 kb (e), were inserted at nucleotide -120 bp upstream of exon 2, which was in turn fused to a sh ble-lacZ fusion reporter gene. (f) To determine the function of the regulatory elements upstream of the core promoter 1/exon 1A a fragment extending from -10 kb to -4.3 kb was fused to the heterologous core promoter of the hsp68 gene. (g, h). By fusing -3/0 kb (g) or -4/0 kb promoter regions (h) to the sh ble-lacZ fusion reporter gene, the region between -4 kb and -3 kb was analysed. tss: translation start site, Sa: Sal I, K: Kpn I, St: Stu I, N: Not I
myelination. They contained fragments of the 6 kb region upstream of exon 1A either in front of the endogenous PMP22 promoter 1 (-10/-4kb Pro1 lacZ, -6.5/-4kb Pro1 lacZ and -10/-6.5kb Pro1 lacZ, Figs. 6-1c, d, e) or in front of a heterologous promoter (-10/-4kb hsp lacZ, Fig. 6-1f). The second group (-3/0kb Pro2 lacZ; PMP-1B-lacZ in Maier et al., 2002a), -4/0kb Pro2 lacZ, Figs. 6-1g, h) contained fragments of the 4 kb region immediately upstream of exon 2 including promoter 2 regions but lacking promoter 1.

6.1 Late myelination Schwann cell specific elements (LMSE) reside in the 6 kb DNA fragment upstream of promoter 1

Four out of the five mouse lines transgenic for the -10/-4kb Pro1 lacZ construct (Figs. 6-3a) showed robust expression of the reporter gene in Schwann cells during postnatal development in spinal nerves (e.g. sciatic nerve; Fig. 6-2) as well as in some cranial nerves (e.g. trigeminal nerve, data not shown). Detailed analysis of the temporal and quantitative expression of the reporter gene by whole mount X-gal staining and in homogenates of the sciatic nerve at postnatal day P4, P12, and P30, revealed an upregulation of the transgene beginning around P12 (Fig. 6-3b). This is rather late compared to the timing previously reported for the endogenous PMP22 mRNA in rat or mouse sciatic nerve (Maier et al., 2002a; Suter et al., 1994). A similar late expression had been observed previously with the -10/0kb lacZ reporter gene (Maier et al., 2002a), suggesting that late myelination Schwann cell specific elements (LMSE) are localized in the -10/-4kb region.

X-gal staining of teased fiber preparations from sciatic nerves showed expression of β-gal in many Schwann cells associated with large caliber axons (Fig. 6-3c). The percentage of β-gal positive internodes varied between 40 and 80% in lines 828 and 830, and between 5 and 15% in lines 827, 829, 831. Typical for the β-gal staining of Schwann cells on teased fiber preparations was the accumulation of cytoplasmic β-gal in regions with substantial Schwann cell cytoplasm, at the paranodes (Fig. 6-3c, filled arrowhead), and in Schmidt-Lantermann incisures or perinuclear regions (Fig. 6-3c, open arrowheads). Consistent with the finding that preferentially Schwann cells associated
**Figure 6-2:** Spatial expression patterns of transgenic reporter constructs in the peripheral nervous system. Presence (+) or absence (-) of reporter gene expression was examined using the histochemical β-galactosidase assay, as described in Materials and Methods. The analysis included samples from the period of maximal myelin gene expression in postnatal development (P21 to P60). "+" was defined by staining that contrasted with failure to stain in age-matched non-transgenic controls. Motor neuron and sensory neuron expression was analyzed on sections and on whole mount preparations of spinal chord tissue, sensory neuron expression in addition by whole mount staining of dorsal root ganglia (DRG). Expression in Schwann cells was judged on whole mount X-gal staining and/or on teased fiber preparations of the sciatic nerve. If no transgene transmission was achieved only the founder animal was analyzed (indicated with F).

Footnotes: ° only observed in founder animal; (+): not consistently observed in different animals of the same line; a: plus 13 transgenic founder mice positive and 6

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negative for β-gal in peripheral nerves on tail sections; b: plus 5 transgenic founder mice negative on whole mount staining; c: plus 23 transgenic founder mice negative on tail sections and 2 negative on whole mount staining; d: Line shows unique pattern of expression outside the PNS, attributed to enhancer trapping at the site of transgene insertion; h: β-gal enzymatic activity measured in homogenates of sciatic nerve, heart, intestine, liver, lung, muscle or brainstem did not show any activity above background; *: published in (Maier et al., 2002a)

with large caliber axons were positive for β-gal, the ventral roots (which contain mainly large caliber axons, arrows in Figs. 6-3d, e, f) were more intensively stained than the dorsal roots (filled arrowheads in Figs. 6-3d, e, f) on whole mounts of the lumbar spinal cord at P21 (Fig. 6-3d) and P90 (Fig. 6-3e). Furthermore, this expression of the transgene is maintained in older animals (in mice of line 830 up to 13 months of age; data not shown)

In contrast to mice with the -10/0kb lacZ transgene, four out of five mouse lines transgenic for the -10/-4kb Pro1 lacZ construct showed no expression in sensory neurons, as determined on whole mount X-gal stainings of dorsal root ganglia (DRG, open arrowhead, Fig. 6-3f), and in motor neurons located in the ventral horn of the grey matter in the spinal cord (asterisk in Fig. 6-3g), along the entire anterior-posterior axis.
Figure 6-3: -10/-4kb Pro1 lacZ transgenic mice show high β-gal expression in Schwann cells of predominantly large caliber fibers in peripheral nerves and in spinal cord nerve roots. (a) schematic depiction of the -10/-4 kb Pro1 lacZ transgene. (b) β-gal enzymatic activity in homogenates of the sciatic nerve at postnatal day P4, P12 and P30 is indicated in the bar diagram. Whole mount X-gal staining of pieces of the corresponding sciatic nerve from -10/-4 kb Pro1 lacZ transgenic mice of line 830 are shown above the graph. Onset of β-gal expression is around P12 and increases dramatically during the late phase of myelination (P30). β-gal activities are shown in relative light unit (RLU)*10^5/µg protein. (c) X-gal staining on teased sciatic nerve of a three week-old mouse of line 828. Mainly large caliber axons are enwrapped with β-gal positive Schwann cells which show the typical accumulation of the cytoplasmic β-gal in regions with increased Schwann cell cytoplasm (open arrowheads: perinuclear, filled arrowheads: paranodal). (d, e) In whole mount X-gal staining of the spinal cord of 21-day old (d) and 90-day old (e) transgenic mice of line 828, β-gal expression is found in ventral roots (arrows) and
to a lesser extent in the dorsal roots (arrowheads) of the lumbar spinal cord (root modality was identified by the site of insertion into the spinal cord). (f) Whole mount staining of dorsal root ganglion (DRG) with attached dorsal (filled arrowhead) and ventral roots (arrow). (g) Staining of crosssection through the thoracic spinal cord of three week old transgenic mice of line 828. In four out of five lines transgenic for the -10/-4kb Pro1 lacZ construct, no neuronal expression of β-gal was detected either in the DRG where the cell soma of sensory neurons are located (f, open arrowhead) or in the ventral horn of the grey matter where the cell bodies of motor neurons can be found (g, asterisks). Scale bars: 50µm (c), 500µm (d,e), 100µm (f,g)

6.2 The LMSE confer Schwann cell specificity to the non-cell type-specific hsp68 promoter and are functional independent of core promoter 1 and exon 1A

To examine whether the 6 kb element contained in the -10/-4kb Pro1 lacZ construct had functions characteristic of an enhancer and could act independently of core promoter 1, exon 1A, and exon 2 sequences, we generated a construct with a slightly shorter segment extending from -10 kb to -4.29 kb fused to the 0.3 kb minimal promoter of the heat shock protein hsp68 (Fig. 6-4a). Thus, the core promoter 1 of PMP22 up to -120 bp from the transcription start site as mapped by Suter et al. (1994), was replaced by the minimal promoter of hsp68. This promoter contains a TATA-box, an SP1 recognition site, a CCAAT box, and three heat shock response elements with no elements contributing to tissue-specific expression (Kothary et al., 1988; Kothary et al., 1989). This promoter has been successfully used before to characterize distal Schwann cell enhancer elements (Forghani et al., 2001; Mandemakers et al., 2000) or a retinoic acid response element (Rossant et al., 1991) in vivo.

All four founder animals carrying this -10/-4kb hsp lacZ transgene expressed β-gal specifically in Schwann cells as determined by whole mount staining of sciatic nerves. The percentage of β-gal positive internodes varied between 44% for founder 853, 11% and 12% for founder 858 and 861, respectively, and about 1% for founder 860. In the founder animal 858 and in F2 animals of this line, robust expression of the transgene could be detected in many Schwann cells mainly associated with large caliber fibers (Fig.
6-4c, d). Thus, this line was used to determine the temporal expression pattern during early postnatal development in homogenates of the sciatic nerve at P4, P12 and P30 (Fig. 6-4b). A late upregulation of the reporter gene around P12 was detected. This timing is comparable to that observed in animals transgenic for the -10/-4kb Pro1 lacZ construct (Fig. 6-3b) and for the -10/0kb lacZ construct (Maier et al., 2002a). Robust ß-gal expression was detected in Schwann cells by whole mount staining of the lumbar spinal cord with attached roots (Fig. 6-4e). No expression was observed in the DRG where the cell soma of sensory neurons are located (empty arrowhead in Fig. 6-4f), whereas the nerves were strongly positive. In contrast to line 858, especially in the F1 and F2 animals of lines 860 and 861, a highly mosaic expression of the transgene in Schwann cells was observed. About 12% of the internodes were positive for ß–gal in the sciatic nerve of the founder animal 861 (Fig. 6-4g), but this number decreased dramatically in later generations (F1 generation: about 1%; Fig. 6-4h), with only some 5-10 positive internodes per sciatic nerve remaining in the F2 generation (Fig. 6-4i).
Figure 6-4: The 6 kb LSME targets expression in the context of a heterologous hsp68 promoter. (a) The 6 kb LSME was ligated to a 0.3 kb hsp68 minimal promoter and used to derive -10/-4kb hsp lacZ transgenic mice. (b) Bar diagram showing the developmental expression of the -10/-4kb hsp lacZ transgene as determined by β-gal enzymatic activity in homogenates of the sciatic nerve at postnatal day P4, P12 and P30. Whole mount X-gal staining of pieces of the corresponding sciatic nerve from transgenic mice of line 858 are shown above the graph. Onset of β-gal expression was around P12 and increased dramatically during the late phase of myelination (P30). β-gal activities are shown in relative light unit (RLU)*10^5/µg protein. (c, d) Teased fiber preparation of the sciatic nerve of the founder animal (c) and of a F2 animal of line 858 (d) with β-gal positive internodal segments mainly detected on large caliber axons. (e, f) β-gal can still be detected in the attached dorsal (arrowhead) and ventral roots (arrow) by a whole mount X-gal staining of the lumbar spinal cord (e) or of the DRG (f) in the 11
month old founder animal of line 858. No neuronal expression was detected in animals with this transgene in the DRG (empty arrowhead in f). (g, h, i) In contrast to line 858, in F1 animals (h) of line 861 only few and in F2 animals (i) only single internodes positive for β-gal could be detected on teased fiber preparations of the sciatic nerve.

6.3 The 4 kb sequence upstream of exon 2, including promoter 2, contains elements directing expression in sensory neurons

As a first step to analyze promoter 2 associated regulatory elements we generated transgenic mouse lines using the -3/0 kb sequence fused to the sh ble lacZ reporter gene (-3/0kb Pro1 lacZ transgene (Maier et al., 2002a; PMP-1B-lacZ); Fig. 6-1g). This construct showed cell-line specific expression in transfection experiments in the mouse Schwann cell line MSC80 compared to the fibroblast NIH 3T3 cell line (cf. part I). Out of 33 PCR-positive founders, only six mice showed weak and unspecific expression of β-gal in cross-sections of tail biopsies (data shown). These six founders and another randomly chosen four founders were mated with B6D2F1 hybrid mice. None of the F1 animals showed expression in Schwann cells or neurons, nor were consistent significant levels of β-gal activity detected with the sensitive luminescence β-gal assay in heart, intestine, lung, muscle and brainstem (data not shown).

In the next step, we wanted to determine the expression pattern directed by the 4 kb between exon 1A and exon 2 in vivo (-4/0kb Pro2 lacZ, Figs. 6-1h and 6-5a). In whole mount X-gal staining of the DRG of three founder mice (lines 818, 820, 825) and four out of five lines (lines 819, 821, 822, 823; Fig. 6-2), we observed large β-gal positive cells suggestive of sensory neurons (Figs. 6-5b, c; arrow). A detailed analysis of the temporal expression pattern of mice from line 822 showed that the transgene was already detected at postnatal day P4 in the DRG (Fig. 6-5b) and was still highly expressed in a one year-old animal (Fig. 6-5e). The temporal expression of this -4/0kb Pro2 lacZ transgene therefore recapitulates the sensory neuron expression pattern of the -10/0kb PMP22 lacZ construct (Maier et al., 2002a).
Figure 6-5: Developmental expression of the -4/0 kb Pro2 lacZ transgene shows specific expression in sensory neurons. (a) Schematic view of the -4 kb Pro2 lacZ transgene. (b) Expression of lacZ detected at postnatal day P4 by whole mount staining of the dorsal root ganglion (DRG, arrow) in transgenic mice of line 822. (c) At P21, lacZ expression is seen both in the DRGs (arrow) and in the dorsal column tract (gt) of the thoracic spinal cord. (d) On a cross-section through the thoracic spinal cord of a 90 day old mouse of line 821 β-gal can be detected in addition in the dorsal horn of the grey matter (d, empty arrowheads). (e) Expression of β-gal is maintained in a one year-old animal (founder of line 822) and can be detected in high amounts in the processes of the sensory neurons by a diffuse X-gal staining in the dorsal roots (dr) of the lumbar spinal cord. (f, g) On a teased fiber preparation of the sciatic nerve of the founder animal of line 822, the diffuse staining seen in dorsal roots and in the sciatic nerve can be localized to a subset of myelinated fibers (f: phase contrast, g: bright field microscopy of the same fibers). The typical accumulation of β-galactosidase reaction product in cytoplasmic compartments of the Schwann cells such as the paranode (arrowhead) is not seen and therefore the weak staining most likely derives from neuronal expression of β-galactosidase. (h) Immunohistochemical staining of dissociated DRG from P4 animals. Neurofilament (NF, red) and β-galactosidase (β-gal, green) were detected in the same cells, gt: gracile tract, vr: ventral roots, dr: dorsal root, Scale bar: 200 µm (b-e), 20µm (f-h)
Additional evidence that the β-gal-positive DRG cells are neurons was provided by the analysis of the β-gal expression of the different cell types in vitro. DRG from P4 animals were dissected, enzymatically dissociated and the cells placed in culture for three days. Staining of the mixed cell population with antibodies against β-gal and neurofilament revealed a co-localization in cells with a neuronal morphology (Fig. 5-5h). In addition, X-gal staining of sister plates showed β-gal positive cells indentified by morphology as neurons (data not shown).

Additional staining was observed in the higher expressing lines, such as 821 and 822, in the dorsal column of the spinal cord as shown for a 90-day old F2 animal of line 822 (Fig. 6-5c). DRG neurons give rise to a peripheral extensions and to central axons. The latter either terminate directly in the dorsolateral region of the spinal cord or ascend ipsilaterally through the dorsal columns of the cord and terminate in the dorsal column nuclei located in the lower medulla. Indeed, in line 821 and in the founder animal of line 822, additional staining was detected in the dorsal horn of the spinal cord (empty arrowheads in Fig. 6-5d), as also observed previously in mice transgenic for the -10/0kb lacZ construct (Maier et al., 2002a). In these high expressing lines 821 and 822 we detected an additional weak and diffuse staining in dorsal roots (Fig. 6-5e), on whole mount stainings of the sciatic nerve (data not shown), and in a subset of fibers in teased fiber preparations (Fig. 6-f, g). However, the typical accumulation of the cytoplasmic β-gal staining in regions with increased Schwann cell cytoplasm such as at the paranodes (arrowhead in Fig. 6-5f, g) was not observed, and the staining was always continuous over several internodes. This pattern is that expected if expression occurs only in the peripheral extensions of the sensory neurons and not in Schwann cells. This is also consistent with the β-gal activity we could detect in the extensions of the DRG neurons of the dorsal columns of the spinal cord.
7 Computational Analysis of Conserved Promoter Elements and Repetitive Genomic Regions

The availability of extensive sequence data for both the human and mouse genome has provided the opportunity to identify regulatory elements using global sequence alignments as functionally important sequences are often conserved during evolution (Hardison et al., 1997; Mandemakers et al., 2000).

As a first step, we compared mouse and human sequences (Fig. 7-1a) using either the VISTA program (Mayor et al., 2000)(see Material & Methods for details concerning software and sequences) or the PIPMaker software (data not shown) to facilitate the delimitation of regulatory elements in the whole PMP22 gene locus. Scanning with a 100 bp window, regions with more than 75% sequence identity were noted between -23 kb and +40 kb (Fig. 7-1a). Homologous regions further upstream (> -30 kb) (data not shown) most likely belong to the mouse homolog of TEKT3, which is the gene upstream of PMP22 on the human sequence (Inoue et al., 2001). In general, regions with more than 75% identity within the PMP22 gene locus were mainly found in the -10/0 kb region but in addition also between exon 2 and +10 kb and upstream of exon 5 at the 3’ end of the gene. With the RepeatMasker program we annotated the mouse DNA sequence for interspersed repeats and low complexity DNA sequences and visualized them with the PIPMaker software as different boxes above the diagram (Fig. 7-1a,b, legend in the figure; for review see Smit, 1996).

In a second step we focused our analysis on the -10/0 kb regulatory region that we have characterized previously in vivo (Fig. 6-1a) (Maier et al., 2002a), and performed a pairwise sequence alignments of three species (murine, human, rat) (Dubchak et al., 2000) using the VISTA program. The human/rat alignment is very similar to the human/mouse alignment (Fig. 7-1b) since the mouse and rat sequences are more than 75% identical over almost the whole region (data not shown). As reported earlier, a high nucleotide sequence conservation surrounding both transcription start sites and regions rich in CpG dinucleotide around promoter 2 can be observed on the PIP (Fig. 7-1b) (Suter et al., 1994; van de Wetering et al., 1999). We grouped the remaining regions of
Figure 7-1: Distribution of conserved DNA segments and repetitive DNA elements in the mouse PMP22 gene locus combined with potential binding sites for transcription factors. (a, b) Percent identity plot (PIP) generated using the VISTA algorithm (Mayor et al., 2000) to compare the murine PMP22 genomic locus (AL 592215) from -26 kb to +40 kb (a) or from -10.5 kb to +1bp (b) (horizontal axis; in relation to the translation start codon (+1)) with the orthologous human sequences. The vertical axis indicates percent identity in a 100 bp window with a 31 bp resolution (a) or in a 50 bp window with a 15 bp resolution (b) of the plot. Regions with >75% identical nucleotides are highlighted in gray. Note that the baseline is 50%. The locations of coding exons (black rectangles), 5'- and 3'-untranslated regions (U, open rectangles) and interspersed repeats (legend in the figure; Smit, 1996) were identified using RepeatMasker software and are shown as different boxes above the profile. Above the alignment for the -10/0kb PMP22 sequence (b) analyzed in the present study, conserved regions (CR) are numbered from 1 to 5. (c)
Potential binding sites for transcription factors for which a binding matrix has previously been defined, and which are known to play a role in myelination or to be expressed in Schwann cells: Krox20/Egr-2, Egr-1, Tst-1/Oct-6, Pax-3, Peroxisome proliferator-activated receptor (PPAR), Progesterone receptor binding site (PRE BS), cAMP-responsive element binding protein 1 (CREBP), Brn-2/Pou3f2. (d) Alignment with the PMP22 promoter driven constructs analyzed in the present study. Sequencing of the -10/0kb PMP22 DNA segment used for the cloning of our constructs revealed that the repetitive region from -8.0 to -7.46 (in a, b) is missing compared to the published mouse PMP22 sequence (AL 592215) (indicated with a dotted line).

The -10/0kb sequence was screened for potential binding sites with the Mat Inspector program (Quandt et al., 1995). Among the 1080 putative elements identified, we selected the specific transcription factors for which a binding matrix once had previously been defined (for references see below and Material and Methods), and which were described to be involved in myelination or to be expressed in Schwann cells (Wegner, 2000a; b).

In CR 1 two peaks of high identity were found, whereas CR 2 and CR 3 mark regions with an overall identity above 50% but without larger regions above 75%.

The sites chosen are: Krox20/Egr-2 and Egr-1 (Swirnoff and Milbrandt, 1995), Tst-1/Oct-6 (He et al., 1991), Pax-3 (Chalepakis and Gruss, 1995), Peroxisome proliferator-activated receptor (PPAR) (Palmer et al., 1995), Progesterone receptor binding site (PRE BS) (Nelson et al., 1999), cAMP-responsive element binding protein 1 (CREBP) (e.g. Benbrook and Jones, 1994; Paca-Uccaralertkun et al., 1994), Brn-2/Pou3f2 (He et al., 1989; Li et al., 1993) (Fig. 7-1c). Interestingly, many potential binding sites for Oct-6, Brn-2, Egr-1 and Egr-2 are found in the -10.5/-9kb region although no longer stretches of sequence similarity were found between the human and mouse sequence. Nevertheless, we found potential binding sites for these transcription factors also on the corresponding human sequence. In conserved regions, a potential binding site for Egr-2 can be found in CR2 and for the progesterone receptor in CR1 and CR4.

In Fig. 7-1d the results from this computer screening are aligned to the PMP22-promoter constructs analysed as transgenes.
8 DISCUSSION AND OUTLOOK

8.1 PART I: MYELIN GENE REGULATION IN VITRO

Commitment, differentiation and maturation of neural cells are dependent on complex programs that determine specific patterns of gene expression. Thus, the elucidation of the regulation of neural gene expression will provide important information on the cellular mechanisms involved in the differentiation and maturation of the nervous system. In this context, I have analyzed the regulation of the PMP22 gene. The gene is of particular interest, because of its gene-dosage sensitivity, leading to hereditary peripheral neuropathies, and because of its pivotal role in Schwann cell biology and myelination.

I tested the suitability of different cell culture systems for reliable studies of PMP22 gene expression. I started with a classical in vitro promoter deletion analysis, by transfection of constructs containing different fragment of the 10kb region upstream of exon 2 fused to a lacZ reporter gene. I found that promoter 1 of PMP22 was active only at very basal levels in the mouse Schwann cell line MSC80 or in cultured Schwann cells. On the other hand, promoter 2 derived PMP22 expression was found in considerable amounts in those cells. This is probably due to the fact that cultured Schwann cells without coculturing with neurons do not myelinate in vitro, so that the Schwann cells do not integrate axonal signals into their transcriptional regulation. This is in contrast to the in vivo situation in the peripheral nerve, where promoter 1 derived 1A-PMP22 message is predominant during myelination (Suter et al., 1994). Indeed, PMP22 expression in cultured Schwann cells can be increased by addition of the adenylate cyclase activator forskolin, which can mimic under certain conditions some aspects of axonal contact of Schwann cells (e.g. Lemke and Chao, 1988; Trapp et al., 1988). This confirms the observation that promoter 1 is the myelin-associated promoter and very likely responsible for the expression levels of PMP22 during myelination, which are much higher than the promoter 2-derived constitutive expression levels in non-myelinating Schwann cells or other cell types. Consequently, in my opinion, it is not reasonable to study promoter 1-associated
expression of \textit{PMP22} in cultured Schwann cells by studying, for example, promoter deletion constructs of promoter 1 in transfections. Nevertheless, an extensive promoter deletion study was performed by Hai \textit{et al.}, (2001) in the RT4-D6P2T rat schwannoma cell line (Imada and Sueoka, 1978; Yamada \textit{et al.}, 1995). In this cell line, they detect some expression of the promoter 1 associated 1A-PMP22 and high expression levels of 1B-PMP22 transcripts in RNase protection assays after addition of high forskolin concentrations (50\(\mu\)M). In a promoter deletion study with constructs ranging from -3451 bp to -43 bp of the human sequence (relative to the transcription start site 1 on exon 1A, Suter \textit{et al.}, 1994) directly fused to the luciferase reporter gene, they detected expression levels in the range of the SV40 promoter activity (if the SV40 construct was also transfected in equimolar amounts). The relative expression levels increased slightly with each additional 5’ deletion up to -105 bp. The last deletion to -43 bp led to a complete loss of reporter gene activity. My present interpretation of these results is, that they reflect basal promoter activity that increases with each additional deletion of – at least in these cells – negative acting ‘myelin specific elements’. This basal promoter activity is abrogated with the deletion of the obviously essential -105/-43bp promoter region, which is just upstream of the core promoter 1. Note that \textit{in vivo} this sequence can be replaced by a \textit{hsp68} minimal promoter without loss of cell type specificity (cf. part III). On the other hand the differences in the results might also be due to the use of the human promoter sequence, to the different cell line used, or due to the direct fusion of the reporter gene to exon 1A so that a splicing event is not required for correct expression.

Another result from my promoter deletion analysis was that expression levels of the \textit{PMP22} promoted reporter constructs were considerably higher in MSC80 cells compared to NIH3T3 cells. For this comparison the SV40 promoter expression levels serve as a standard with the assumption that the SV40 promoter is expressed at similar levels in both cell types. If these different relative expression levels indeed can be confirmed by measurement of the absolute expression levels, one could conclude that the MSC80 cells contain certain factors which increase the low levels of \textit{PMP22} expression and which are not abundant in NIH3T3 cells. It would be very interesting to identify these factors which are differentially expressed in only one cell line. This could be done for example on RNA levels using microarray technology or on protein levels using 2D-gel electrophoresis and other proteomic approaches.
Another cell culture system in which PMP22 expression has been studied involves *in vitro* myelination of DRG neurons by Schwann cells (Pareek *et al.*, 1997). Unfortunately, *in vitro* myelination systems of the PNS, especially with mouse tissue, are not very reliable and nearly as time-consuming as *in vivo* experiments. In addition from my own experience, they are not established to the degree that additional manipulations or modifications are tolerated or can be reliably evaluated in a quantitative manner. Therefore I did not present my initial *in vitro* myelination experiments to characterize the -10/0kb PMP22 lacZ transgenic mice in the present thesis.

Since we did not have a cell culture system in which both promoters of *PMP22* were reliably expressed, I sought another setting in which *PMP22* expression could be induced e.g. by exogenously altered expression of transcription factors. This was done successfully for the myelin protein zero (MPZ) in cotransfection experiments (Monuki *et al.*, 1990; Zorick *et al.*, 1999) or with doxycycline induction of transcription factors in N2A cells (Peirano *et al.*, 2000). Unfortunately, I did not observe any upregulation of *PMP22* promoter-directed reporter gene expression by cotransfection of various transcription factors (see chapter 4.1) nor did I see a co-regulation of PMP22 with P0 by Sox10 in N2A cells (see chapter 4.3). At least in this setting, PMP22 seems not to be regulated by Sox10. This is astonishing, since these two myelin genes have a comparable temporal and spatial expression pattern, at least postnatally in myelinating Schwann cells.

One may speculate why these systems are not working, but it must be realized that complex interactions and combinations of known and of probably still unknown regulatory factors finally lead to the correct temporal and spatial expression in a complex organ like the PNS. In addition the cotransfected transcription factors are usually strongly overexpressed and may therefore “overload” the physiology of the cell (e.g. the RNA splicing machinery), and additionally required endogenous factors may not be available in sufficient amounts.

Even so, Nagarajan and coworkers found a way to circumvent some of these problems. Infection of cultured Schwann cells with adenovirus expressing Krox20 led to an upregulation of myelin genes, with steady-state mRNA levels rising between 5-fold
(MBP) and 60-fold (for MPZ) \cite{Nagarajan2001}. This means that transcription of myelin genes in their endogenous chromosomal context can be induced by the overexpression of Krox20. I could reproduce this up-regulation of myelin genes, at least in the absence of serum. The presence of 10% FCS in the cell culture medium prevented a strong upregulation of PMP22. Possibly mitogens in the serum prevent the Schwann cells from attaining a pro-myelinating-like phenotype and concomitant upregulation of *PMP22/growth arrest specific gene 3 (gas-3)* \cite{Zanazzi2001}.

The expression levels of the myelin genes in cultured Schwann cells overexpressing Krox20 are still much below the expression levels in myelinating Schwann cells. This points to the fact that additional factors or signals are required for increased expression of the myelin genes. A screening for those additional components could be performed, for example by expression cloning, or also by treatment of Schwann cells with membrane- and soluble fractions of DRG neurons since neuronal contact is described to upregulate *PMP22* expression. In any event, Krox20-infected Schwann cells may allow a dissection of the regulatory network involved in the initiation of myelin gene expression *in vitro* by studying co-regulated or target genes of Krox20. I tried to confirm by quantitative RT-PCR the up-regulation of certain candidates previously identified in gene expression profiling experiments \cite{Araki2001,Nagarajan2001}. I could confirm only the upregulation of the POU domain transcription factor Brn-2 by Krox20. Unfortunately, I could not confirm the upregulation of our additional candidates. This could be due to slightly different cell culture conditions, a PCR which is not sensitive enough, or to false positives in the gene chip analysis (the data presented in Nagarajan *et al.*, 2001 seem to derive from a single chip hybridisation experiment).

Indeed, in the meantime it has been shown that Brn-2 has an expression pattern similar to that of Oct-6 during Schwann cell myelination and after nerve crush \cite{Sim2002}. This makes Brn-2 an interesting candidate for further studies. That could be used to dissect this regulatory network of Krox20-induced myelin gene regulation. For example adenoviral overexpression in cultured Schwann cells could be used to ask whether Brn-2 is able to directly regulate myelin gene expression or whether it has a cooperative effect if coexpressed with Krox20. Finally the target genes of Brn-2 could be identified using microarray technology, as has been done for Krox20 \cite{Nagarajan2001}. New candidates for further analysis might thus be discovered. Another possibility would be to
identify the pathways that are involved in the initiation of the PMP22 gene expression by the addition of specific inhibitors, as has been done by Awatramani et al., (2002). Once interesting pathways are identified, specific candidates could be blocked, for example by RNA interference experiments.

8.2 PART II: REGULATORY ELEMENTS ON THE -10/0KB PMP22 LACZ TRANSGENE

The development and proper function of peripheral nerves in vertebrates depend on intimate interactions and continued signalling between Schwann cells and the associated axon(s). In recent years much progress has been made in identifying components of cell-cell interactions necessary in early stages of peripheral nerve development (Mirsky and Jessen, 1999; Mirsky et al., 2002). In contrast, little is known about extracellular signals and intracellular signalling pathways that initiate and regulate myelination. Earlier work has indicated that the myelination program of Schwann cells is under the control of the associated axon and correlates with axonal diameter (Aguayo et al., 1976a; b; Voyvodic, 1989). Whatever the exact nature of these signals might be, finally they must be relayed to the Schwann cell nucleus where transcription factors coordinate the regulation of sets of genes.

To start the dissection of the coordinate regulation of PMP22 in vivo I have produced transgenic mice carrying both the lacZ reporter and the zeomycin resistance (sh ble) genes driven by PMP22 promoter 1 and promoter 2 plus additional potential regulatory domains in the 10 kb region 5’ to the coding sequence of the PMP22 gene. I examined the role of this region in the spatiotemporal transcriptional regulation of PMP22 with a lacZ reporter gene in vivo during development, regeneration, and in animal models of hereditary peripheral neuropathies. Consistent with the endogenous expression of PMP22, by far the highest expression levels of the -10/0kb PMP22 lacZ transgene were observed in Schwann cells of peripheral nerves during myelination (Haney et al., 1996; Snipes et al., 1992; Spreyer et al., 1991; Welcher et al., 1991). Dramatic upregulation of
the transgene was observed around P8-10 during the most active phase of myelination. Upregulation of the endogenous PMP22 gene, however, starts already with the initiation of PNS myelination shortly after birth. The delayed upregulation of the transgene may have various causes. First, reliable quantitative detection of the very low lacZ mRNA levels in the sciatic nerve in the first postnatal days was not possible probably due to a general low abundance of the lacZ mRNA as it has also been observed in other studies (Feltri et al., 1999; Wight et al., 1993). This might be the result of decreased mRNA stability or different post-transcriptional regulation of the lacZ mRNA compared to the PMP22 mRNA (Bosse et al., 1999). Alternatively, regulatory elements for the early PMP22 transcription could be missing within the 10 kb 5' flanking region used in our reporter study. This interpretation offers the hypothesis that the upregulation of PMP22 during myelination (and possibly also of other genes encoding myelin components) is controlled on the molecular level by various distinct factors. On a speculative view, one might envisage that some specific factors are important at myelination initiation while others contribute to the exceptionally high gene expression that is required during the peak of myelination or later during adulthood in myelin maintenance (cf. part III). Another possibility would be that the PMP22 transgene might interfere with endogenous PMP22 expression, but I have not observed any evidence for this. In particular, I have also not observed signs of delayed myelination as analysed at postnatal day P4 on semithin sections through the sciatic nerve (data not shown). Furthermore the ratio of 1A-LacZ to 1B-lacZ mRNA (transgenic) was similar to the ratio of 1A-PMP22 to 1B-PMP22 mRNA (endogenous) indicating that no important regulatory elements are missing for similar relative promoter activities of the two transgenic promoters compared to the two endogenous promoters at postnatal day 21.

The expression of the -10/0kb PMP22 lacZ transgene after nerve lesions and during remyelination follows the spatio-temporal expression pattern of the endogenous PMP22 gene. These experiments strongly suggest that PMP22 transcriptional regulation in Schwann cells is dependent on axonal contact and that full activity of the PMP22 promoters is dependent on myelination (Gupta et al., 1993; Spreyer et al., 1991). The observations from cell culture experiments with -10/0kb PMP22 lacZ transgenic mice
are consistent with this notion: Schwann cells isolated from postnatal sciatic nerves showed only very low β-gal activity (data not shown).

To analyse the regulation of the -10/0 kb PMP22 lacZ transgene in settings with impaired myelination, demyelination and only limited remyelination (without actively severing the axon, but see also Sancho et al., 1999), mice carrying the transgene were crossed with Trembler (Tr) and PMP22-deficient mice. The drastically reduced levels of the -10/0 kb PMP22 lacZ transgene expression in the sciatic nerve of the resulting animals (Fig.5-8) was likely due to the presence of fewer myelinating Schwann cells (Adlkofer et al., 1995; Suter et al., 1992a), consistent with the reduced levels of PMP22 mRNA levels in Tr mice (Garbay et al., 1995). Thus, the mutant Schwann cells are not capable of maintaining the normal program regulating myelin gene expression, possibly due to impaired axon-Schwann cell signalling (Sancho et al., 1999) or to an intrinsic failure to differentiate.

The detection of the reporter gene product in sensory and motoneurons of peripheral and cranial nerves confirms the expression of PMP22 mRNA found in motor neurons (Parmantier et al., 1995) and in the DRG during early postnatal development (Parmantier et al., 1997). These findings indicate that the control elements required for neuronal expression are contained within the -10/0 kb PMP22 lacZ transgene and are consistent with the detection of low level PMP22 immunoreactivity in the DRG and the dorsal horn of the spinal cord (De Leon et al., 1994).

The expression of the transgene during embryonic development was roughly consistent with the previously described expression pattern of PMP22 mRNA (Baechner et al., 1995; Parmantier et al., 1997). However, the relevance of the prominent β-gal staining in the outer ear, the limb muscle, and in the ventricular epithelium of the rhombencephalon remain to be determined.

One of the potential benefits of this study was the generation of an experimental tool that could be used to direct foreign gene expression reliably in Schwann cells and their associated neurons to examine myelination and dysmyelination. The PMP22 gene
regulatory region used here proved to be very consistent in its expression pattern between different lines, and was robust in the number of expressing founder animals. Importantly, I could not detect consistent transgene expression in postnatal non-neural tissues such as small intestine or lung, despite the fact that low levels of PMP22 mRNA had been found before (Fig. 6-6; Patel et al., 1992; Taylor et al., 1995). This finding indicates that, on one hand, the regulatory elements required for this non-neural PMP22 expression are probably missing on our transgene (or that I am below the detection limit of the assay). On the other hand, it makes this PMP22 regulatory region an even more valuable tool due to its strict tissue specificity.

Furthermore, taking into account the strong upregulation of the reporter gene during myelination, the -10/0kb PMP22 lacZ animals are a valuable tool to investigate potential myelin gene regulatory factors that might also be involved in the pathogenesis of hereditary peripheral neuropathies. Such an approach is currently being followed in collaboration with R. Melcangi and his group. Currently, they are analysing the effect of progesterone derivatives on the transgene expression and on PMP22 mRNA levels in vivo. Previous in vitro and in vivo experiments have shown that certain progesterone derivatives are able to influence PMP22 and P0 gene expression (Chan et al., 2000; Desarnaud et al., 1998; reviewed by Magnaghi et al., 2001; Melcangi et al., 1999; 2001). Indeed, initial experiments show, that the -10/0kb PMP22 lacZ transgene can be upregulated by repeated administration of tetrahydroprogesterone (R. Melcangi, personal communication). Since some conserved potential progesterone receptor binding sites are found in the -10/0kb sequence (cf. part III), progesterone derivatives potentially could act directly on the PMP22 gene promoter. To narrow down the sequence of the progesterone responsive regions of the PMP22 promoter, different reporter transgenes (cf. part III) could be tested for their response to progesterone derivatives. Since the reporter gene allows a screening for different PMP22 regulatory factors in vivo, further studies will focus on potential pathways involved in the progesterone-mediated PMP22 and PMP22 reporter transgene up-regulation in vivo.
8.3 PART III & IV: PMP22 PROMOTER DELETION ANALYSIS IN VIVO

The results of the detailed characterization of the -10/0kb PMP22 lacZ transgene show a specific regulation and expression in Schwann cells and in a subset of peripheral neurons. This justified further effort in delineating PNS specific elements.

To this end I generated a first group of transgenic mice containing different fragments of the 6 kb region 5’ of Promoter 1 fused to both a lacZ reporter and a zeomycin resistance (sh ble) gene. The transgenes contained the regulatory elements sufficient for expression of the reporter gene during myelination in Schwann cells. In contrast to the expression pattern of the previously characterized -10/0kb PMP22 lacZ transgenic mice I did not observe neuronal expression of the reporter gene (cf. part II, Maier et al., 2002a). With the fusion of this 6 kb fragment to a lacZ reporter gene with a minimal promoter of the hsp68 gene, I showed that these cis-acting elements have enhancer-like properties, since they are sufficient for expression of the reporter gene independent of the core promoter 1 and sequences of exon 1A. I analysed a second group of transgenic mice which contained a 3 kb or a 4 kb fragment 5’ to exon 2 fused to the sh ble lacZ reporter gene. Although I did not observe expression in the PNS with the 3 kb fragment, the 4 kb fragment showed expression in sensory neurons of the PNS.

I observed strong upregulation of the transgenes with the myelin-specific Schwann cell elements (-10/-4kb Pro1 lacZ and -10/-4kb hsp lacZ) around postnatal day 12, during the most active phase of myelination and of expression of the endogenous PMP22 gene (Haney et al., 1996; Snipes et al., 1992; Spreyer et al., 1991; Welcher et al., 1991). In comparison with the endogenous PMP22 gene which begins upregulation with the initiation of PNS myelination shortly after birth, the reporter constructs have a delayed upregulation. This phenomenon was already observed with the 4 kb longer -10/0kb PMP22 lacZ construct (Maier et al., 2002a) and could be due to missing regulatory elements responsible for the early induction of PMP22. This interpretation offers the exiting hypothesis that different regulatory mechanism are involved in different phases of myelination and that these late myelinating specific pathways converge on this regulatory DNA element. Consequently I termed this DNA element a late myelinating Schwann cell
specific element (LMSE), which I located in this study within the 6 kb region in front of promoter 1. In the previous part (part II) I showed that the -10/0kb region in the -10/0kb PMP22 lacZ construct contains the regulatory regions needed to reflect the spatiotemporal expression of the endogenous PMP22 also after nerve lesions and during remyelination. I have shown that the Schwann cells upregulate the transgene specifically during myelination and have only very basal expression of the transgene without axonal contact. So far I did not observe any difference in spatiotemporal expression of the -10/-4kb Pro1 lacZ construct compared to the -10/0kb PMP22 lacZ transgene. In accordance with this observation, the -10/-4kb Pro1 lacZ transgene is still highly expressed in one year old animals. Therefore I assume that the LMSE contain the regulatory regions and may bind transcription factors specifically involved in myelin maintenance and probably also regeneration.

So far little is known about transcription factors specifically involved in remyelination or myelin maintenance. Presumably these factors would be expressed at high levels in myelinating Schwann cell. Recently, the POU domain transcription factor Brn-5 has been shown to be expressed in increasing amounts in late Schwann cell development (Wu et al., 2001), with an expression pattern inverse to that of SCIP/Oct-6. Therefore Brn-5 could be involved in these regulatory mechanisms. So far no regulatory elements have been described to be responsible specifically for expression in the late phase of myelination. Only the previously characterized MBP Schwann cell enhancer (SCE1) has been described to maintain expression of the reporter gene also in old adult animals, at least in some lines (Forghani et al., 2001). For the myelin specific element (MSE) of the Krox20 gene, which leads to specific expression in myelinating Schwann cells, it would be very interesting to follow this aspect of expression since it has not been addressed so far in old adult animals (Ghislain et al., 2002). However, the existence of elements responsible for early- versus late expression of myelin genes has been discussed in the case of oligodendrocyte-specific expression of MBP promoter-lacZ reporter genes. The expression of certain constructs declined in old adult animals, whereas it was maintained in constructs with different regulatory sequences (reviewed by Ikenaka and Kagawa, 1995). In the PNS, a similar phenomenon has been observed for CNP promoted lacZ reporter gene expression, which only partially recreated endogenous CNP expression.
since lacZ was only detected at early stages of development, but not in older adult mice (Gravel et al., 1998; reviewed by Wegner, 2000a). Since not many transcription factors involved in this type of regulation are known, a combination of the obtained spatiotemporal expression pattern of the LMSE reporter constructs with whole genome expression studies in the peripheral nerve, selecting for co-regulated genes with similar expression profiles, may help to reveal new candidates involved in the late phase of myelination, in myelin maintenance or regeneration.

The fact that the LMSE is sufficient for specific expression in Schwann cells in front of a minimal hsp68 promoter in the -10/-4kb hsp lacZ construct shows that the LMSE has enhancer-like characteristics and that the endogenous promoter 1 and exon 1A sequences are not necessary for tissue specific expression since they can be replaced by the unspecific hsp68 minimal promoter. By comparing at least three different lines of a given construct I have tried to average out effects due to different expression levels of the randomly integrated transgene, which depend on copy number and in many cases also on the integration site. From such comparison, I observed one difference between the spatial expression of β-gal on teased fiber preparation of mice transgenic for the -10/-4kb Pro1 lacZ construct with animals transgenic for the -10/-4kb hsp lacZ. It was more difficult to establish stable transgene-expressing lines with the latter construct since two out of three lines showed a highly mosaic expression (variegation) in F1 and F2 generations. In addition, I got the impression that mosaic expression also of the -10/-4kb Pro1 lacZ was more common than with the -10/0kb PMP22 lacZ transgene. The interpretation of these observations may involve different explanations and mechanisms which are not clearly distinguishable. Traditionally, enhancers have been thought to function by increasing the rate of transcription initiation from a linked promoter. In recent years, it has been shown that in some cases enhancers not so much influence the rate of transcription initiation, but instead increase the chance that a linked promoter is activated at all. In this probabilistic model, enhancers are thought to function through a mechanism that involves modifications to the local chromatin configuration or relocation to an active centre within the nucleus (Fiering et al., 2000; Hume, 2000). This model predicts that an enhancer increases the percentage of cells in a population expressing the gene. In transgenic mice experiments, such a mechanism might explain the often observed variegated expression
of the transgene (Elliott et al., 1995; Milot et al., 1996). Consequently, the loss of enhancer activity could lead either to a smaller number of Schwann cells expressing the reporter gene rather than a generally lower expression of the reporter gene in internodes resulting in a less intense staining. So far intensities of different β-gal stainings can be compared, this is in fact what I observed, both, in the -10/-4kb Pro1 lacZ compared to the -10/0kb PMP22 lacZ and in the -10/-4kb hsp lacZ compared to the -10/-4kb Pro1 lacZ transgenes. In the first case, one can speculate that the -10/0 kb region acts as a stronger enhancer than the -10/-4kb LMSE in Schwann cells by modulating the chance that the linked promoter 1 is activated. Alternatively, one can argue that the LSME acts on both PMP22 promoters instead of just promoter 1. In the second case, one may speculate that some enhancer activity was lost due to the replacement of the endogenous promoter 1, exon 1A and exon 2 sequences by the hsp68 promoter (cf. Fig. 7-1). Alternatively, one can not exclude an effect due to the different reporter cassettes used. However, finally we do not have enough data and the appropriate system to determine which of the mechanisms discussed above dominate. The effects probably overlap, especially due to the removal of the enhancer from its native context (Graubert et al., 1998; Sutherland et al., 1997). These mechanisms can be taken apart to a certain degree with a system as introduced in the section below.

A reproducible integration of the transgene always as a single copy at the same defined genomic locus would have the advantage that absolute expression levels of different transgenes can be compared (Guillot et al., 2000; Misra et al., 2001). This can be achieved in ES cells, modified with a partially deleted HPRT locus. A transgene can be inserted into the genome with a construct that allows reconstitution of the HPRT locus at the same homologous recombination site in multiple transformants. This system may allow to distinguish the different aspects of enhancers as mentioned above. In collaboration with the groups of Alan C. Peterson and G. Jackson Snipes ES cells containing a -21/0kb lacZ and a -11/0kb lacZ constructs are currently being tested for the spatiotemporal expression pattern of the reporter gene in mice. Interestingly, preliminary analysis of the -21/0kb lacZ construct indicates that there is no high expression levels of β-gal in sciatic nerves (G. Snipes, personal communication). The analysis of the -11/0kb lacZ construct in mice will show whether this is due to the integration of a single copy of
the reporter gene, which might be expressed at levels too low to be detected, or whether this is due to additional inhibitory elements upstream of the -10/0 kb fragment analysed in the present study. The most rigorous way to test the function of these transcriptional control elements in their native context would require specific deletion by homologous recombination in embryonic stem cells. Such deletion could result in the generation of a hypomorphic allele in case of the deletion of a cell-type specific enhancer (for example see Ghazvini et al., 2002 with the Oct-6^{ASCE/ASCE} mice). For PMP22, it would be of interest to see whether mice with a deletion of the LMSE develop a myelination phenotype caused by low expression of PMP22. This is likely since a reduction of PMP22 expression leads to HNPP in humans (Chance et al., 1993) and to a HNPP-comparable phenotype in mice heterozygous for the PMP22 deletion (Adlkofer et al., 1997a).

To continue the dissection of the regulatory network of PMP22, it would be interesting to assess whether the Oct-6^{ASCE/ASCE} mice (Ghazvini et al., 2002) show delayed induction of PMP22 mRNA during their delayed myelination. This is likely since Ghazvini et al., (2002) have shown that also Krox-20 and P0 expression is delayed in these mice. In this case, it would be of interest to see if these animals completely compensate for the lower PMP22 levels in old adult animals. This would provide some evidence that a different set of regulatory factors is involved in myelin maintenance, or at least that Oct-6 is not the rate-limiting transcription factor. Similar questions could be addressed with a tamoxifen-inducible creERT2 transgene expressed in adult myelinating Schwann cells (e.g. a PLP creERT2, a P0Cx32 creERT2 or a -10/0kb PMP22 creERT2 which all are under construction) combined with a “floxed” allele for a transcription factor such as the Krox20 (Taillebourg et al., 2002). These mice would recombine the Krox20 allele in Schwann cells upon tamoxifen induction, which then should lead to a tissue-specific ablation of Krox20 in adult myelinating Schwann cells.

Most mouse lines with the -10/-4kb hsp lacZ or -10/-4kb Pro1 lacZ transgene show expression mainly in Schwann cells associated with large calibre fibres. This is in contrast to the -10/0kb PMP22 lacZ construct, where ß-gal positive Schwann cells are also associated with middle and small calibre fibres, albeit with clearly reduced ß-gal
levels. In agreement with this observation, the ventral roots of the lumbar spinal cord, which contain only large calibre motor axons were more strongly stained than the dorsal roots which contain the sensory axons (Figs. 6-3d, e). This could be explained by lower expression levels of the transgenes, which would not be detectable in Schwann cells of small calibre fibres. The latter already showed lower expression levels in -10/0kb PMP22 lacZ mice (Maier et al., 2002a). Alternatively this finding might be due to the lack of additional positive regulatory elements outside the 6 kb LMSE element. If correct, this may suggest a novel level of heterogeneity among Schwann cells that would be correlated with the fiber diameter of the associated axon. Such molecular differences between Schwann cells associated with motoneurons versus sensory neurons have been described before (Martini et al., 1994).

The majority of transgenic mice lines carrying the -4/0kb Pro2 lacZ construct show expression of the transgene specifically in sensory neurons. Since the -3/0kb Pro2 lacZ construct does not give rise to expression in the PNS (Maier et al., 2002a), it can be concluded that regulatory elements located between -4 and -3 kb are necessary for expression of the transgene in sensory neurons. In contrast to the neuronal expression in the PMP -10/0 kb lacZ transgenic mice, no expression in motor neurons was observed, suggesting that expression in motor neurons probably needs additional regulatory elements located further upstream.

At this point it can be asked whether promoter 2-associated expression of PMP22 alone is responsible for neuronal expression of PMP22, since in the CNS mainly 1B-PMP22/SR13 messages are detected by RT-PCR (Parmantier et al., 1995). This could be addressed by the isolation of RNA from purified DRG neurons which still show expression of the -4/0kb lacZ transgene - at least in mixed cultures of dissociated DRG. With the sensitive PMP22 TaqMan PCR a determination of the probably low expression levels might be possible, although D'Urso et al., (1997) failed to detect PMP22 by conventional RT-PCR in purified rat DRG neurons.

It is also possible that the -3/0 kb lacZ construct, which does not show any specific expression as transgene, is silenced in vivo, for example by methylation. The previously
reported fact that many conserved CpG islands can be found around promoter 2 (van de Wetering et al., 1999) and in front of exon 2 is consistent with such a possibility, since methylation was proposed to be one regulatory mechanisms of promoter 2. In addition, with the presence of CpG islands and the absence of a CCAAT box, promoter 2 shows a structure typically found in promoters of house-keeping genes (van de Wetering et al., 1999). It may therefore be that the -3/0kb Pro2 lacZ construct does not contain the elements necessary to prevent silencing of the transgenic promoter 2.

Up to now, we focused our attention on the PNS expression of PMP22. But promoter 2 of PMP22 is active also in many non-neural tissues. Due to the broad PMP22 expression pattern and due to the promoter structure as described above, promoter 2 was proposed to be a constitutively active promoter (Suter et al., 1994, van de Wetering et al., 1999). So far, I did not observe a consistent specific expression pattern of the transgenes analyzed in the present in vivo promoter deletion study in non-neural tissues. This shows that the transgenes did not contain the elements necessary for transgene specific expression in the non-neural tissue in postnatal animals (data not shown). In combination with the results from the -10/0kb PMP22 lacZ construct, which also did not show specific postnatal expression in organs with high PMP22 mRNA levels such as small intestine and lung (cf. Fig. 6-6, Suter et al., 1994), this indicates that I am missing regulatory elements responsible for non-neural expression of PMP22 in the -10/0 kb region. As an inverse interpretation I argued in the previous part (cf. part II), that this also could be due to the presence of negative acting elements. In such a case, one would anticipate that a dissection of the -10/0kb promoter region into subfragments would abrogate the effect of negative acting elements in certain constructs. I did not observe such a phenomena and therefore this interpretation is unlikely.

So far, no PMP22 promoter driven reporter construct completely reflected endogenous PMP22 expression. The characterization of transgenic reporter constructs which contain additional regions 5’ or 3’ to the previously characterized -10/0 kb genomic fragment, will hopefully lead to the discovery of additional regulatory elements. Of special interest would be those involved in the initiation of PMP22 expression in early development. The detection of regions with high homology between mouse and human PMP22 genomic
sequences outside of the characterized -10/0 kb genomic fragment supports the hypothesis that additional regulatory elements, for example between exon 2 and 3 or downstream of exon 3 may contribute to PMP22 expression in early Schwann cells or in non-neural tissues.

Our collaborators from the group of J. Snipes have shown that a -8.5/0kb PMP22 lacZ-IRES-luciferase transgene with the rat promoter sequence shows only weak expression in Schwann cells, but considerable expression in DRG (J. Snipes, personal communication). The fact that many potential binding sites for Oct-6 and Brn-2 are found in the -10/-8.5kb region and that the rat and mouse sequences are highly conserved, make this element an attractive sequence to screen for binding of myelin specific transcription factors. Further delineation of the 6kb LMSE with the -6.5/4kb Pro1 lacZ and the -10/-6.5kb Pro1 lacZ construct is ongoing in our laboratory and will hopefully answer the question of whether the LMSE can be taken apart further into smaller segments. However, if it turns out that the -10/-6.5kb element leads to Schwann cell expression, the indications from potential binding site would be confirmed and this -10/-6.5kb or even the shorter -10/-8.5kb element indeed could be used to screen for binding factors, for example with a yeast-one-hybrid system. Since this system was successfully used only with short sequence elements (oligonucleotides)(Lehming et al., 1994; Wang and Reed, 1993) the screening has to be performed only with subfragments of the -10/-8.5kb element. If it is not possible to further narrow down the 6kb LSME, potentially important regulatory regions on large sequence elements could be determined by screening for DNaseI Hypersensitive sites (HSS). The challenge of these experiments is to obtain enough starting material, since the most reasonable way to perform these experiments is to take cell nuclei from myelinating Schwann cells. So far, at least the isolation of nuclear extract from myelinating Schwann cells seems to be possible (Forghani et al., 1999), and could be used for example also for electromobility shift assays (EMSA). Classical EMSA permit the characterization of one or maybe a few transcription factor at a time and for a DNA sequence usually between 20 bp and 300 bp. Alternatively, if one can isolate sufficient amounts of nuclear extracts a new developed system called “TranSignal Protein/DNA array” (Panomics, CA, USA) may allow profiling the activities of multiple transcription factors simultaneously. With this system
one screens for the binding of transcription factors to a mixture of oligonucleotides with known consensus-binding site sequences. Nuclear extracts are incubated with a mixture of biotin-labelled oligonucleotides, DNA/protein complexes allowed to form, and DNA/protein complexes are isolated. The recovered DNA is hybridized to a prespotted membrane with complementary sequences to those of the oligonucleotides. However, this system has the disadvantage that one is limited to known consensus binding sequences. Further evaluation of this system will reveal how reliable and useful it is. Alternatively, one may use chromatin immunoprecipitation assays combined with PCR amplification of potential target regions to confirm a binding of a specific transcription factor to a certain promoter region (Sasaki et al., 2002).

In summary, I have used a transgenic approach to characterize the sequences within the PMP22 gene that are involved in its tissue- and cell-type specific as well as temporal regulation. It will be interesting to further delineate the regions of the PMP22 gene and its binding partners required for cell-type specificity and for regulation by neuron-Schwann cell interactions. This will be supported by ongoing complementary in vitro approaches, including the mapping of PMP22 promoters by transfection, by analyzing DNA-proteins interactions (Hai et al., 2001; Saberan-Djoneidi et al., 2000), or by using adenoviral infections of Schwann cells with Krox20 to study the regulatory network involved in the initiation of PMP22 transcriptional expression. I anticipate that such studies will provide additional insights into the coordinate regulation of myelin genes in conjunction with related studies analyzing other genes encoding myelin components. Furthermore, taking into account the strong upregulation of the -10/-4kb Pro1 lacZ reporter gene during myelination and its Schwann cell-specificity, this promoter segment is a valuable tool to specifically express potential myelin gene regulatory factors that might also be involved in the pathogenesis of hereditary peripheral neuropathies, or to regulate PMP22 expression levels by antisense approaches (Hai et al., 2001; Maycox et al., 1997).
9 EXPERIMENTAL METHODS

9.1 Generation of reporter constructs

For the generation of a construct expressing sh ble-lacZ under the control of PMP22 regulatory regions (−10/0kb PMP22 lacZ), a 10 kb Sal I/Not I fragment from cosmid pTCF-6.1 (Magyar et al., 1996) was fused to a Not I-Nco I PCR fragment containing the first 42 bp of the exon 2 and a Nco I-Sac II fragment of the sh ble-LacZ fusion gene (Cayla, Toulouse, France) reconstituting the original Kozak sequence and translation start on exon 2. The sequence of the PCR fragment was controlled by DNA sequencing. The resulting 14 kb reporter construct (−10/0kb PMP22 lacZ; SNlacZ; SNE2zeolacZ; PMP22- lacZ) was excised from the vector backbone with Sal I and Sac II.

Four fragments were ligated for the generation of the -10/-4kb Pro1 lacZ (1AlacZ) reporter construct: 1) a 3.5kb Sal I (-10kb)/Kpn I (-6.5kb) fragment derived from a Sal I /Not I PMP22 subclone that was used to generate the -10/0kb PMP22 lacZ construct (Figs. 5-1, 6-1, PMP22lacZ in Maier et al., 2002a) a 2.6kb Kpn I (-6.5kb)/Not I fragment using a Not I site introduced by PCR at -3.91 kb with the same Sal I/Not I subclone as template 3) a Not I (-0.12kb)/Bgl II fragment, containing exon 2 sequences and the sh ble-LacZ fusion gene (pUT111, Cayla, Toulouse, France); and 4) a Sal I/Bgl II fragment containing the Bluescript vector II KS+ (Stratagene). Fragments (3) and (4) were derived from the PMP22 -10/0kb lacZ (PMP22lacZ) construct. The resulting reporter construct was released from the vector backbone with Sal I and Sac II digestion for pronuclear injection.

The -6.5/-4kb Pro1 lacZ (Δ1AlacZ) was generated by an intramolecular deletion of a 3.5kb Kpn I(-10.1kb)/Kpn I(-6.5kb) fragment of the -10/-4kb Pro1 lacZ construct. To generate the -10/-6.5kb Pro1 lacZ (ΔPro1lacZ) construct a Sal I (-10kb)/Kpn I (overhang blunt-ended with T4 DNA Polymerase) fragment was fused to a Stu I (-4.33kb)/Sal I fragment of the -10/-4kb Pro1 lacZ construct. The -10/-6.5kb Pro1 lacZ and the -6.5/-4kb Pro1 lacZ constructs were released with a Kpn I and Sac II digest.

The -10/-4kb hsp lacZ (hspAlacZ) construct was derived from a fusion of three fragments: a 3.5 kb Sal I/Kpn I fragment (the same as used for the -10/-4kb Pro1 lacZ construct), a 2.2 kb Kpn I/Sal I fragment in which the Sal I site was introduced by PCR...
at -4.29kb and a third Sal I/Sal I fragment containing the minimal 0.3kb hsp68 promoter ligated to lacZ (clone p610ZA; R. Kothary, Universit of Ottawa, Ottawa, Ontario, Canada; (Forghani et al., 2001; Mandemakers et al., 2000) ). The sequence of all PCR fragments was controlled by DNA sequencing. The resulting reporter construct was excised from the vector backbone with Sma I and Sph I. 

For the generation of the -3 kb Pro2 lacZ (PMP22-1B-lacZ; Maier et al., 2002) or -4 kb Pro2 lacZ vector (1ABlacZ), a 7 kb respectively a 6 kb Sal I /Sac I fragment was removed by Sal I and partial Sac I digest from the 5’ end of the -10/0kb PMP22 lacZ (PMP22-lacZ) construct. The overhang was blunt-ended using T4 DNA polymerase and the vector was intramolecularly ligated reconstituting the Sal I site. Both reporter constructs were released with a Sal I and Sac II digest.

For the generation of the Del1AlacZ construct a Avr II(blunted)/Stu I deletion for promoter 1 and Exon 1A was performed on a Kpn I/BamH I subclone of the -10/0 kb Sal I/Not I fragment from cosmid pTCF-6.1 (Magyar et al., 1996) resulting in the plasmid KBDe11A. The deletion was controlled by sequencing of the plasmid. In a next step a 3.0kb Kpn I/BamH I fragment of this KBDe11A plasmid was fused to a 6.3kb BamH I/BamH I fragment, a 2.9kb BamH I/Sal I (containing the BS vector) and a 3.8 kb Sal I/Kpn I fragment of the PMP22 -10/0kb lacZ construct.

For the generation of the Del1BlacZ construct a Bgl II/Avr II deletion for promoter 2 and Exon 1B was performed on a BamH I/BamH I subclone (clone Bam2.3) of the PMP22 cosmid pTCF-6.1 (Magyar et al., 1996) resulting in the plasmid Bam2.3De11B. The deletion was controlled by sequencing of the plasmid. In a next step a BamH I/Not I fragment of this Bam2.3De11B plasmid was fused to a Not I/BamH I fragment (containing BS vector and Exon1A) and a Not I/Not I fragment (containing the Ex2lacZ cassette) of the PMP22 -10/0kb lacZ construct.

9.2 Generation of transgenic animals

The resulting reporter constructs were excised from the vector backbone as indicated, purified by agarose gel electrophoresis, and electroeluted. The DNA was microinjected into the pronucleus of fertilized eggs from B6D2F1 hybrid mice using standard
EXPERIMENTAL METHODS

9.3 β-gal histochemical analysis

Expression of the lacZ gene was monitored by standard histochemical staining reactions with 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal; AppliChem, Germany). In the spinal cord and brainstem analysis, mice were anesthetized and perfused intracardially with 0.9% NaCl followed by 0.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4). Tissues were dissected out and postfixed for 10 (sciatic nerve) to 60 min at 4°C in 2% formaldehyde, 0.2% glutaraldehyde in phosphate buffered saline (PBS), pH7.4. The specimens were washed three times with 1x PBS, equilibrated in 30% sucrose overnight, and embedded in O.C.T. compound (Tissue Tek) for cryostat sectioning. 30 µm-cryostat sections were stained for several hours (sciatic nerve) or overnight (embryos, spinal cord sections) in the X-gal staining solution (5mM K3[Fe(CN)6], 5mM K4[Fe(CN)6], 2mM MgCl2 , 1mg/ml X-Gal, in 1x PBS).

Embryos were fixed for 1 hour in 2% formaldehyde, 0.2% glutaraldehyde, 0.1% sodium deoxycholate, 0.02% NP-40 in 1xPBS, washed three times in 1x PBS, cryoprotected over night in 30% sucrose, and stored at –80°C. Whole mount stainings were performed before cryoprotection in modified X-Gal staining solution (5mM 5mM K3[Fe(CN)6], 5mM K4[Fe(CN)6 ], 2mM MgCl2 , 0.1% sodium deoxycholate, 0.02% NP-40, 2mg/ml X-Gal in 1x PBS). E19, P1 and P5 animals were decapitated and the head and two pieces of the body were processed individually.

Sciatic nerves were dissected, fixed, teased in PBS buffer as described in (Neuberg et al., 1999), transferred to glass slides, briefly air-dried, and incubated in the X-gal staining solution.
**EXPERIMENTAL METHODS**

*Differences for part III:*

Tissues were dissected out and fixed for 10 (sciatic nerve) to 45 min (spinal cord) in 2% formaldehyde, 0.2% glutaraldehyde in phosphate buffered saline (PBS), pH 7.4, at 4°C. The specimens were washed three times with PBS and stained overnight in the X-gal staining solution (5mM K₃[Fe(CN)]₆, 5mM K₄[Fe(CN)]₆, 2mM MgCl₂, 0.1% sodium deoxycholate, 0.02% NP-40, 1mg/ml X-Gal, in 1x PBS) at 37°C. For X-gal staining of teased sciatic nerve the nerves were fixed, the epineurium of the nerve was removed to ensure penetration of the staining solution and stained overnight at 37°C. In a next step the nerve was teased in PBS buffer as described in (Neuberg et al., 1999) and transferred to glass slides and air-dried. Finally, teased fiber preparations were mounted in AF1 (Citifluor, Canterbury, UK) supplemented with DAPI (1:1000 Roche Diagnostics, Switzerland). For the quantification of β-gal positive internodes at least 100 DAPI-stained nuclei corresponding to 100 internodes were counted for each animal and analysed for associated X-Gal staining.

### 9.4 β-gal solution assay

Organ samples (approx. 100mg) or 10-15mm of sciatic nerve from transgenic and control mice were homogenized with a Polytron homogenizer in lysis buffer (0.1M potassium phosphate buffer, pH 7.8; 0.2% Triton X-100, 0.5mM dithiothreitol). Cell debris was removed by centrifugation and aliquots of each homogenate were stored at –80°C. β-gal activity was assayed in triplicates with the Galacto-Star™ kit (Tropix) according to the manufacturers’ instructions. In brief, 10 µl of the lysate (or of a 1:50 dilution with lysis buffer in cases of very high β-gal levels) were mixed with 100 µl of reaction buffer. After 30-45 min the chemiluminescence was determined in a scintillation counter (Canberra Packard SA). The amount of protein in each sample was measured using the Bio-Rad DC protein assay in triplicate with BSA as a standard. The light emission representing the enzymatic β-gal activity (in relative light units RLU) was then normalized to the amount of protein.

*Differences for part III*
For each time point 4 - 6 sciatic nerves from transgenic and control mice were homogenized individually with a Polytron homogenizer, β-gal activity was assayed in triplicates with the Galacto-StarTM kit (Tropix) according to the manufacturers’ instructions and as described above.

9.5 Quantitative analysis of PMP22 mRNA levels

Total RNA was isolated using TRIzol reagent (Gibco BRL) according to the manufacturer’s recommendations. Briefly, nerves were homogenized in the presence of TRIzol with a Polytron homogenizer (PT 1200, Kinematica AG, Switzerland). Insoluble material was removed from the homogenate by centrifugation for 10 min at 4°C, the supernatant extracted with chloroform and precipitated with isopropanol. 500 ng of total RNA was reverse transcribed in a 20 µl reaction using 140U MMLV reverse transcriptase (Promega), 2.5 µM random hexamer primers, 1 mM dNTP, 30U RNasin in 1x Promega reverse transcription buffer.

Five different PCR reactions were performed with the TaqMan PCR system to analyze five different transcripts (Fig. 5-1): The 1A-PMP22 transcript with the forward primers Pr1A (5’-GAGGAAGGGGTTACACCATTG-3’) located on exon 1A and the PMP22 sequence specific backward primer Pr2PMP22 (5’-GCAACACTAGC-ACCGCGAT-3’) located in the second half of exon 2, the 1B-PMP22 message with Pr1B (5’-TGTGCCTGAGGCTAATCTGC-3’) located in exon 1B and the primer Pr2PMP22, the 1A-lacZ transcript with Pr1A and the Pr2lacZ (5’-GTGAGCACCGGAACGGC-3’) which is specific for the sh ble-lacZ message, and the 1B-lacZ transcript with primers Pr1B and Pr2lacZ. To standardize between different samples, GAPDH mRNA levels were analyzed with the primers GAPDH-f (5’-TGTGTCCCGTTCGATCGTCA-3’) and GAPDH-b (5’-CTGCTTTCACC-ACCTTCTTGA-3’). To measure in real time the amount of amplified PCR product, the TaqMan system (AP Applied Biosystems) was used with the following sequence specific probes: 5’-TCCTCTGATCCCAGCCCACTCC-3’ (with 5’ FAM reporter and 3’ TAMRA quencher dye modifications) located in the first half of exon 2 in the common sequence for the 1A-PMP22, 1B-PMP22, 1A-lacZ and 1B-lacZ message, and 5’-
CCGCTGGAGAACCTGCCAAGTATG-3' (5' VIC, 3' TAMRA) specific for the GAPDH message. Purified PCR products were prepared as serial dilutions from 10^{-9} to 10^{-15} M for the construction of the standard curves. Amplifications were performed in triplicate 25 µl reactions for 40 cycles (denaturation at 95°C for 20s, annealing at 53°C for 30s, elongation at 60°C for 60s) on the ABI Prism 7700 sequence detection system. The PCR reaction contained 1mM MgCl2, 2.8% DMSO, 500nM forward and backward primers, 200nM TaqMan probe and 1 µl template from the RT reaction in addition to 12.5 µl 2x TaqMan Universal PCR Master Mix (AP Applied Biosystems). The quantitation of gene expression was performed as described in the User Bulletin #2, ABI PRISM 7700 Sequence Detection System, PE Applied Biosystems.

**9.6 -10/0kb PMP22 lacZ x PMP22^{-/-} and -10/0kb PMP22 lacZ x Tr mice**

To obtain -10/0kb PMP22 lacZ transgenic mice on a PMP22-deficient background (-10/0kb PMP22 lacZ; PMP22^{-/-}) in a first breeding -10/0kb PMP22 lacZ transgenic animals Line 48.4 were mated with PMP22^{-/-} females (Adlkofer et al., 1995). In a second breeding -10/0kb PMP22 lacZ transgenic mice heterozygous for PMP22 (-10/0kb PMP22 lacZ; PMP22 wt/-) were mated with PMP22^{-/-} females. Homogenates of single sciatic nerves of -10/0kb PMP22 lacZ / PMP22^{-/-} animals were prepared as described and compared with age-matched -10/0kb PMP22 lacZ transgenic animals without PMP22 mutations. PCR analysis of PMP22-deficient mice has been reported elsewhere (Sancho et al., 2001).

-10/0kb PMP22 lacZ transgenic animals with the Tr point mutation in the PMP22 gene (Suter et al., 1992b) were obtained by mating -10/0kb PMP22 lacZ transgenic mice Line 48.4 with females heterozygous for the Tr mutation (Tr/+). β-gal solution assays were performed if possible with siblings otherwise with age-matched animals with or without the PMP22 Trembler mutation at postnatal day 21 and in adult P60 and P90 animals. PCR analysis of Tr mice has been reported elsewhere (Adlkofer et al., 1997b).
9.7 Sciatic nerve transection and crush

Using aseptic technique, the sciatic nerve of anesthetized (Ketamine 80mg/kg and Xylazine 4mg/kg) adult (9-12 weeks old) -10/0kb PMP22 lacZ transgenic mice were exposed at the sciatic notch. Nerves were doubly ligated, transected with fine scissors and the nerve-stumps were sutured at least five millimeter apart to prevent regeneration. Nerve crush was produced by tightly compressing the sciatic nerve at the sciatic notch with flattened forceps twice, each time for 10s; this technique causes the axons to degenerate, but allows axonal regeneration. At varying times after nerve injury, 3-4 animals for each time point were sacrificed, the distal nerve-stumps were removed, the most proximal 4-5 mm trimmed off and further processed as described earlier. For transsected nerves, the entire distal nerve-stump was taken just below the lesion. At each time point the homogenate of the distal part of the lesioned nerve was compared with the equivalent part of the contralateral unlesioned nerve.

9.8 Immunocytochemistry of dissociated dorsal root ganglia (DRG)

Dorsal root ganglia of E19 embryos were isolated, digested with 0.2% trypsin for 20 min, triturated after the addition of DMEM/10%FCS, washed once with DMEM/10%FCS, and plated on poly-L-lysin coated plastic dishes (Corning) in 10%FCS/DMEM/NGF (50ng/ml, Sigma). After 3 days, cells were washed, fixed for 10 min with 4% formaldehyde, incubated for 4-6 hours with blocking solution (10% NGS, 0.1% Triton X-100, 1% BSA in 1x PBS) and stained with rabbit polyclonal antibodies against β-galactosidase (1:300, CN Kappel) for 12-14 hours and with mouse monoclonal antibody against NF160 (1:500, Sigma) for 1 hour, washed with PBS and incubated for 1 hour with the secondary antibodies goat anti-mouse Cy3 and goat anti-rabbit FITC (1:300, Jackson Laboratories). Immunoreactivity was visualized by conventional fluorescence microscopy using a Hamamatsu Colour Chilled 3CCD Camera in conjunction with Adobe Photoshop 5.0.
**Differences for part III**

Dorsal root ganglia of postnatal day P4 mice were isolated and digested with 0.25% trypsin and 0.3mg/ml collagenase type I (Worthington) in Ca\(^{2+}\)/Mg\(^{2+}\) free Hank’s Balanced Salt Solution for 45 min at 37°C. After the addition of 100µl FCS/ml to the digestion mix, cells were triturated and further processed as described above.

**9.9 Cell culture, transfection and reporter assays**

*Promoter deletion study in MSC80 and NIH3T3 cells*

The mouse Schwann cell line MSC80 (Boutry *et al.*, 1992) and the NIH 3T3 cell line were transfected using SuperFect reagent (Qiagen, Switzerland) according to the manufacturer’s recommendations. Equimolar amounts of -10/0kb PMP22 lacZ, -3/0 kb Pro1 lacZ, -4/0 kb Pro1 lacZ, Del1AlacZ, Del1BlacZ, pSV-β-galatosidase control vector (Promega) or empty lacZ vector (pUT111, Cayla, Toulouse, France) were used as reporter constructs and total amount of plasmids was kept constant by addition of empty Bluescript Vector (Stratagene). The plasmid SV40Luciferase (pGL3-Promoter Vector, Promega) was used as an internal control to assess transfection efficiency and for normalization. Cells were maintained in DMEM supplemented with 10% FCS and 10µM forskolin if indicated. Forty hours after transfection the cells were washed once with 1x PBS, lysed in 1x reporter lysis buffer (Promega) and extracts were assayed for β-gal activity in microtiter plates in triplicate according to (Sambrook *et al.*, 1989) and for luciferase activity with the LucLite Plus Luminescence kit (Packard Biosciences).

*Incucible Sox10 expression in N2A cells*

RNA was extracted with the Trizol Method from N2A cells stably transfected with the reverse tetracycline-controlled transactivator (rtTA, harboring the G418 resistance) and the cDNA of Sox10 under a tetracycline-regulatable promoters (harboring a hygromycin selection cassette). The cells were maintained in DMEM + 0.5% FCS and Sox10 expression was induced for 48 hours with 5µg/ml Doxycylin. The generation of this N2A cell line, the PCR Primers and cDNA Synthesis is described in (Peirano *et al.*, 2002).
To standardize between different samples, 18S rRNA levels were measured with a predeveloped assay reagent from Applied Biosystems.

**Screening for upregulated transcription factors with quantitative PCR**

Subconfluent (40-60%) rat Schwann cells were infected overnight with the Adenovirus Adegr2GFP and AdGFPSJS2 or AdGFPR1* (Ehrengruber et al., 2000) as control virus either in DMEM Medium in the presence of 10% FCS or in defined N2 medium after 48 hours. Total RNA was isolated from the cells after 30, 24 or 48 hours as indicated with the RNeasy Total RNA Purification Mini Prep Kit (Qiagen, Switzerland) according to the manufacturers’ instructions. Homogenisation of cell lysates was done using the syringe method by passing the lysate three to four times through a sterile plastic syringe fitted with a 18-21 gauge needle. Reverse transcription was performed as described in section 9.5 above. SYBR Green PCR were performed at standard conditions as recommended by Applied Biosystems (25mM MgCl2, 12mM dNTP (containing dUTP), Amp Erase, AmpliTaq Gold, 500nM forward and backward primer, 3µl of 1:5 diluted RT reaction). To standardize between different samples, 18S rRNA levels were measured with a predeveloped assay reagent from Applied Biosystems with TaqMan PCR. The Oligonucleotides used in the quantitative SYBR Green PCRs are:

<table>
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<tr>
<th>Oligo#</th>
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<td>rKZF1-b</td>
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9.10 Sequence analysis and determination of potential binding sites

Global alignments of orthologous PMP22 loci were done with available murine (accession number AL 592215.12) and human (AC 005703) sequences containing the PMP22 gene loci, using VISTA homology plot software (http://www-gsd.lbl.gov/vista/index.html; Dubchak et al., 2000; Mayor et al., 2000). The results obtained were compared with the percent identity plot (PIP) constructed with Pipmaker software at http://bio.cse.psu.edu/pipmaker (data not shown). The three species comparison of the -10/0kb region was done with VISTA homology plot software with orthologous sequences of mouse, human, and rat (AC 108967).

To screen the murine genomic DNA sequences for interspersed repeats and low complexity DNA sequences, the RepeatMasker program was used (Smit, AFA & Green, P RepeatMasker at http://ftp.genome.washington.edu/RM/RepeatMasker.html and references therein).

Screening for potential binding site was performed on the mouse -10/0kb fragment (AL 592215.12) with the MatInspector software (Quandt et al., 1995) with all available vertebrate matrices (core similarity: 0.75; matrix similarity: optimized; Matrix Family Library Version 2.4 May 2002). The software is available online at http://www.genomatix.de and is based on the TRANSFAC databases (Heinemeyer et al., 1998) (http://transfac.gbf.de/).


REFERENCES


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transmembrane domain of the 22-kDa peripheral myelin protein in the trembler-J mouse. 


REFERENCES


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Degree obtained: Matura type C (natural science)

1991 Award of the Swiss Organisation "Science and Youth" for a thesis in the area of ecological investigation of dragonflies

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May 1998 Diploma, Dipl. Natw. ETH in cell biology, immunology, molecular biology, biochemistry and genetics at the Swiss Federal Institute of Technology (ETH) Zürich, Switzerland

1999-2002 Ph.D. student, Institute of Cell Biology, ETH Zürich, Switzerland
Area of research: Molecular and Cellular Neurobiology
Title of the Project: Transcriptional regulation of the CMT1A-disease gene Peripheral Myelin Protein (PMP22)

2002 ASN Young Investigator Education Enhancement Award for the American Society of Neurochemistry (ASN) Meeting

**Scientific Training and Research experience**

1999-2002 Postgraduate courses and exams in different areas of neurobiology at the Neuroscience Center Zürich, University of Zürich, Switzerland
1999-2002 Ph. D. training in laboratory techniques and methods:
molecular biology: standard DNA cloning techniques, RNA techniques, DNA extractions, Northern and Southern blot, quantitative real time (RT)-PCR (TaqMan), In situ RNA Hybridisation

cell biology: immunohistochemistry, classical histological stainings, semithin sections, electron microscopy

cell culture: standard cell culture techniques, primary mouse Schwann cell cultures, DRG explant cultures/in vitro myelination, cotransfection studies & reporter gene assays, adenoviral infections

animal experiments: establishing and maintaining transgenic mouse and rat lines, performing sciatic nerve crushes

2001
Training in laboratory animal experiments (LTK Module 1) at the Institute of Laboratory Animal Sciences, University of Zürich, Switzerland: Handling and treatment of animals, welfare and ethical issues

Additional professional skills and activities

1993-1996 Leader of Boy Scout troop with ca. 50 members

September - October 1996 Training in clinical chemistry and analysis at the Hospital Lachen, Switzerland during military service.

December 1998 - February 1999 General assistant in the Management unit of the information technology department at Union Bank of Switzerland (UBS), Zürich

Languages

German: mother tongue

English: fluent written and spoken; 5 years during high school; First Certificate 1993; 1996: 1 month English language course in San Diego, USA

Spanish: intermediate; 1 year during high school, 1998: 1 month Spanish language course in Couzco, Peru

French: intermediate; 6 years during High School

Conferences and presentations


Publications


Maier M., Castagner F., Berger P. and Suter U. (2003). Dissection of the Peripheral Myelin Protein 22 (PMP22) Promoter in vivo Reveals a Late Myelinating Schwann Cell Specific Element, in preparation

Referees

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