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Control of myelination in Schwann cells: a *Krox20* *cis*-regulatory element integrates Oct6, Brn2 and Sox10 activities

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Myelination in Schwann cells is governed by several transcription factors, including the POU proteins Oct6 and Brn2, the high mobility group protein Sox10 and the zinc-finger protein Krox20. How the function of these factors is integrated in the control of myelination has not been established. Previously, we identified an enhancer element controlling *Krox20* expression throughout myelination in Schwann cells. In this paper, cell culture experiments were combined with transgenesis to identify transcription factors acting directly upstream of *Krox20*. The results show that during the promyelin–myelin transition, *Krox20* expression is directly activated by Oct6 and Brn2 acting on this enhancer. In addition, the enhancer-dependent synergism between these POU proteins and Sox10 suggests that *Krox20* expression requires this combination of factors. These results resolve previous controversy concerning the mechanism of action of Oct6 and Brn2 during myelination and provide an explanation for myelin deficiencies in Waardenberg–Hirschsprung disease patients whereby *Sox10* mutations could lead to a loss of *Krox20* expression.

Keywords: *Krox20/Egr2*; *Oct6/Tst1/SCIP/Pou3f1/Oft-6*; *Brn2/N-Oct-3/Pou3f2/Oft-7*; myelination; peripheral nervous system; transcriptional regulation
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INTRODUCTION

The myelin sheath, which serves to increase nerve conduction velocities, is deposited around axons by specialized cells in the central and peripheral nervous systems of higher vertebrates. In the peripheral nervous system, myelin is synthesized by Schwann cells and, so far, several transcription factors participating in the onset of myelination have been described. A pivotal factor is the zinc-finger transcription factor gene *Krox20* (*Egr2*), the mutation of which in the mouse blocks Schwann cells at the promyelinating stage (Topilko *et al*, 1994). Consistently, *Krox20/Egr2* mutations have also been identified in patients suffering from peripheral neuropathies (Warner *et al*, 1998). Taken together with cell culture experiments showing that myelin genes are induced by Krox20 (Nagarajan *et al*, 2001), these data suggest that *Krox20* has characteristics of a master regulator of myelination. Previously, our studies into the regulation of *Krox20* identified a transcriptional enhancer, designated the myelinating Schwann cell element (MSE), which is under the control of the POU domain transcription factor *Oct6* (Ghislain *et al*, 2002).

Oct6 is transiently expressed in Schwann cells, peaking at the promyelinating stage (Jaegle *et al*, 2003). The analysis of *Oct6* loss-of-function alleles indicated that mutant Schwann cells show a transient delay in myelination (Jaegle *et al*, 1996). More recently, the related POU gene, *Brn2*, expressed in Schwann cells in a manner similar to *Oct6*, was shown to compensate for the *Oct6* mutation, the combined loss of both Oct6 and Brn2 provoking a more severe delay in myelination (Jaegle *et al*, 2003). Although a role for *Oct6* and *Brn2* in promoting myelination is established, their mechanism of action remains controversial, as no data providing a satisfactory molecular explanation have been put forward.

The SRY-related high-mobility group (HMG) domain protein Sox10 is expressed throughout Schwann cell development (Kuhlbrodt *et al*, 1998). However, the early loss of Schwann cells in *Sox10* mutant mice precluded the analysis of a possible function in myelin formation (Britsch *et al*, 2001). Nevertheless, Waardenberg–Hirschsprung disease patients carrying dominant *Sox10* mutations often show peripheral neuropathies characterized by normal Schwann cell numbers in the absence of myelin (Inoue *et al*, 2002), which suggests that Sox10 is involved in myelination. Indeed, the capacity of Sox10 to directly regulate myelin genes in Schwann cells provides a possible basis for this phenotype (Peirano *et al*, 2000). The paradigm of Sox–Oct cooperation in gene regulation (Kamachi *et al*, 2000) could provide a further explanation. However, whereas cell culture transfection studies have shown that Sox10 can cooperate with Oct6 (Kuhlbrodt *et al*, 1998), this activity has not been linked to Schwann cell myelination.

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In this paper, transgenic and cell culture experiments were combined to show that the related POU proteins Oct6 and Brn2 activate transcription of *Krox20* by directly binding to the MSE. In addition, Sox10 was found to synergize with these POU proteins on this element. Overall, these data suggest a molecular model that integrates the principal myelin transcription factors, in which Oct6 and Brn2 cooperate with Sox10 in driving *Krox20* expression and thereby controlling myelination in Schwann cells.

RESULTS

Oct6 and Brn2 activate *Krox20* by binding to the MSE

In previous studies, a 1.3 kb *cis*-regulatory element located at +35 kb relative to the start site of transcription of the mouse *Krox20* gene was identified (Ghislain *et al*, 2002). This element, designated the MSE, directs *Krox20* expression throughout myelination in Schwann cells. To facilitate the search for transcription factor-binding sites in the enhancer, orthologous sequences from human and chicken genomes were identified (Fig 1).

Previously, we showed that *Oct6* acts upstream of the MSE to control *Krox20* expression during Schwann cell myelination (Ghislain *et al*, 2002). Here, the possibility that *Krox20* transactivation is mediated by the direct binding of Oct6 to sequences in the MSE was tested. Co-transfection of an *Oct6* expression vector and the mouse 1.3 kb MSE fused to a minimal promoter/*lacZ* reporter led to a significant increase in reporter gene activity in both the glioblastoma cell line U138 and HeLa cells when compared with controls (Fig 2A). To identify regions responding to Oct6 in the MSE, a series of 5′ deletions were generated. A significant decrease in activity was observed on deletion of the *Pst*I–*Ban*II region, with a further weak but reproducible contribution of the *Psp*1406I–*Pst*I region in HeLa cells (Fig 2A). These results indicate that essential *cis*-regulatory information for Oct6 transactivation is located between *Psp*1406I and *Ban*II. Direct binding of Oct6 to this region was tested in a bandshift assay. The addition of bacterial extracts containing the Oct6 protein produced several specific complexes that were absent in the control extracts (Fig 2B; data not shown). The Oct6-binding sites in this region were then delimited in DNase I footprinting assays. Two subregions, *Psp*1406I–*Pst*I and *Pst*I–*Ban*II, were assayed, showing four footprints with extracts containing Oct6 that were absent with control extracts (Fig 2C, I–IV; data not shown). Sequence comparisons between human, mouse and chick confirmed the presence of several conserved sequences similar to the Oct6 consensus binding site in these footprints (Li *et al*, 1993; Fig 1). AT to GC substitutions were then introduced to specifically eliminate the putative Oct6-binding sites (Fig 1), resulting in the elimination of the Oct6-binding activity of the *Psp*1406I–*Ban*II fragment (Fig 2B).

Having defined several Oct6-binding sites in the MSE, the role of these sites in the activity of the MSE by Oct6 was tested both *in vitro* and *in vivo*. In addition, as the related POU protein Brn2 has been shown to compensate for the myelination defect in *Oct6* mutant nerves (Jaegle *et al*, 2003), the possible involvement of these sites in transactivation by Brn2 was also investigated. Mutations in Oct6-binding sites II–IV or I–IV were introduced into the full-length MSE fused to a minimal promoter/*lacZ* reporter (Fig 3A). Whereas strong stimulation of reporter activity was detected with the wild-type MSE following co-transfection of the Oct6 expression vector in both HeLa and U138 cells, both mutant MSE constructs showed behaviour similar to the enhancerless control construct (Fig 3B). Similar results were obtained following co-transfection of the Brn2 expression vector (Fig 3B). The POU factors Brn5 and Oct1 were inactive on the wild-type MSE (data not shown), which suggested that MSE-dependent reporter activity is specific to Oct6 and Brn2. The effects of the Oct6 binding site mutations were then analysed by mouse transgenesis. Whereas the wild-type MSE led to 54% of lines expressing a high level of β-galactosidase activity in sciatic nerve Schwann cells after birth (Ghislain *et al*, 2002; Fig 3A,C), mutation of the Oct6-binding sites II–IV and I–IV reduced this frequency to 22% and virtually eliminated this activity, respectively (Fig 3A,D; data not shown), disclosing important roles of Oct6-binding sites II–IV and of site I in the activity of the MSE during myelination. In the mature nerve, although the wild-type element led to 30% of high-expressing lines (Fig 3A,E), such levels of expression were never observed with either of the mutant constructs (Fig 3A,F; data not shown).

Overall, these results strongly suggest that during myelination, *Krox20* expression is specifically controlled by the direct binding of Oct6 and Brn2 to the MSE. Interestingly, although both *Oct6* and *Brn2* are only transiently expressed after birth (Jaegle *et al*, 2003), their binding sites are still required for MSE-dependent reporter expression in the mature myelinating cells, suggesting the involvement of other POU genes.

Synergism between Sox10 and POU factors

As the HMG protein Sox10 is expressed throughout Schwann cell development and Sox10 has been shown to cooperate with Oct6 (Kuhlbrodt *et al*, 1998), we suggested that *Krox20* expression depends on a cooperation between these factors acting on the MSE. Indeed, interspecies sequence comparisons identified several conserved putative Sox10-binding sites in the MSE (Fig 1). In the transactivation assay using the wild-type 1.3 kb MSE, although both Oct6 and Sox10 moderately activated reporter gene expression, combining these factors led to a strong synergistic activation in both U138 and HeLa cells (Fig 4A,B). Interestingly, although the responsiveness of 5′ deletions of the MSE to Sox10 alone correlated with the presence of putative Sox10-binding sites, the synergism between Sox10 and Oct6 required a core region, *Stu*I to *Ban*II, containing the demonstrated Oct6-binding sites and most of the putative Sox10-binding sites (Fig 4B). As Brn2 acts similar to Oct6, its activity on the wild-type MSE was also tested. Similar to Oct6, Brn2 strongly synergized with Sox10 in HeLa cells (Fig 4C). Interestingly, neither Oct6 nor Brn2 cooperated with Sox2, although this factor has been shown to synergize with other POU proteins (Fig 4C; Kamachi *et al*, 2000). Finally, given the synergism between Oct6/Brn2 and Sox10 on the wild-type MSE, we tested the responsiveness of the MSE Oct6 binding site mutants. Consistent with their loss of activity in transgenic experiments, both mutant constructs II–IV and I–IV, although they largely retained their responsiveness to Sox10 alone, were not cooperatively activated by Oct6 or Brn2 with Sox10 (Fig 4C). The addition of Oct6 or Brn2 actually led to a repression of the Sox10 inducing activity, which may be due to Sox10 sequestering.

Overall, these data show a specific cooperation between Sox10 and the POU proteins Oct6 and Brn2 in MSE-dependent enhancer activity and suggest that Sox10 participates in myelin formation by controlling *Krox20* expression.

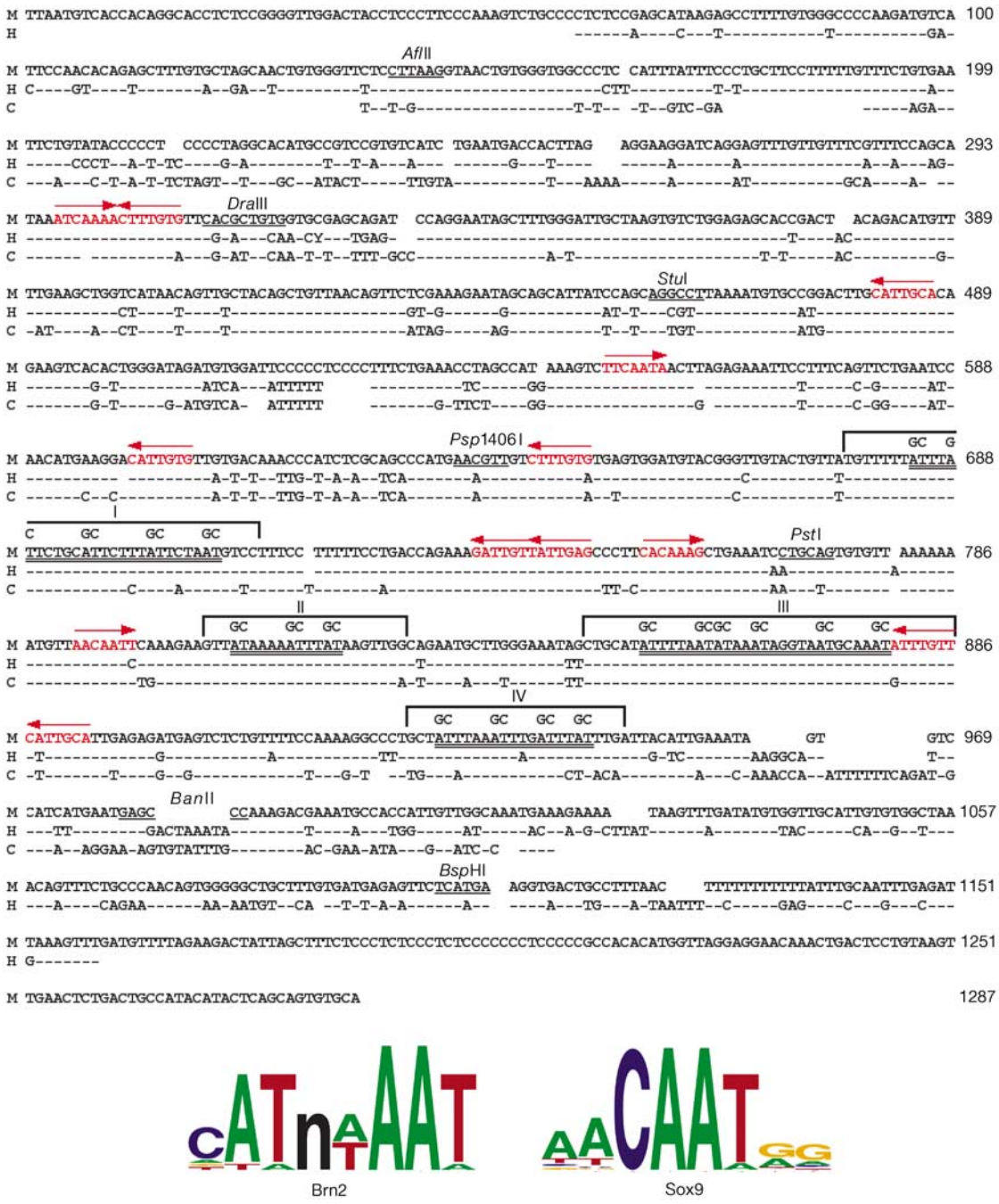


Fig 1 | Alignment of human and chick sequences orthologous to the mouse myelinating Schwann cell element (MSE) shows several conserved transcription factor-binding sites. The nucleotide sequence of the mouse 1.3 kb MSE is shown (Ghislain *et al*, 2002). Nucleotide numbering corresponds to the mouse sequence. Restriction sites used in deletion studies are indicated. Conserved residues identified in the human and chick genomes are aligned to the mouse sequence and indicated as dashes. Oct6 footprints are indicated (I–IV; see Fig 2C). In these regions, conserved candidate binding sites for Oct6 and Brn2 are double underlined. These sites are based on the Brn2 binding site matrix (lower left; *n* can be either 0, 2 or 3 nucleotides) and Oct6 and Brn2 show similar binding characteristics (Li *et al*, 1993). The AT to GC substitutions introduced to inactivate these sites are shown. Conserved candidate Sox10-binding sites are shown in red with arrows. These sites are based on the Sox9 binding site matrix (lower right; Mertin *et al*, 1999), and Sox9 and Sox10 have similar DNA-binding characteristics (Peirano *et al*, 2000). Binding site matrices were generated using the WebLogo program (Crooks *et al*, 2004). Stack height reflects conservation and symbol height indicates frequency of residue. C, chick; H, human; M, mouse.

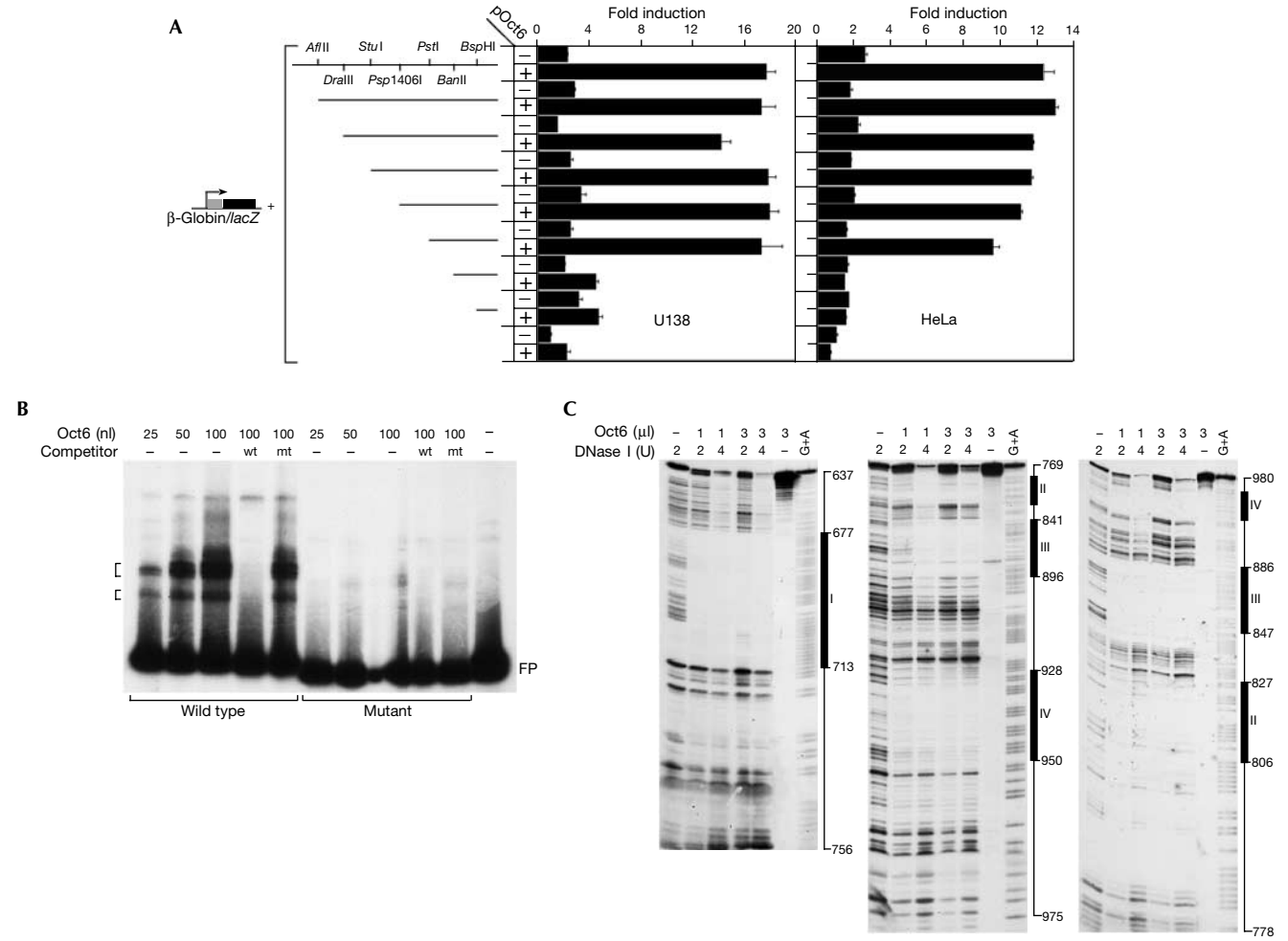
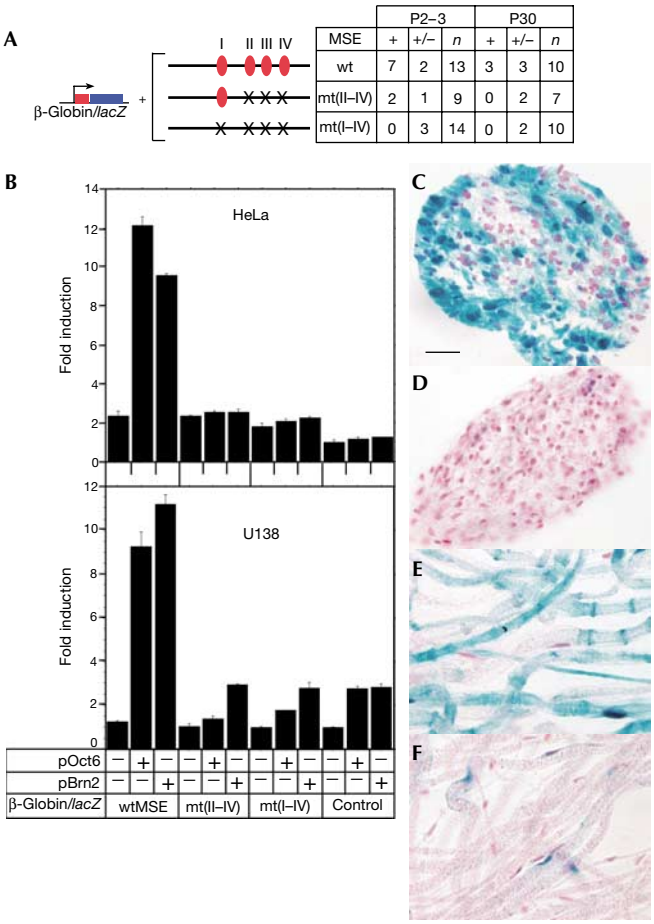


Fig 2 | Identification of Oct6-binding sites in the myelinating Schwann cell element. (**A**) 5′ deletions of the 1.3 kb myelinating Schwann cell element (MSE) fused to a minimal β-globin promoter/*lacZ* reporter were transfected into U138 (left) and HeLa (right) cells, with 300 or 6 ng/well, respectively, of the expression vector, empty (−) or carrying the *Oct6* coding sequence (+). The data show the mean β-galactosidase activity of two independent, normalized experiments carried out in duplicate. Values for transfections with the empty promoter/reporter and expression plasmids were set to one. Data from all other transfections are presented as the fold induction over this level. Error bars represent the standard error. (**B**) The wild-type MSE subfragment, *Psp*1406I–*Ban*II (left), or a mutant version containing the AT to GC substitutions indicated in Fig 1 (right) were used as probes in bandshift experiments with increasing amounts of Oct6-containing bacterial extracts. As a control, both probes were combined without the bacterial extract (far right). To identify specific complexes, unlabelled competitor oligonucleotides corresponding to a high-affinity Oct6-binding site (wt) or a mutant version unable to bind to Oct6 (mt) were included in the binding reaction at a 200-fold molar excess. Specific complexes are indicated with brackets. FP, free probe. (**C**) The upper (left) and the upper (centre) and lower (right) strands of the *Psp*1406I–*Pst*I and the *Pst*I–*Ban*II fragments, respectively, were analysed for Oct6 binding in DNase I footprinting assays using extracts from Oct6-expressing bacteria. Nucleotide numbering corresponds to the mouse 1.3 kb MSE sequence (Fig 1). The positions of the Oct6 footprints are indicated (I–IV).

DISCUSSION

In this paper, transcription factors acting directly upstream of *Krox20* during myelination in Schwann cells have been identified. The results indicate that *Krox20* expression depends on a synergistic interaction of the POU proteins, Oct6 and Brn2, with Sox10 on sequences in the *Krox20* MSE enhancer. As Krox20 directs myelination in Schwann cells, the MSE has a central role in this process by integrating the activities of Oct6, Brn2 and Sox10.

Our results establish that Oct6 activates transcription of *Krox20* by binding to the MSE, providing a molecular explanation for the *Oct6* mutant phenotype. However, Oct6 is required only transiently (Jaegle *et al*, 1996), whereas Krox20 is necessary throughout myelination (Topilko *et al*, 1994; L. Decker, E. Taillebourg & P.C., unpublished data). Consistent with this, *Krox20* is expressed in *Oct6* mutants as myelination resumes (Ghazvini *et al*, 2002). Previously, *Brn2* was shown to act redundantly to *Oct6*: their combined deletion leads to a more severe block in myelination than that of *Oct6* mutants alone and *Brn2* can substitute for *Oct6* in rescue experiments of *Oct6* mutant Schwann cells (Jaegle *et al*, 2003). As shown in this study, the capacity of Brn2 to substitute for Oct6 and directly activate *Krox20* provides a molecular explanation for



MSE	P2–3 +	P2–3 +/−	P2–3 *n*	P30 +	P30 +/−	P30 *n*
wt	7	2	13	3	3	10
mt(II–IV)	2	1	9	0	2	7
mt(I–IV)	0	3	14	0	2	10

Fig 3 | Oct6-binding sites are essential for the *in vivo* activity of the myelinating Schwann cell element. (**A**) Schematic representation of the wild-type (wt) 1.3 kb myelinating Schwann cell element (MSE) and mutant MSE carrying mutations in the Oct6-binding sites II–IV (mt(II–IV); Fig 1) and I–IV (mt(I–IV); Fig 1) fused to a minimal β-globin promoter/*lacZ* reporter. Results of transgenic experiments are shown. *n*, the number of transgene positive mice analysed for β-galactosidase activity in the sciatic nerve at postnatal day (P) 2–3 or 30; +, strongly β-galactosidase-positive sciatic nerves with levels similar to those shown in (**C,E**); +/−, weakly β-galactosidase-positive sciatic nerves with levels similar to those shown in (**D,F**). (**B**) The wild-type 1.3 kb MSE and mutant constructs were transfected into HeLa (upper) and U138 (lower), with either 6 or 75 ng/well, respectively, of the expression vector, empty (−) or carrying the murine *Oct6* or *Brn2* coding sequence (+). Control, promoter/reporter plasmid without MSE. Presentation of transfection data is described in the legend of Fig 2. (**C–F**) β-Galactosidase-positive sciatic nerves from P2–3 (**C,D**; transverse section) and P30 (**E,F**; teased nerve) mice carrying the wild-type 1.3 kb MSE (**C,E**, (+) in **A**) or mt(I–IV) mutant construct (**D,F**, (+/−) in **A**). Scale bar, 25 μm.

the redundancy of these factors. Is *Krox20* the sole major target of Oct6 and Brn2 in Schwann cells? The mutant phenotypes of *Oct6* and *Brn2* are similar to that of *Krox20*, suggesting that this may be the case. However, confirmation of this hypothesis awaits the forced expression of *Krox20* in *Oct6*/*Brn2* double-mutant nerves.

Myelination in the *Oct6* and *Brn2* double mutant, although severely delayed, resumes (Jaegle *et al*, 2003). Assuming that as myelination proceeds in this mutant, *Krox20* is expressed (as in the *Oct6* single mutant), then which factors are controlling its expression? An involvement of Oct6 cannot be totally excluded, as the *Oct6* mutant allele used in these studies is hypomorphic (Ghazvini *et al*, 2002). However, as *Krox20* expression is maintained in the mature myelinating nerve, in which both *Oct6* and *Brn2* have been downregulated (Jaegle *et al*, 2003), we favour the involvement of other factors. As we have shown that the Oct6 binding site MSE mutants were significantly less active in the adult compared with the wild type (Fig 3), the factors required for *Krox20* adult expression are likely to belong to the POU family. Although Brn5 and the ubiquitous Oct1 are expressed in myelinating Schwann cells (Wu *et al*, 2001; Jaegle *et al*, 2003), their inactivity on the MSE, either alone or in combination with Sox10, suggests that these are not appropriate candidates (data not shown). The involvement of other POU proteins in the adult activity of the MSE therefore needs investigation.

Our data suggest that both Oct6 and Brn2 cooperate with Sox10 to activate *Krox20* transcription through the MSE, identifying for the first time a natural *cis*-element responding synergistically to these factors. These results are consistent with previous findings identifying a synergism between Oct6 and Sox10 on the FGF4 enhancer (Kuhlbrodt *et al*, 1998) and extend these studies to show that Brn2 can also synergize with Sox10. Although a direct role of Sox10 in myelin gene regulation may in part be responsible for Schwann cell myelin deficiencies in Waardenberg–Hirschsprung disease patients carrying mutations in *Sox10* (Inoue *et al*, 2002), our results provide an alternative explanation. Owing to the synergistic role of Sox10, mutations reducing its activity could lead to a marked loss of *Krox20* expression, in turn blocking myelination. Although we have found numerous candidate Sox10-binding sites in the MSE (Fig 1), they do not present the particular dimeric organization characteristic of the P0 promoter (Peirano *et al*, 2000). This suggests that other types of dimeric site, as well as monomeric sites, are functional, at least in the context of the MSE.

In conclusion, the pivotal role of *Oct6*, *Brn2*, *Sox10* and *Krox20* in Schwann cell myelination and the importance of both *Krox20* and *Sox10* in peripheral neuropathies emphasize the importance of this study. By showing a direct link between these factors, our work provides a first insight into the hierarchy of transcription factors controlling myelination in Schwann cells and a better understanding of the molecular mechanisms underlying myelin disorders in humans.

METHODS

Cell culture transfection and transgenic mice. U138 and HeLa cell lines were maintained in DMEM supplemented with 10% FCS. Transfection and assay techniques have been described previously (Ghislain *et al*, 2003). The murine *Oct6*, *Brn2* and *Brn5* expression vectors (Jaegle *et al*, 2003) were gifts from D. Meijer. The murine *Sox10* expression vector was obtained from RZPD (clone ID: IRAVp968C0325D). The murine *Sox2* (Yuan *et al*, 1995) and human *Oct1* (Tanaka & Herr, 1990) expression vectors were gifts from L. Dailey and W. Herr, respectively. Transgenesis, identification of transgenic mice by PCR, 5-bromo-4-chloro-3-indolyl-β-D-galactoside staining of sciatic nerves and sectioning were carried out, as described previously (Ghislain *et al*, 2002).

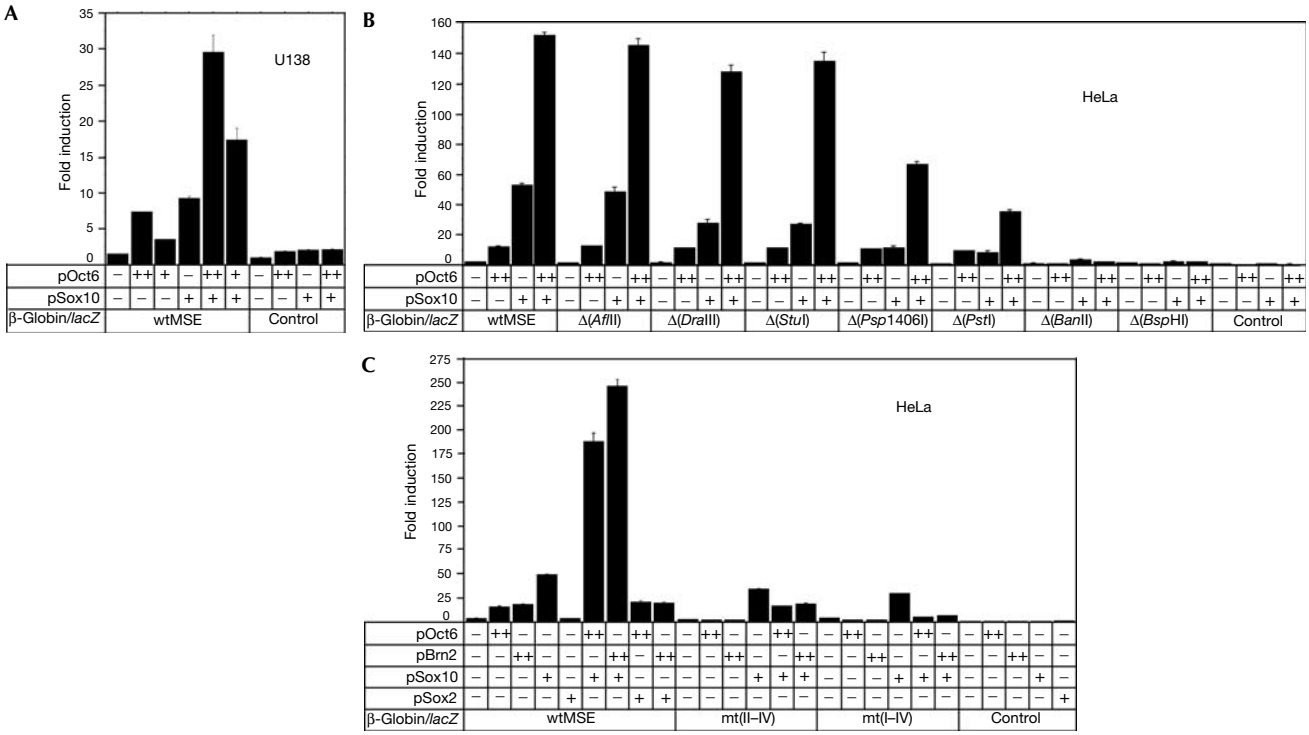


Fig 4 | Myelinating Schwann cell element (MSE)-dependent enhancer activity involves synergism between Sox10 and the POU proteins Oct6 and Brn2. The wild-type and mutant 1.3 kb MSE constructs (Fig 3A) and 5′ deletion constructs (Fig 2A) fused to a minimal β-globin promoter/*lacZ* reporter were co-transfected with the expression vector, empty (−) or carrying the murine *Oct6* or *Brn2* coding sequence (++, 6 ng/well; +, 0.6 ng/well) and/or *Sox10* or *Sox2* coding sequence (+, 60 ng/well), as indicated, into U138 (**A**) and HeLa (**B**,**C**) cells. Control, promoter/reporter plasmid without MSE. Presentation of transfection data is described in the legend of Fig 2.

Sequence analyses and isolation of the chick myelinating Schwann cell element. Sequence analyses were carried out, as described previously (Ghislain *et al*, 2003). The GenBank accession numbers for the human and mouse contigs containing sequences shown in Fig 1 are AC067751 and AC068424, respectively. Chick sequences containing homology to these sequences have been submitted to GenBank (AY519467). This sequence was isolated from a chicken genomic BAC clone carrying the *Krox20* gene (Giudicelli *et al*, 2001) by low-stringency hybridization, using the mouse 1.3 kb MSE as a probe.

DNase I footprinting, bandshift assays and mutagenesis. The pET11a (Novagen, La Jolla, CA, USA) bacterial expression plasmid containing the full-length murine *Oct6* coding sequence was a gift from D. Meijer. Protein extraction from Oct6-expressing and control bacteria, DNase I footprinting and bandshift experiments were carried out, as described previously (Ghislain *et al*, 2003). The mutant *Pst*I–*Ban*II restriction fragment of the mouse MSE carrying the AT to GC substitutions (Fig 1, sites II–IV) was synthesized chemically. The AT to GC substitutions in the *Psp*1406I–*Pst*I fragment (Fig 1, site I) were introduced using the Exsite™ PCR-based site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). All mutated subfragments were verified by sequencing. MSE mutants were generated by replacing the *Pst*I–*Ban*II fragment of either the wild-type or the mutant 1.3 kb MSE carrying the mutation in site I with the mutant version to generate MSE mutants in sites I–IV and II–IV, respectively. The wild-type and mutant 1.3 kb MSE were cloned into pBGZ40 (Ghislain *et al*, 2003).

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