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Characterisation of *cis*-acting sequences reveals a biphasic, axon-dependent regulation of *Krox20* during Schwann cell development

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SUMMARY

In Schwann cells (SC), myelination is controlled by the transcription factor gene *Krox20*/*Egr2*. Analysis of *cis*-acting elements governing *Krox20* expression in SC revealed the existence of two separate elements. 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 to 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. Genetic studies showed that both elements were active in a *Krox20* mutant background, while the activity of the MSE, but likely not of the ISE, required the POU domain transcription factor Oct6 at the time of myelination. The MSE was localised to a 1.3 kb fragment, 35 kb downstream of *Krox20*. The identification of multiple Oct6 binding sites within this fragment suggested that Oct6 directly controls *Krox20* transcription. Taken together, these data indicate that, although *Krox20* is expressed continuously from 15.5 dpc in SC, the regulation of its expression is a biphasic, axon-dependent phenomenon 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.

Key words: *Krox20*/*Egr2*, *Oct6*/*Tst1*/*SCIP*/*Pou3f1*, Schwann cell, Boundary cap, Myelination, Transcriptional regulation, Peripheral nervous system

INTRODUCTION

Myelin in the peripheral nervous system (PNS), which accelerates nerve conduction velocities, is elaborated by a specialised glial cell population known as Schwann cells. Immature Schwann cells arise from precursors in the mouse peripheral nerves between 13-15 days post coitum (dpc) (Dong et al., 1999). Those associated with large diameter axons develop after birth into promyelinating Schwann cells (Jessen and Mirsky, 1999). These cells express high levels of myelin genes such as those for myelin basic protein (MBP) and protein zero (P0) and over the next 30 days in the mouse deposit a multilamellar structure known as the myelin sheath [reviewed by Braun (Braun, 1984)]. However, not all Schwann cells are myelinating, and in the adult sciatic nerve approximately 20% of the axons remain unmyelinated, the associated Schwann cells retaining characteristics of their immature counterparts (Jessen et al., 1990; Arroyo et al., 1998).

In the PNS, development, maintenance and regeneration are dependent on an intimate dialog between axons and Schwann cells (Jessen and Mirsky, 1999). This is most potently illustrated in degeneration/regeneration experiments where damaged nerves undergo a complex process characterised by the dedifferentiation of myelinating Schwann cells distal to the site of lesion and removal of myelin and axon debris, a process known as Wallerian degeneration (Stoll and Muller, 1999). Dedifferentiation in Schwann cells is thought to be a consequence of the loss of axonal contact and has been described at the molecular level where the genes for late myelin markers such as MBP and P0 are downregulated (LeBlanc and Poduslo, 1990). Concomitantly these cells express markers typical of nonmyelinating Schwann cells, such as the glial fibrilliary associated protein (GFAP) and the low-affinity nerve growth factor receptor ($NGFR^{P75}$) (Jessen et al., 1990). Progressively axons begin to regenerate into the distal stump where axon-Schwann cell interactions initiate the process of myelination in a manner that is thought to recapitulate development.

The identification of several transcription factors that exert a pivotal role in Schwann cells has shed light on the molecular mechanisms governing myelination. These include the zinc finger protein Krox20/Egr2 and the POU protein Oct6/SCIP/Tst1/Pou3f1 (Chavrier et al., 1988; Joseph et al., 1988; Monuki et al., 1989; Meijer et al., 1990; He et al., 1991). In the mouse

PNS *Krox20* is first expressed in a specialised population of glial cells, the boundary cap, located at the entry and exit points of spinal and cranial nerve roots, beginning at 10.5 dpc (Wilkinson et al., 1989; Schneider-Maunoury et al., 1993; Niederlander and Lumsden, 1996; Golding and Cohen, 1997). At 15.5 dpc, coincident with the transition of precursors to immature Schwann cells, *Krox20* is activated throughout the PNS (Murphy et al., 1996). After birth, at the onset of myelination, *Krox20* expression reaches a peak and is subsequently expressed in myelinating Schwann cells throughout life (Zorick et al., 1996; Topilko et al., 1997). *Oct6* is first expressed at 16.5 dpc, and like *Krox20*, reaches a peak at the promyelinating stage (Blanchard et al., 1996; Arroyo et al., 1998; Mandemakers et al., 2000). However, in contrast to *Krox20*, *Oct6* is expressed only transiently in the Schwann cell lineage, being largely undetectable in the adult nerve. Both factors are regulated by axons in nerve degeneration/regeneration experiments (Zorick et al., 1996). In vitro studies involving the coculture of primary Schwann cells with dorsal root ganglia (DRG) neurones also revealed a requirement for axonal contact in the expression of both of these genes (Scherer et al., 1994; Murphy et al., 1996).

The generation of null mutant mice has revealed important roles for these factors in the process of myelination. In both *Krox20* and *Oct6* mutants, Schwann cells are blocked at the promyelinating stage (Topilko et al., 1994; Bermingham et al., 1996; Jaegle et al., 1996). Detailed analysis of *Krox20* mutants revealed that, whereas Schwann cells express early markers such as S100 and myelin associated glycoprotein (MAG), the late myelin markers, P0 and MBP, do not accumulate. Mutant cells were also shown to remain in a proliferative state, in contrast to wild-type mice in which these cells rapidly exit the cell cycle at the onset of myelination (Zorick et al., 1999). Although *Oct6* mutant Schwann cells exhibit a similar phenotype to *Krox20* mutants during the first week after birth, myelination subsequently resumes (Jaegle et al., 1996). In *Krox20* mutants, however, this block appears to be permanent, Schwann cells remain undifferentiated at the latest stage that has been tested, postnatal day 15 (P15) (Topilko et al., 1994). This has led to the suggestion that *Oct6* 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 et al., 2001). Indeed these results are reinforced by clinical studies on patients with hypomyelinating neuropathies including congenital hypomyelinating neuropathy, Charcot-Marie-Tooth type 1 and Dejerine-Sottas syndrome. These patients carry dominant or recessive point mutations affecting two domains of the Krox20 protein, the zinc finger DNA binding domain or the NAB repressor interaction domain (Warner et al., 1998; Timmerman et al., 1999). The late onset in some of these patients suggests that *Krox20* is also involved in myelin maintenance in the adult, consistent with its expression throughout life.

Although *Krox20* and *Oct6* have been shown to be important in myelination, the axon-dependent molecular events involved in their expression are only beginning to be defined (Mandemakers et al., 2000). To elucidate this regulatory network we have undertaken to identify *cis*-acting sequences controlling *Krox20* in Schwann cells using transgenesis. We have found that, expression is controlled in an axon-dependent manner by regulatory sequences acting independently in immature and myelinating stages. Together the activity of these elements reproduces the profile of *Krox20* during development and regeneration. We have also shown, using a genetic approach, that the myelinating stage element is regulated by *Oct6* at the time of myelination. The delimitation of these elements to a 1.3 kb fragment revealed the presence of multiple candidate Oct6 binding sites supporting a direct role for this factor. Our results provide insight into the molecular mechanisms regulating *Krox20* during Schwann cell development in addition to generating essential tools for the molecular dissection of this process.

MATERIALS AND METHODS

Mutant mice and genotyping

The *Krox20*^{lacZ}, *Krox20*^{cre} and *Oct6*$^{\beta\text{-}geo}$ alleles have been described previously (Schneider-Maunoury et al., 1993; Jaegle et al., 1996; Voiculescu et al., 2000). All alleles were maintained in a mixed C57Bl6/DBA2 background. Genotyping was performed by PCR on extracted tail DNA using primers described previously for the *Krox20*, *Krox20*^{lacZ} and *Krox20*^{cre} alleles (Schneider-Maunoury et al., 1993; Voiculescu et al., 2000). *Oct6* and *Oct6*$^{\beta\text{-}geo}$ alleles were identified using a common primer (sense: 5′-ggactggcgctgggcaccctctac-3′) and specific primers (antisense: 5′-tggcgcattctggatcttccgctg-3′ and 5′-cggcctcaggaagatcgcactcca-3′, respectively).

DNA constructs and transgenesis

For the generation of the transgenic lines –31/+7 *Krox20/lacZ* and –4.5/+40 *Krox20/lacZ*, equimolar amounts of the –4.5 kb to +7 kb *Krox20/lacZ* reporter were mixed with the –31 kb to –2.3 kb or the +3 kb to +40 kb cosmid inserts, respectively. In enhancer delimitation studies cosmid subfragments were cloned upstream of a minimal *β-globin* promoter/*lacZ* reporter, plasmid pBGZ40, described previously (Yee and Rigby, 1993). Inserts were purified by agarose gel electrophoresis and Elutip-d (Schleicher & Schuell) and suspended in TE (10 mM Tris, pH 7.5; 0.1 mM EDTA) for microinjection. Transgenesis was performed as described previously (Sham et al., 1993). Briefly, fertilised eggs from superovulated B6D2 × B6D2 were injected with DNA at a final concentration of 1.5 ng/μl. Injected embryos were reimplanted at the one cell stage into pseudopregnant B6/CBA mice and allowed to develop to term. For enhancer delimitation studies, founder mice were analysed, otherwise lines were established.

PCR and Southern analysis of transgenic mice

Tail DNA was analysed by PCR to screen for founder mice. PCR primers were generated to detect *lacZ* (sense: 5′-caccgatcgcccttcccaacagt-3′; antisense: 5′-gccgctcatccgccacatatcct-3′) and pcos2EMBL cosmid (Chavrier et al., 1989) sequences present in the –31 kb to –2.3 kb insert (sense: 5′-gcccagtcctgctcgcttcgcta-3′; antisense: 5′-gaatgcccatggccaccttcctg-3′) and the +3 kb to +40 kb insert (sense: 5′-gatcctctacgccggacgcatcg-3′; antisense: 5′-atggtgcatgcaaggagatggcg-3′). Of 27 mice born for the –31/+7 *Krox20/lacZ* transgene, 4 contained both vector and *Krox20/lacZ* sequences. Of 21 mice born for the –4.5/+40 *Krox20/lacZ* transgene, 3 contained both vector and *Krox20/lacZ* sequences. Mice containing both vector and *lacZ* sequences were analysed for recombination by Southern analysis. 10 μg of tail DNA, was digested with restriction enzymes and resolved on a 0.8% agarose gel. Enzymes cutting outside of the recombination site and within the *lacZ* were chosen. Following gel electrophoresis, DNA was transferred under alkaline conditions to Hybond-N+ membrane and hybridised according to the manufacturers

instructions (Amersham Pharmacia Biotech). The probe was labelled with [α-^{32}P]dATP by random priming.

Semiquantitative RT-PCR analysis

Total RNA was prepared from sciatic nerves of 18.5 dpc embryos using TRIzol reagent (Gibco BRL). RNA, approximately 350 ng, was reverse transcribed using the Superscript first strand cDNA synthesis kit with random hexamers (Gibco BRL). Oligonucleotides corresponding to *Krox20* sequences (sense: 5′-ctgggaggccccttgaccagatga-3′; antisense: 5′-tggcggcgataagaatgctgaagga-3′) and *lacZ* sequences (antisense: 5′-cggcctcaggaagatcgcactcca-3′) were used in PCR amplification of 10 ng total RNA to quantify *Krox20* and *Krox20/lacZ* transcripts. Oligonucleotides directed against 18S rRNA sequences (sense: 5′-ctcggcgccccctcgatgctcttag-3′; antisense: 5′-ctgcccggcgggtcatgggaataac-3′) were included to monitor the relative amount of cDNA synthesised. Samples were taken every two cycles and resolved on a 2% agarose gel to identify the linear range of amplification. The number of amplification cycles used were chosen from within this range and were 35 cycles for *Krox20* and *Krox20/lacZ* and 18 cycles for 18S.

X-gal staining of whole mounts, sections and teased nerves

Embryos were stained for β-galactosidase activity, whole (10.5 dpc) or partially dissected (13.5-18.5 dpc), following fixation in 2% paraformaldehyde (PFA) and 0.2% gluteraldehyde in PBS for 30 minutes to 1.5 hours at 4°C. P2/3 sciatic nerves were fixed in Kryofix reagent (Merck) for 15 minutes at 4°C prior to equilibration in 30% sucrose in PBS for the preparation of cryosections. Nerves were cryo-imbedded and 15 μm sections were prepared. P30 or older teased nerves were prepared following fixation in 4% PFA for 5 minutes at 4°C. Transgenic embryos or sciatic nerves were stained in 1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ and 0.1% NP-40 in PBS at 30°C for 2-15 hours. All tissues were postfixed in 4% PFA in PBS. Embyos at 13.5 dpc to 18.5 dpc were dehydrated and either clarified in benzyl benzoate:benzylic acid (2:1) or embedded in paraffin wax for the preparation of sections. Cryosections, paraffin sections and teased nerve preparations were stained with Nuclear Fast Red (1 mg/ml) for 10 seconds, dehydrated and mounted in Eukitt (Merck).

Immunohistochemistry

Following X-gal staining, teased nerves were post-fixed in 4% PFA for 10 minutes and nonspecific binding sites blocked with PBS containing 0.1% Triton X-100 (PBT), 5% FCS and 2% BSA. Subsequently, the nerves were incubated with rabbit antibodies directed against either GFAP at 1:2000 dilution (DAKO) or MBP at 1:500 dilution (gift from F. Lachapelle) in PBT containing 2% BSA. A secondary peroxidase-conjugated goat anti-rabbit antibody was used at 1:200 dilution (BioRad). Peroxidase activity was detected using 3, 3′-diamine benzidine tetrahydrochloride (DAB) according to the manufacturer's instructions (Sigma). For Oct6 and Krox20 immunolabelling, nerves were fixed overnight at 4°C in 35% acetone, 35% methanol and 5% acetic acid in water. After dehydration and paraffin embedding, 5 μm sections were cut and mounted on Superfrost glass/plus slides (Menzel Glaser). Sections were dewaxed, rehydrated and subjected to a microwave antigen retrieval procedure (Evers et al., 1998). All subsequent procedures have been described (Jaegle et al., 1996; Zwart et al., 1996). Affinity-purified rabbit polyclonal anti-Oct6 serum (Zwart et al., 1996) and rabbit polyclonal anti-Krox20 serum (Vesque and Charnay, 1992) antibodies were used at 1:200 dilutions. A FITC-coupled goat anti-rabbit secondary antibody was used at 1:200 dilution (Nordic). Sections were examined using epifluorescence microscopy (Leitz).

Sciatic nerve regeneration and transection

For degeneration/regeneration experiments adult mice were anaesthetised and the right sciatic nerve was cryolesioned by crushing 6 times for 10 seconds using blunt forceps that were cooled in liquid nitrogen. Sutures were placed in the adjacent muscle to identify the location of the lesion. The left nerve was not operated on and used as a control. For transection experiments the sciatic nerve was cut and suttered back in order to prevent regeneration. At least two mice were tested for each time point and condition.

Sequence alignment

The GenBank accession numbers for human and mouse genomic contigs containing the *Krox20* gene and flanking sequence are AL133417 and AC068424, respectively. Mouse sequences were aligned to human sequences using the VISTA and Dialign 2 programs (Morgenstern et al., 1998; Mayor et al., 2000). Repetitive sequences were masked using RepeatMasker (A. F. A. Smit and P. Green at http://ftp.genome.washington.edu/RM/RepeatMasker.html).

RESULTS

Construction of transgenic lines

In order to identify regulatory sequences controlling *Krox20* in the PNS, we utilised a modified transgenic approach whereby genomic fragments containing homologous ends are coinjected into murine zygotes. These sequences recombine prior to insertion within the genome, which facilitates the introduction of reporter genes into large transgenes, a process known as extrachromosomal homologous recombination (ECHR) (Pieper et al., 1992). We generated transgenes containing large regions surrounding a *Krox20* allele carrying a reporter *lacZ* gene inserted in-frame in exon 2. This is similar to the *Krox20/lacZ* knock-in allele (*Krox20^{lacZ}*) generated previously in our lab, which has been shown to reproduce the expression profile of the endogenous gene (Schneider-Maunoury et al., 1993). Murine genomic cosmid inserts were used as a source of extragenic sequences (Chavrier et al., 1989). Inserts from a 5′ cosmid, containing sequences extending to –31 kb relative to the start site of transcription of the *Krox20* gene, and a 3′ cosmid, extending to +40 kb, were prepared such that they overlapped with a *Krox20/lacZ* reporter construct containing genomic sequences from –4.5 kb to +7 kb (Fig. 1A). Previous transgenic studies have shown that this latter construct is inactive in Schwann cells before birth (M. F., unpublished data).

Two sets of transgenic lines were generated by injecting each cosmid insert independently with the *Krox20/lacZ* reporter construct. The first set, designated –31/+7 *Krox20/lacZ*, contained extragenic sequences extending from –31 kb to +7 kb. The second set, designated –4.5/+40 *Krox20/lacZ*, contained sequences extending from –4.5 kb to +40 kb. For both sets we identified three lines in which recombination had occurred as established by Southern analysis (Fig. 1B). For the purpose of detailed analyses we have maintained two –31/+7 and three –4.5/+40 *Krox20/lacZ* lines.

Distinct regulatory elements function at immature and myelinating Schwann cell stages

The expression of *Krox20* begins in the PNS at approximately 10.5 dpc (Schneider-Maunoury et al., 1993; Wilkinson et al., 1989). The profile of β-galactosidase activity in the –31/+7 *Krox20/lacZ* lines beginning at this stage and each day of development thereafter until birth was analysed. This transgene was active at 10.5 dpc, similar to the endogenous gene, in the cranial nerve roots and the dorsal and ventral spinal cord entry

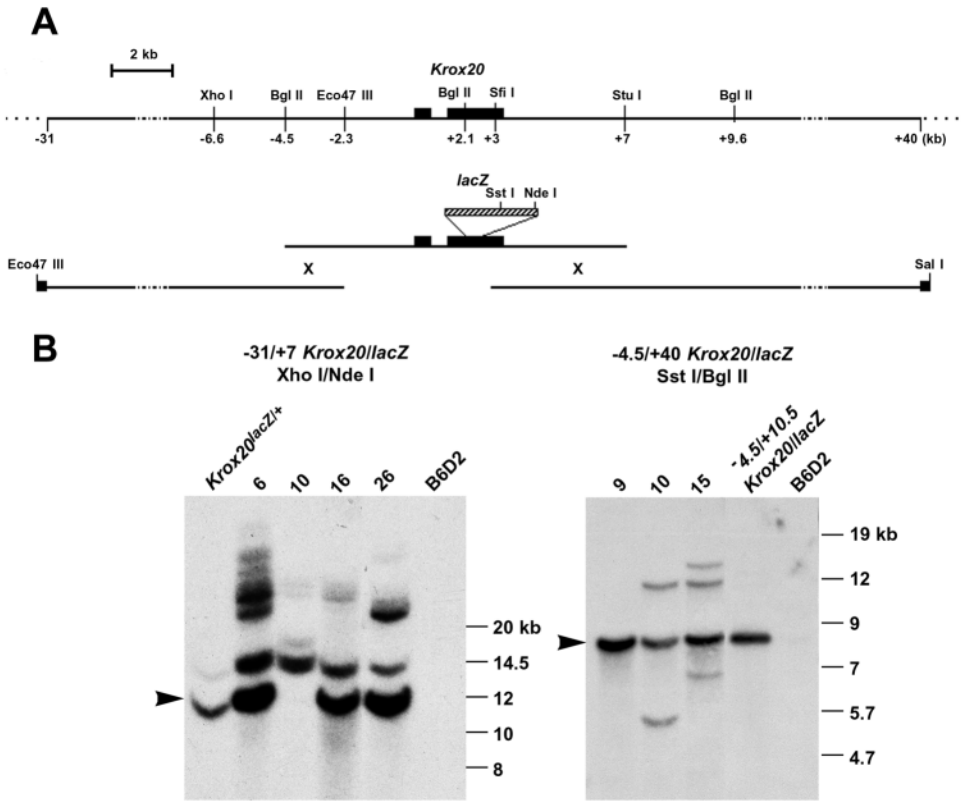

Fig. 1. Construction of transgenic lines. (A) *Krox20* gene and extragenic regions extending from –31 kb to +40 kb relative to the start site of transcription (upper). Cosmid inserts extending from –31 kb to –2.3 kb and +3 kb to +40 kb as well as the –4.5 kb to +7 kb *Krox20/lacZ* construct used to generate the –31/+7 *Krox20/lacZ* and –4.5/+40 *Krox20/lacZ* transgenic lines (lower). Restriction enzymes used to prepare these fragments for transgenesis and for Southern analysis are shown. *Krox20* exons are indicated as solid black rectangles. Solid squares identify vector sequences. (B) Southern analysis of genomic DNA. A *lacZ Sst*I/*Nde*I fragment was used as a probe. Enzymes used to diagnose recombination are indicated. Four –31/+7 *Krox20/lacZ* founders that were positive in PCR for both *lacZ* and cosmid vector sequences were analysed (left panel). DNA from *Krox20*$^{lacZ/+}$ mice was included to identify the size of the recombinant band as the *lacZ* sequence was inserted at the *Bgl*II site in both the knock-in and transgenic mice (Schneider-Maunoury et al., 1993). Three –4.5/+40 *Krox20/lacZ* transgenic founders positive in PCR for both fragments injected were analysed (right panel). The size control consisted of a *Krox20/lacZ* plasmid with genomic sequences extending from –4.5 kb to +10.5 kb. The arrowheads indicate the size of bands consistent with recombination, which are 11.7 kb and 8.5 kb for the –31/+7 *Krox20/lacZ* and –4.5/+40 *Krox20/lacZ* transgenes, respectively. In both panels DNA from the B6D2 genetic background was included as a negative control.

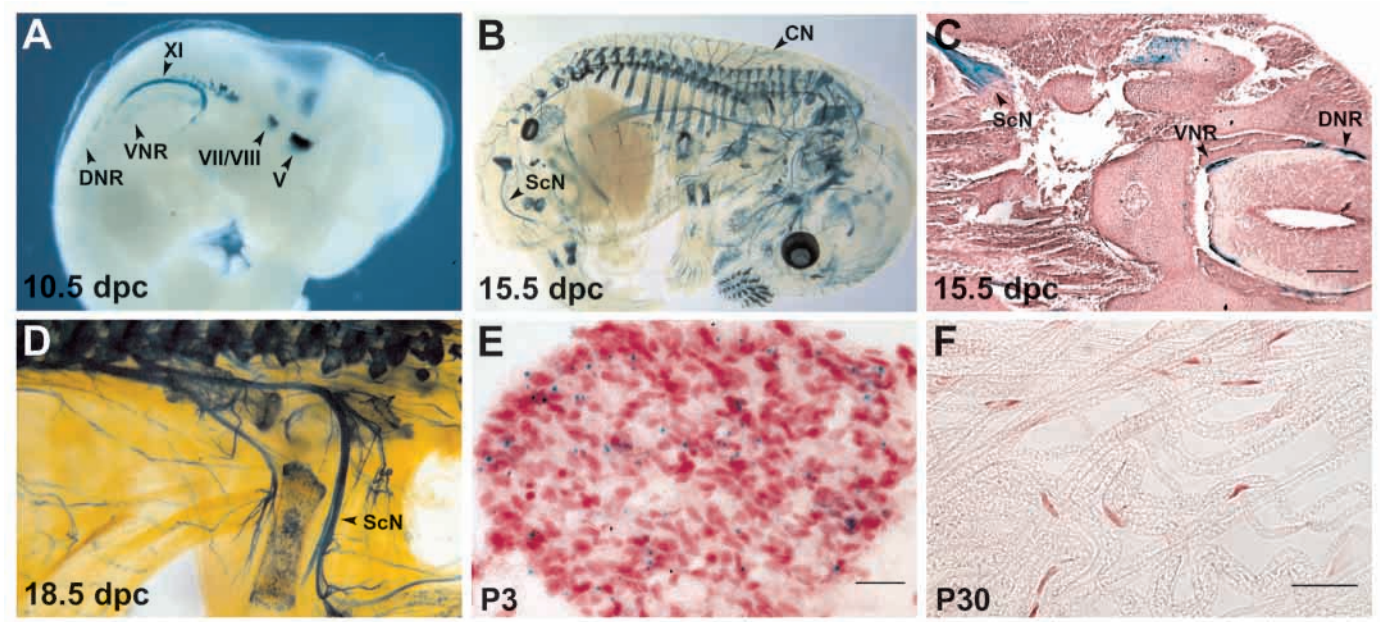

Fig. 2. Expression profile of the –31/+7 *Krox20/lacZ* transgenic line. (A,B) Transgenic embryos stained for β-galactosidase activity at 10.5 and 15.5 dpc, respectively. (C) Transverse section through the lumbar region of the embryo shown in B (dorsal to the right). The scale bar represents 200 μm. (D) Hindlimb view of a 18.5 dpc transgenic embryo stained for β-galactosidase. (E,F) Transverse cryosection and teased preparation of the distal sciatic nerve of transgenic mice at P3 and P30, respectively, stained for β-galactosidase. The scale bars represent 25 μm. The preparations shown in C, E and F were counterstained with Nuclear Fast Red. V, trigeminal nerve; VII/VIII, facial/acoustic nerve; XI, accessory nerve; VNR, ventral nerve root; DNR, dorsal nerve root; ScN, sciatic nerve; CN, cutaneous nerve.

and exit points (boundary cap cells; Fig. 2A). At 15.5 dpc, this transgene was activated along the length of the motor and sensory nerves, including the sciatic, similar to the endogenous gene (Fig. 2B,C) (Topilko et al., 1994). However, unlike *Krox20*$^{lacZ/+}$ embryos (data not shown), this transgene was also active throughout the cutaneous nerves possibly reflecting the much higher level of β-galactosidase activity observed in the transgenic lines compared to *Krox20*$^{lacZ/+}$ (Fig. 2B). The expression in the bone and hair follicles, detected at this stage, reflects the expression of the endogenous gene and corresponds to *cis*-acting elements contained within the –4.5/+7 reporter construct (Levi et al., 1996) (M. F., unpublished data). However, β-galactosidase activity was also detected in several other sites not consistent with *Krox20* expression. These sites were not shared among the two lines tested and therefore likely reflect the site of integration rather than transgene-specific profiles (data not shown). Although expression remained high throughout the PNS at 18.5 dpc, in contrast to the endogenous gene, expression in the distal sciatic nerve was greatly diminished by P3 (Fig. 2D,E) (Topilko et al., 1994). At this stage many Schwann cells are in the promyelinating phase suggesting that this transgene is only active prior to myelination, during the immature Schwann cell stage. By P30 expression was undetectable in the sciatic nerve (Fig. 2F).

We next analysed the expression pattern in the –4.5/+40 *Krox20/lacZ* transgenic lines. Although these lines expressed in the bone, because of the presence of proximal *cis*-acting sequences (M. F., unpublished data), and in other tissues linked to the sites of integration, no expression was detected in either the boundary cap cells or immature Schwann cells at either 10.5 or 15.5 dpc (Fig. 3A,B). In contrast, at 18.5 dpc high levels were observed in the sciatic and intercostal nerves (Fig. 3C,D). At this stage β-galactosidase was also detected with the –31/+7 *Krox20/lacZ* transgene, suggesting that the activity of the two elements overlaps temporally in the Schwann cell lineage. At P3, unlike the –31/+7 *Krox20/lacZ* transgene, the –4.5/+40 *Krox20/lacZ* lines expressed at high levels in the sciatic nerve Schwann cells consistent with the expression of the endogenous gene (Fig. 3E). At this stage the majority of the Schwann cells are in the promyelinating phase and progressively differentiate to give rise to the myelinating and nonmyelinating cells, a process that is largely complete by P30. Nonmyelinating Schwann cells can be distinguished from their myelinating counterparts by the expression of markers such as GFAP (Jessen et al., 1990). *Krox20* is expressed exclusively in the myelinating Schwann cells (Zorick et al., 1996; Topilko et al., 1997). Consistent with this, at P30 *lacZ* was detected only in GFAP-negative Schwann cells in the sciatic nerve of –4.5/+40 *Krox20/lacZ* transgenic mice, a profile that continued throughout life (Fig. 3F and data not shown).

In summary the analysis of the –31/+7 and –4.5/+40 *Krox20/lacZ* transgenes has revealed the presence of at least two independent *cis*-acting regulatory elements controlling *Krox20* at different phases of Schwann cell development. One element located 5′ of the *Krox20* gene, designated immature Schwann cell element (ISE), was active in the immature Schwann cells from the time of their appearance at 15.5 dpc until after birth. The other, located 3′ of the *Krox20* gene, designated myelinating Schwann cell element (MSE), was active before the onset of myelination and indeed throughout life in myelinating cells. An additional activity, associated with the –31/+7 *Krox20/lacZ* transgene, directed expression to the nerve roots (boundary cap cells) beginning at 10.5 dpc. Together the two transgenes completely reproduce the expression profile of the endogenous gene during PNS development.

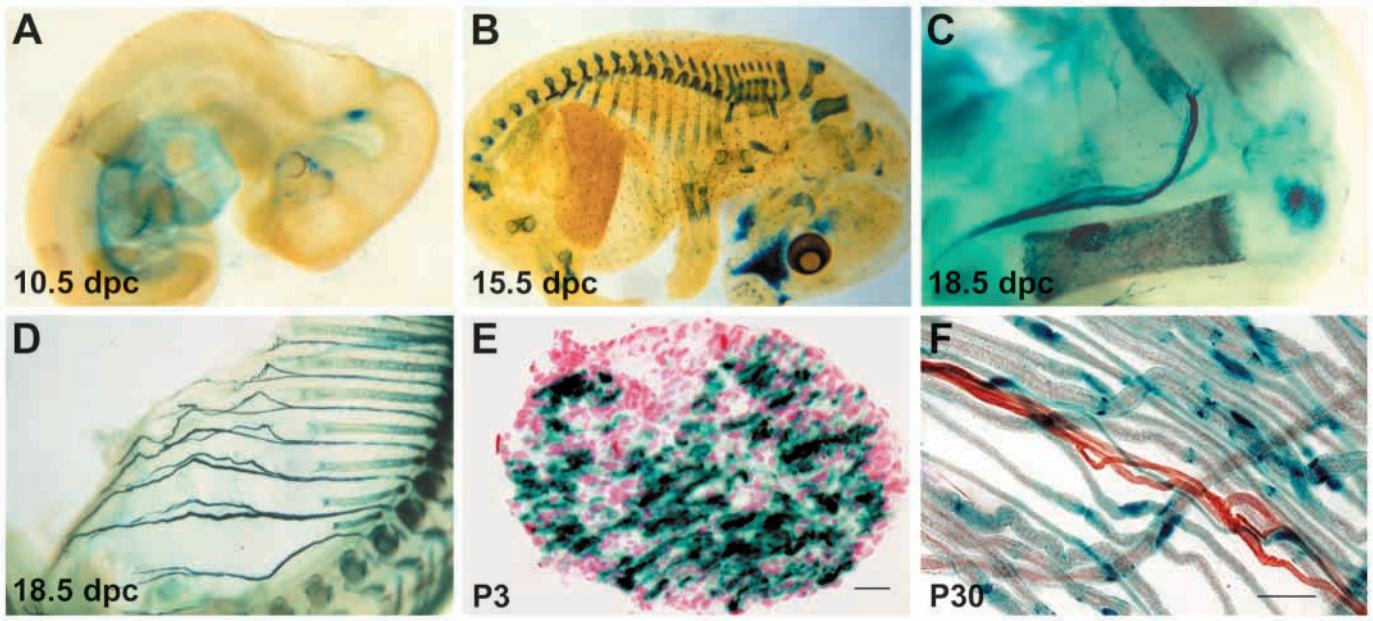

Fig. 3. Expression profile of the –4.5/+40 *Krox20/lacZ* transgenic line. (A-D) Transgenic embryos stained for β-galactosidase activity at 10.5 (A), 15.5 (B) and 18.5 (C,D) dpc (hindlimb, C; thorax, D). (E) Transverse section from the distal sciatic nerve at P3 prepared by cryosectioning and subsequent staining for β-galactosidase. (F) Teased preparation of the sciatic nerve from a P30 mouse stained for β-galactosidase and immunolabelled with an antibody against GFAP (red-brown deposit). Preparations in E and F were counterstained with Nuclear Fast Red. The scale bars represent 25 μm.

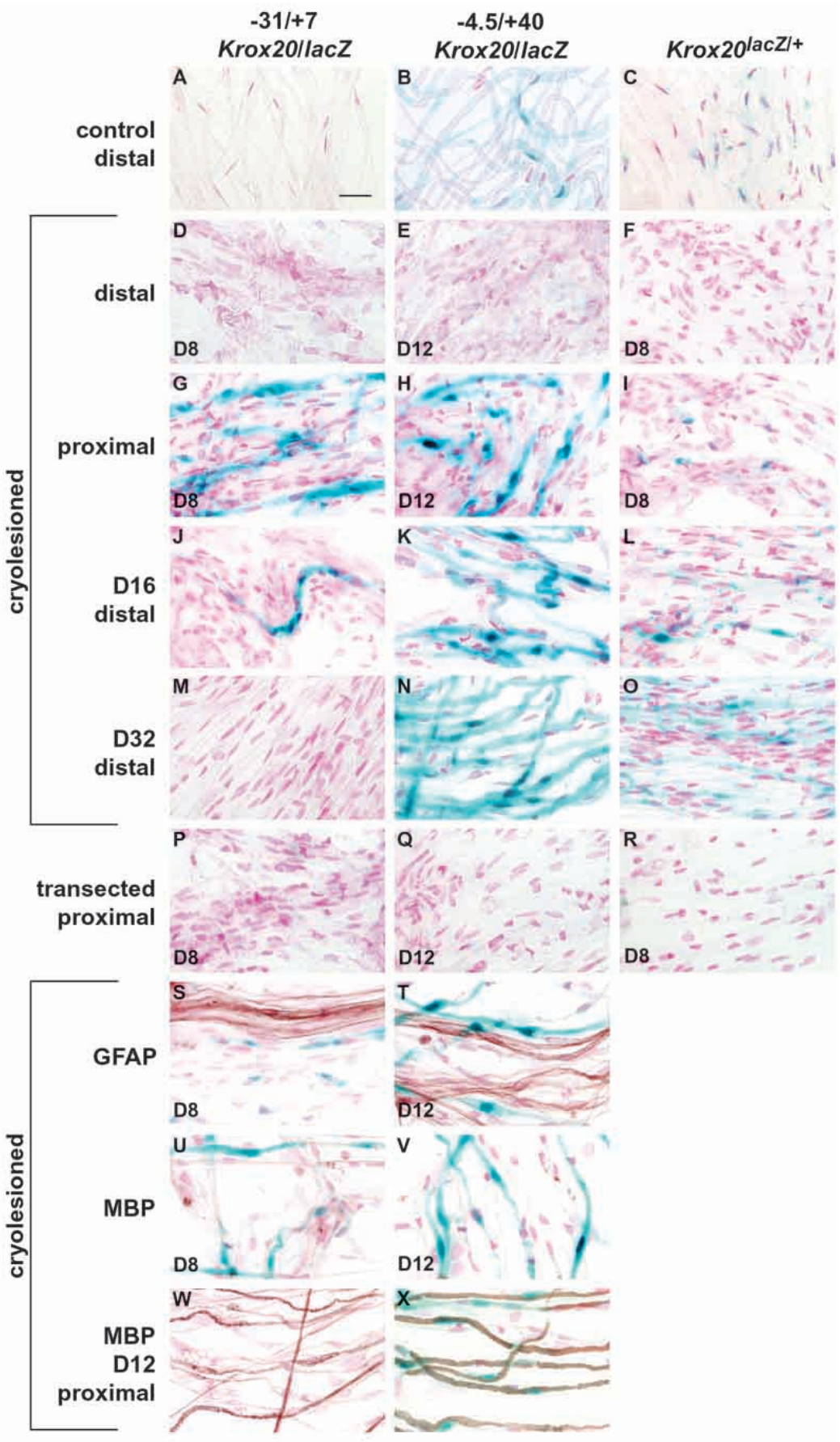

Fig. 4. Axon-dependent regulation of the –31/+7 and –4.5/+40 *Krox20/lacZ* transgenes during nerve regeneration. (A-O) The sciatic nerves from adult –31/+7 and –4.5/+40 *Krox20/lacZ* transgenic and *Krox20*$^{lacZ/+}$ mice, either untreated (A-C), or cryolesioned (D-O). –31/+7 *Krox20/lacZ* transgenic and *Krox20*$^{lacZ/+}$ nerves after development for 8 days (D,G,F,I), 16 days (J,L) and 32 days (M,O) post-cryolesion. (E,H,K,N) –4.5/+40 *Krox20/lacZ* transgenic nerves after development for 12 days (E,H), 16 days (K) and 32 days (N). Teased nerves of the distal region of the sciatic nerve femural branch (A-F, J-O; distal) and proximal to the lesion on the distal side (G-I; proximal). (P-R) Nerves were also transected and teased from the proximal region of the distal stump (proximal) at 8 days for –31/+7 *Krox20/lacZ* transgenic (P) and *Krox20*$^{lacZ/+}$ (R) and 12 days for –4.5/+40 *Krox20/lacZ* transgenic (Q). Cryolesioned nerves from both the –31/+7 and –4.5/+40 *Krox20/lacZ* lines were analysed by immunohistochemistry for GFAP at 8 and 12 days, respectively (S,T; red-brown deposit). MBP from –31/+7 *Krox20/lacZ* lines was analysed at 8 (U) and 12 (W) days and at 12 days (V,X) from –4.5/+40 *Krox20/lacZ* lines. β-galactosidase activity was analysed in teased nerves originating from the most distal side of the lesion (S-V) where significant activity was detected, or adjacent to the lesion (W,X). All preparations were stained for β-galactosidase activity and counterstained with Nuclear Fast Red. The scale bar represents 25 μm.

Both the –31/+7 and –4.5/+40 *Krox20/lacZ* transgenes are regulated in an axon-dependent manner during nerve regeneration

Previous studies have shown that the expression of *Krox20* in Schwann cells is axon dependent (Murphy et al., 1996; Zorick et al., 1996). As the –31/+7 and –4.5/+40 *Krox20/lacZ* transgenes were active at distinct stages during Schwann cell development we investigated whether both were regulated by axons in the adult nerve. To address this question we performed nerve cryolesion experiments to induce degeneration/regeneration in the sciatic nerve of both the –31/+7 and the –4.5/+40 *Krox20/lacZ* transgenic lines.

These experiments were performed on adult mice at which stage the –31/+7 *Krox20/lacZ* transgene is inactive (Fig. 4A). At day 8 post cryolesion this transgene remained silent distally at the sciatic nerve femeral branch, the point furthest from the lesion (Fig. 4D). However, at this stage, just adjacent to the lesion on the distal side, multiple fibres exhibited high levels of β-galactosidase activity in Schwann cells (Fig. 4G). Some positive fibers were also detected distally at day 16 (Fig. 4J), however, by day 32 levels had decrease to that of the unoperated control nerve (Fig. 4M). In the –4.5/+40 *Krox20/lacZ* line, cryolesion resulted in a dramatic loss of β-galactosidase activity distally as early as day 8 and remained low up to day 12 when compared to the control nerve (Fig. 4B,E and data not shown). However, adjacent to the lesion multiple β-galactosidase-positive fibres were detected at day 12 (Fig. 4H). Positive fibres were also abundant distally at day 16 and more numerous at day 32 (Fig. 4K,N). For comparison $Krox20^{lacZ/+}$ mice were included in the analysis. Consistent with previous studies, β-galactosidase activity was greatly diminished distally 8 days post cryolesion, when compared to the unoperated control nerve (Fig. 4C,F) (Zorick et al., 1996). However, adjacent to the lesion, similar to the –31/+7 *Krox20/lacZ* transgene, several β-galactosidase-positive fibres were detected (Fig. 4I). Much like the –4.5/+40 *Krox20/lacZ* transgene expression was detected distally at day 16 and increased further at day 32 (Fig. 4L,O).

In order to establish the requirement of axons in the activity of the transgenes nerve transection experiments were conducted to prevent their regrowth into the distal nerve stump. No activation was detected in either of the transgenic or $Krox20^{lacZ/+}$ lines adjacent to the lesion at stages when cryolesioned nerves showed strong activation, consistent with the notion that *Krox20* is under axonal control (compare Fig. 4G with P, H with Q and I with R) (Murphy et al., 1996; Zorick et al., 1996).

The nerve degeneration/regeneration studies provided an opportunity to correlate the activity of the transgenes with the expression of various markers of Schwann cell development. Cryolesioned nerves from both the –31/+7 and –4.5/+40 *Krox20/lacZ* lines were analysed for the expression of the nonmyelinating Schwann cell marker GFAP, and the myelin marker, MBP. Although GFAP is expressed in Schwann cells following nerve degeneration (Jessen et al., 1990) (data not shown), the activity of both transgenes during regeneration was restricted to GFAP-negative cells (Fig. 4S,T). In addition, although both transgenes were active prior to the accumulation of MBP (Fig. 4U,V), only the –4.5/+40 *Krox20/lacZ* transgene was coexpressed with MBP, the –30/+7 *Krox20/lacZ* transgene being largely inactive in MBP positive cells (Fig. 4W,X).

In conclusion, both transgenes are reactivated following nerve degeneration/regeneration in an axon-dependent manner. In addition, their activity is restricted to the myelinating lineage during this process, the –31/+7 and –4.5/+40 *Krox20/lacZ* transgenes being active in immature and myelinating stages, respectively. As the –31/+7 *Krox20/lacZ* transgene is downregulated before myelination begins, this suggests that the chronological order observed during development for the activation of the two transgenes may be maintained during regeneration. Overall the combination of the β-galactosidase expression profiles of these transgenes during degeneration and regeneration reproduced that of the $Krox20^{lacZ/+}$ mice.

The POU domain transcription factor Oct6 regulates *Krox20* at the time of myelination by acting upstream of the MSE

In order to begin to characterise the transcription factors controlling *Krox20* in Schwann cells we first wanted to determine whether the elements we had isolated, present in the –31 kb to +40 kb region, were dependent on autoregulatory mechanisms. This was addressed by assessing the activity of both the –31/+7 and –4.5/+40 *Krox20/lacZ* transgenes in a *Krox20* mutant background at the time of their activation. In these studies we used a null allele in which the *cre* gene was inserted into the *Krox20* locus ($Krox20^{cre}$) (Voiculescu et al., 2000). Both the –31/+7 *Krox20/lacZ* transgene at 15.5 dpc and the –4.5/+40 *Krox20/lacZ* transgene at 18.5 dpc were activated normally in the absence of a functional Krox20 protein (Fig. 5A), indicating that autoregulatory mechanisms are not required for the activity of the ISE and MSE.

As both *Krox20* and *Oct6* are important in the onset of myelination we tested for a possible genetic interaction between these factors (Topilko et al., 1994; Bermingham et al., 1996; Jaegle et al., 1996). Sciatic nerve sections from P0 control and $Krox20^{lacZ/lacZ}$ mice were tested for Oct6 protein. High levels were detected in both wild-type and mutant nerves, indicating that *Oct6* expression can occur independently of *Krox20* (Fig. 5B, upper panels). In contrast, when sciatic nerves of control and $Oct6^{β\text{-}geo/β\text{-}geo}$ mice at P8 were tested for Krox20, although wild-type nerves expressed the protein, mutant nerves were largely negative (Fig. 5B, lower panels). These results indicate that *Oct6* is required for the high level of Krox20 characteristic of postnatal Schwann cells.

Given that the MSE is responsible for *Krox20* expression during myelination it seemed likely that this element is regulated by *Oct6*. In order to establish this we tested the activity of the –4.5/+40 *Krox20/lacZ* transgene in $Oct6^{β\text{-}geo/+}$ and $Oct6^{β\text{-}geo/β\text{-}geo}$ sciatic nerves by semiquantitative RT-PCR. These studies were performed on embryos at 18.5 dpc, a stage where the ISE is still active and shortly after the activation of the MSE (Fig. 2, Fig. 3). These studies revealed an unequivocal requirement of *Oct6* for the activation of the MSE (Fig. 5C). Interestingly, analysis of endogenous *Krox20* transcripts revealed only a minor decrease in *Oct6* mutant nerves at this stage (Fig. 5C). This is consistent with the possibility that the ISE operates independently of *Oct6*.

In summary, our genetic studies revealed that both the ISE and the MSE are activated independently of *Krox20* and that the high level of Krox20 characteristic of myelinating Schwann cells is dependent on the action of *Oct6* on the MSE.

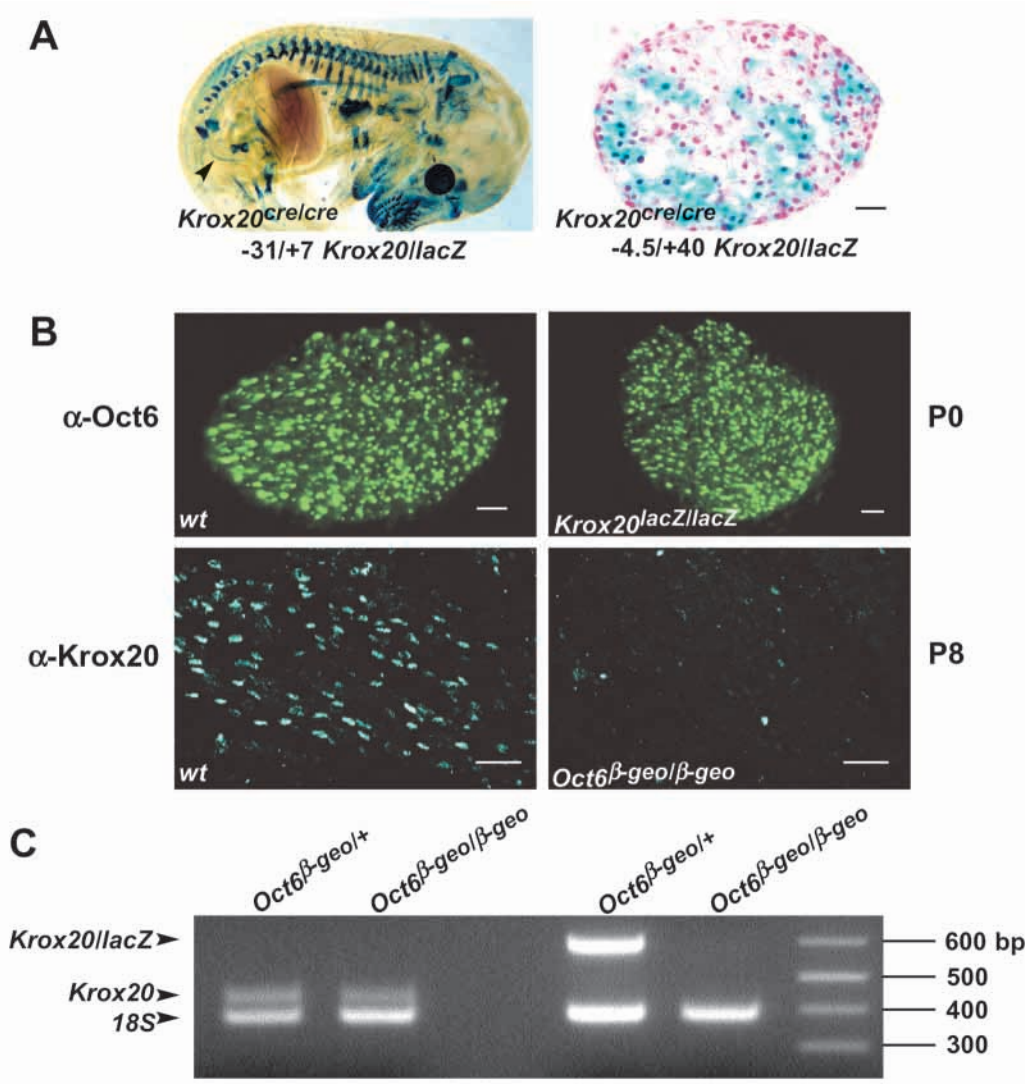

Fig. 5. The POU domain transcription factor Oct6 acts upstream of the MSE to regulate *Krox20*. (A) Analysis of the –31/+7 and –4.5/+40 *Krox20/lacZ* transgenes in a *Krox20* null-mutant background. (Left) 15.5 dpc *Krox20*$^{cre/cre}$ embryo carrying the –31/+7 *Krox20/lacZ* transgene stained for β-galactosidase activity. The arrowhead identifies the sciatic nerve. (Right) Section of a β-galactosidase-stained sciatic nerve from a 18.5 dpc *Krox20*$^{cre/cre}$ embryo carrying the –4.5/+40 *Krox20/lacZ* transgene. The section was counterstained with Nuclear Fast Red. (B upper) Immunofluorescent analysis of sections of P0 wild type (left) and *Krox20*$^{lacZ/lacZ}$ mutant (right) nerves using an antibody to Oct6 (α-Oct6). (B lower) Immunofluorescent analysis of sections of P8 wild-type (left) and *Oct6*$^{β–geo/β–geo}$ mutant (right) nerves using an antibody to Krox20 (α-Krox20). The scale bars in A and B represent 25 μm. (C) Semiquantitative RT-PCR analysis of endogenous *Krox20* transcripts (447 bp) and transcripts from the –4.5/+40 *Krox20/lacZ* transgene (607 bp) from 18.5 dpc sciatic nerves from *Oct6*$^{β–geo/+}$ and *Oct6*$^{β–geo/β–geo}$ embryos without transgene (left) or carrying the –4.5/+40 *Krox20/lacZ* transgene (right). Analysis of the 18S rRNA (389 bp) was included as a control. The results are representative of two independent experiments.

Characterisation of a 1.3 kb conserved sequence containing the MSE

As a first step to identify direct upstream regulators of *Krox20* in Schwann cells we undertook the characterisation of *cis*-elements. The analysis of the –31/+7 and –4.5/+40 *Krox20/lacZ* transgenic lines suggested that the ISE and MSE were located between –31 kb and –4.5 kb and +7 kb and +40 kb, respectively. We tested a series of overlapping fragments from these regions in mouse transgenesis by fusion to a *β-globin* minimal promoter/*lacZ* reporter. Four constructs carrying upstream sequences extending from –31 kb to –23.5 kb, –25.7 kb to –14 kb, –16 kb to –10.5 kb and –11 kb to –4 kb were tested for ISE activity at 16.0 dpc. Although, regulatory sequences controlling *Krox20* in the nerve roots were identified, none of the constructs tested showed expression distally in the peripheral nerves (data not shown). This suggests that ISE activity requires the cooperation between distant regulatory sequences. Downstream constructs were tested for expression in the sciatic nerve of founders at P2/3 and P30. These experiments identified a single fragment extending from +30 kb to +40 kb that was active at both of these stages in 3/7 cases, indicating the presence of the MSE (Fig. 6A, construct #4 and data not shown).

The availability of extensive sequence data for both the human and mouse genomes has provided the opportunity to identify regulatory elements using global sequence alignments, as these are usually conserved during evolution (Hardison et al., 1997). We performed human-mouse sequence comparisons using the VISTA program to facilitate the delimitation of regulatory elements present in fragment #4 (Fig. 6A) (Mayor et al., 2000). A largely contiguous 1 kb segment was identified with >80% identity within this fragment (Fig. 6B). Regions outside of this showed significantly weaker similarity. As the conserved sequence showed no homology to either EST or cDNA sequences, nor did it contain repetitive sequences, we tested this region in transgenesis. A 1.3 kb fragment spanning this region, fused to the *β-globin/lacZ* reporter (construct #5) was found to express in the sciatic nerve at P2/3 and P30 in 7/13 cases in a manner indistiguishable from the original –4.5/+40 *Krox20/lacZ* transgene (Fig. 6A,C,D).

As *Oct6* was found to act upstream of the MSE at the time of its activation (Fig. 5C) we searched for Oct6 binding sites

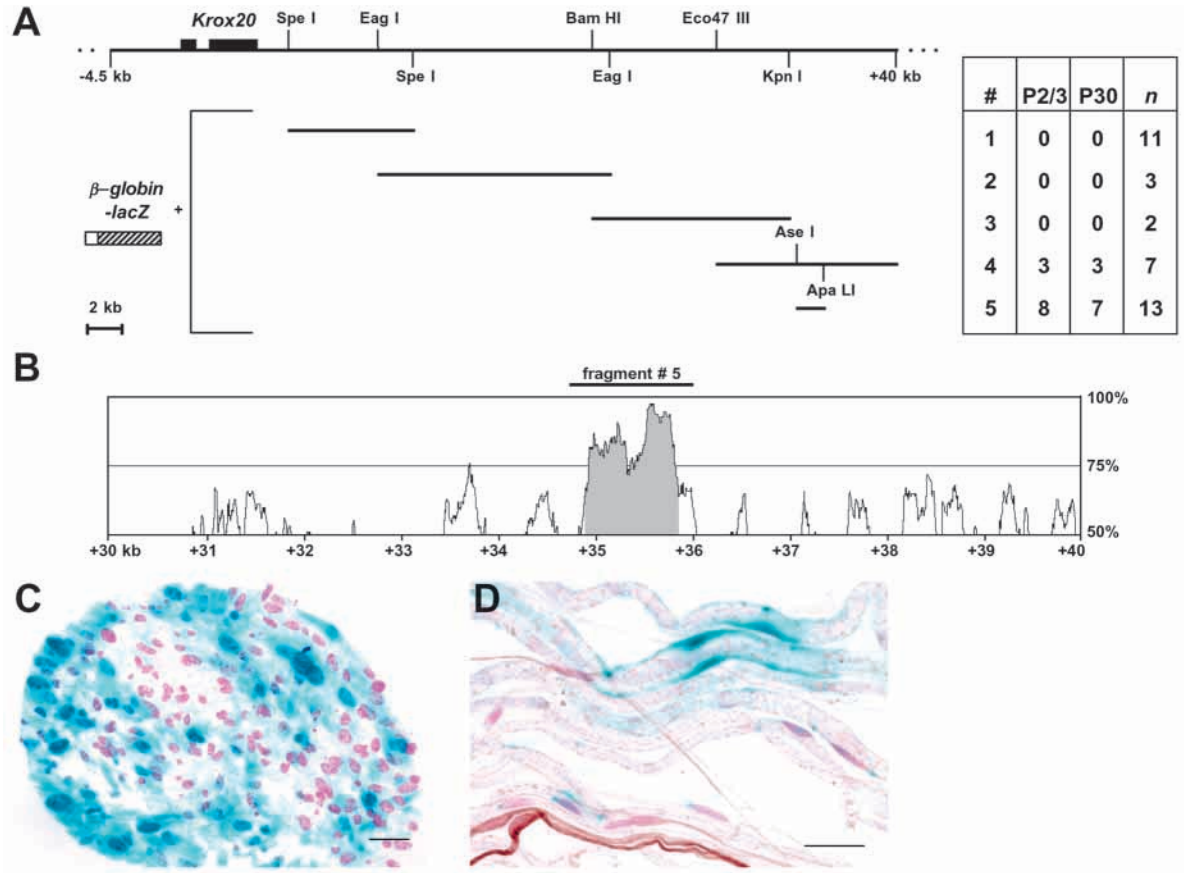

Fig. 6. Isolation of the MSE on a conserved 1.3 kb element. (A) Murine *Krox20* gene and extragenic sequences extending from –4.5 kb to +40 kb (upper). Restriction enzymes used to generate overlapping subfragments are shown. These fragments were fused to a *β-globin* minimal promoter/*lacZ* reporter and tested in transgenesis (lower). The number of mice that showed β-galactosidase activity in sciatic nerve biopsies at P2/3 and P30 and the total number of transgenic mice analysed (*n*) is indicated. For fragments #4 and #5 the mice that expressed β-galactosidase at P30 were among those that expressed at P2/3. (B) Homology plot generated using the VISTA algorithm (Mayor et al., 2000) between murine fragment #4 (horizontal axis; numbering in kb is relative to the *Krox20* gene) and corresponding human sequences. The vertical axis indicates percent homology in a window of 100 bp with a resolution of 7 bp. Homology >80% is highlighted in gray. Note the base homology shown is 50%. Mice transgenic for construct #5 were tested for β-galactosidase activity in the sciatic nerve at P3 (C) and P30 (D) as described in Fig. 3. Nonmyelinating Schwann cells were detected using an antibody to GFAP (D; red-brown deposit). The scale bars represent 25 μm.

within fragment #5. This analysis revealed the presence of multiple candidate binding sites for Oct6 in two highly conserved domains, the majority of which were located in the 3′ domain (Fig. 7, domain B). The capacity of Oct6 to bind the sites within domain B was determined by footprinting analysis. In these studies Oct6 footprinted regions were identified that overlapped all of the conserved sites (data not shown).

In summary, by using transgenic analysis in combination with human/mouse sequence comparison we have isolated a 1.3 kb element controlling the expression of *Krox20* from the time of myelination to adulthood, a profile that was identical to the original –4.5/+40 *Krox20/lacZ* transgene. Genetic analyses indicating that *Oct6* participates in the activation of the MSE and the presence of multiple Oct6 binding sites within the 1.3 kb fragment suggest that Oct6 could constitute a direct transcriptional activator of *Krox20*.

DISCUSSION

This report describes an analysis of *Krox20* regulation during Schwann cell development and the characterisation of the *cis*-acting regulatory elements. We have identified two distinct elements that function in an axon-dependent manner: the ISE, active in immature Schwann cells and the MSE, active at the onset of myelination and in mature myelinating Schwann cells. In addition, genetic studies and sequence analysis suggest that Oct6 acts to drive *Krox20* expression at the time of myelination by interacting with sequences in the MSE. The significance of these findings are discussed in the context of axon signals and the transcription factor regulatory network controlling myelination.

Krox20 expression in immature and myelinating Schwann cells is controlled by distinct regulatory elements

The analysis of the *Krox20* profile in the PNS has revealed expression in the nerve roots (boundary cap cells) and in immature and myelinating but not nonmyelinating Schwann cells (Wilkinson et al., 1989; Schneider-Maunoury et al., 1993; Murphy et al., 1996; Zorick et al., 1996; Topilko et al., 1997). The analysis of *Krox20* extragenic sequences extending from

–31 kb to +40 kb, presented in this study, identified regulatory elements controlling *Krox20* in all of these sites (Fig. 2, Fig. 3). Interestingly, our results revealed that *Krox20* expression in Schwann cells is due to the combined activities of distinct regulatory elements, the ISE and MSE, which function during development and regeneration in the myelinating lineage at immature and myelinating stages, respectively (Fig. 2, Fig. 3, Fig. 4).

Previous studies have revealed that *Krox20* expression during Schwann cell development is dynamic, beginning at low levels in immature Schwann cells, peaking during myelination and dropping again in mature cells (Blanchard et al., 1996; Zorick et al., 1996; Topilko et al., 1997). These fluctuations may be explained by the different regulatory elements we have identified. The increase after birth likely reflects a shift from ISE to MSE-dependent regulation. However, as the early phase of MSE activity is dependent on *Oct6*, which is expressed only transiently during myelination (Arroyo et al., 1998), the high levels observed at this stage likely give way to lower levels as other factors acting in mature cells take over.

The identification of distinct regulatory elements acting in immature and myelinating Schwann cells raises the question as to the role of *Krox20* at each of these stages. The analysis of mutant mice and mutations in *Krox20* associated with human hypomyelinating diseases and more recently the global analysis of Krox20 target genes in Schwann cells indicate that *Krox20* is important in both the initiation and maintenance of myelination, which correlate with the activity of the MSE (Topilko et al., 1994; Warner et al., 1998; Timmerman et al., 1999; Nagarajan et al., 2001). However, these data do not reveal a role for *Krox20* during its early expression in immature Schwann cells when it is regulated by the ISE. Moreover, we have found that the MSE is active in a *Krox20* mutant background indicating that the MSE is presumably not dependent on the ISE (Fig. 5A). Nevertheless, the existence of an early Schwann cell *Krox20* profile in other species as well (Golding and Cohen, 1997) suggests that the ISE is conserved and supports a role for Krox20 in immature Schwann cells. The existence of separate regulatory elements could also allow *Krox20* to be regulated independently during immature and myelinating stages. Consistent with this, we have found that whereas the activities of the ISE and the MSE overlap in the sciatic nerve after birth, in the sympathetic trunk the ISE is not active during this early phase of MSE activity (data not shown). This suggests that myelinating Schwann cells may develop differently in the sympathetic trunk. Ultimately the determination of the precise role of each stage of *Krox20* expression may require enhancer knock-out studies.

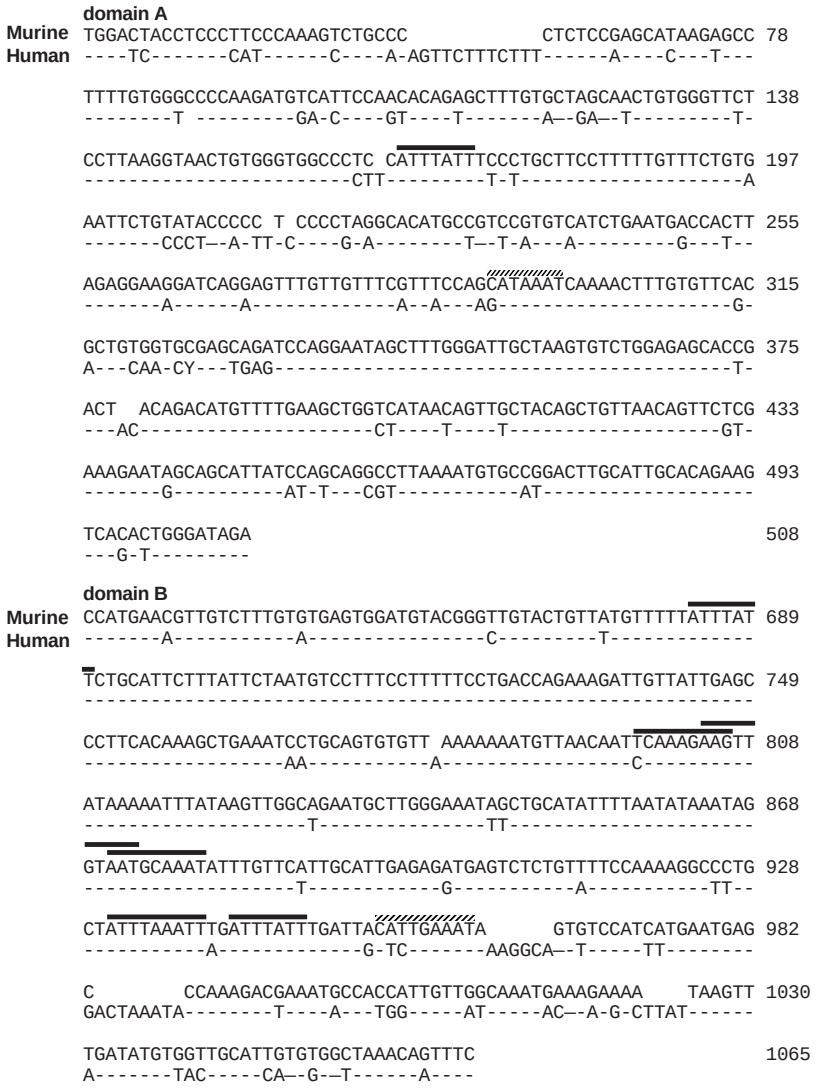

Fig. 7. Conserved sequences within the 1.3 kb MSE contain multiple candidate Oct6 binding sites. Two regions of highest sequence conservation between human and mouse fragment #5 (Fig. 6B) were analysed for candidate Oct6 binding sites. These consisted of 478 nt (domain A) and 436 nt (domain B) showing 84% and 90% sequence identity, respectively. Nucleotide numbering corresponds to fragment #5. Conserved residues are indicated with a dash in the human sequence. Nucleotide sequences corresponding to candidate Oct6 binding sites were identified by searching for sequences satisfying the Brn2 binding site consensus, C/AATnT/AAAT, where n=0,2,3 nt (Li et al., 1993). Brn2 and Oct6 are closely related class III POU proteins that are thought to exhibit similar binding characteristics (Schreiber et al., 1997). Conserved and non-conserved sites are overlined with a solid and hatched bar, respectively.

Axon-dependent regulation of both phases of *Krox20* expression

In vitro Schwann cell-neuron coculture studies and in vivo nerve regeneration experiments presented in this study and elsewhere have revealed that *Krox20* expression is axon dependent (Fig. 4) (Murphy et al., 1996; Zorick et al., 1996; Topilko et al., 1997). Similarily, regeneration studies on the –31/+7 and –4.5/+40 *Krox20/lacZ* lines revealed a requirement for axons in the expression of these transgenes (Fig. 4). Interestingly, these results are also consistent with a recapitulation of the events during development as the –31/+7 and –4.5/+40 *Krox20/lacZ* transgenes, possessing distinct regulatory elements active at different stages of Schwann cell development, were both reactivated during regeneration.

The characterisation of distinct regulatory elements acting at different stages of Schwann cell development raises the question of the nature of the axonal signal(s) involved. Although multiple, developmentally regulated signals may control the different phases of *Krox20* expression, an interesting alternative view is that axons may provide a constitutive signal, the Schwann cell response being determined by their stage of development. Immature Schwann cells would respond to axons acting through the ISE, whereas myelinating Schwann cells would respond through the MSE. In this senario the downregulation of β-galactosidase activity in the –31/+7 *Krox20/lacZ* lines after birth would be explained by the loss of an activator or the accumulation of a repressor in myelinating Schwann cells. The analysis of the genetic interactions involved in *Oct6* expression in Schwann cells suggests a similar mechanism acting on this gene. Although *Oct6* is normally downregulated in adult myelinating Schwann cells, in *Oct6*$^{-/-}$ mice the mutant gene continues to be transcribed suggesting that the signal is present on the axon throughout life (Jaegle and Meijer, 1998). Previously, we showed that 12.5 dpc DRG neurones can activate *Krox20* in immature Schwann cells indicating that a signal is present on the axon before *Krox20* is normally expressed (Murphy et al., 1996). The analysis of Schwann cells derived from both the –31/+7 and –4.5/+40 *Krox20/lacZ* transgenic lines in this assay may provide support for this model.

Oct6 acts upstream of *Krox20* at the time of myelination

We performed genetic studies that revealed that the POU domain transcription factor Oct6 acts upstream of the MSE (Fig. 5C). The importance of this pathway is illustrated by the very low levels of Krox20 protein detected in the postnatal nerve of *Oct6* mutant mice (Fig. 5B). The presence of Krox20 protein in some Schwann cells may be due to the continued activity of the ISE in *Oct6* mutants and/or a later activation of the MSE by other factors (see below). Given that both *Krox20* and *Oct6* mutant Schwann cells are blocked at the same stage, the *Oct6* mutant phenotype could be explained by the low level of Krox20 protein (Topilko et al., 1994; Bermingham et al., 1996; Jaegle et al., 1996). As this block in myelination is only transient in *Oct6* mutants (Jaegle et al., 1996), Krox20 would be expected to accumulate once myelination proceeds. Experiments to rescue the *Oct6* mutant phenotype by expressing Krox20 at the time of myelination will help to resolve this question.

The identification of multiple candidate binding sites for Oct6 in the MSE (Fig. 7) allows us to speculate on the regulatory interactions controlling *Krox20* in myelinating Schwann cells. (i) Oct6 likely directly regulates *Krox20* at the time of myelination. However, unlike the activity of the MSE, *Oct6* is not restricted to myelinating Schwann cells (Zorick et al., 1996). In addition, whereas *Oct6* is transiently expressed in the myelinating cells of the central nervous system, the oligodendrocytes, *Krox20* is not expressed in this lineage (Collarini et al., 1991; Topilko et al., 1994). This suggests that additional factors must be involved in restricting the activity of the MSE. Sox10, a high mobility group (HMG) domain protein, has been shown to synergise with Oct6 by binding to adjacent sites in an artificial enhancer (Kuhlbrodt et al., 1998a; Kuhlbrodt et al., 1998b). Although this interdependency may explain why the activity of Oct6 is largely restricted to the glial lineage, it cannot account for the absence of *Krox20* expression in oligodendrocytes or indeed in nonmyelinating Schwann cells, as *Sox10* is expressed in both of these lineages. Two other transcription factors, the paired domain protein, Pax3 and the zinc finger protein Krox24/Egr1 are expressed in a manner complementary to *Krox20* during Schwann cell development and ultimately restricted to nonmyelinating Schwann cells (Kioussi et al., 1995; Topilko et al., 1997). Indeed, Pax3 has been implicated in the suppression of myelin-specific gene expression (Kioussi et al., 1995). Such factors may be involved in suppressing the activity of the MSE in the nonmyelinating Schwann cells. (ii) Another factor(s) is responsible for maintaining the activity of the MSE once *Oct6* is downregulated in the adult nerve. This latter factor(s) may be responsible for initiating myelination, albeit delayed, in the *Oct6* mutant Schwann cells (Jaegle et al., 1996). Brn5/Pou6f1, a POU domain protein distantly related to Oct6, may account for this activity as it is expressed in myelinating Schwann cells (Wu et al., 2001). Indeed, as these two factors have similar binding specificities (Andersen et al., 1993), Brn5 could function by interacting with the Oct6 binding sites that have been identified within the MSE. The complex regulatory interactions involved in the expression of *Krox20* and, indeed, in the onset of the myelination program, will be revealed by the detailed dissection of the MSE.

In conclusion, we have shown that distinct regulatory sequences, the ISE and MSE, control *Krox20* in an axon-dependent manner at different stages of Schwann cell development. We also presented evidence that Oct6 initiates the activity of the MSE. Together, these studies provide insight into the mechanisms controlling Schwann cell development and myelination. In addition, the isolation of the MSE will facilitate the identification of additional upstream regulatory factors. Finally, the availability of regulatory elements with restricted activities in Schwann cells may be useful in future gene therapy approaches in the treatment of hypomyelinating diseases.

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