

## The regulation of *Krox-20* expression reveals important steps in the control of peripheral glial cell development

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### SUMMARY

The zinc finger transcription factor gene *Krox-20* is expressed in Schwann cells and is required for the myelination of peripheral nerves. We show that the regulation of *Krox-20* expression in peripheral glial cells reveals three important steps in the development and differentiation of these cells. (i) Expression of *Krox-20* in Schwann cells requires continuous neuronal signalling via direct axonal contact. Therefore *Krox-20* appears to be a key component of the transduction cascade linking axonal signalling to myelination. (ii) *Krox-20* inducibility is acquired by Schwann cells at the time that they are formed from their precursors. Diffusible factor(s) synthesised by the neural tube can mediate this transition and can be mimicked by

NDF $\beta$  or a combination of CNTF and bFGF. Furthermore, the neural tube activity is blocked by a hybrid protein containing the NDF-binding domain of the ErbB4 receptor, strongly implicating NDF in the physiological transition. (iii) In sensory ganglia, the microenvironment is capable of negatively regulating *Krox-20*, presumably by preventing the conversion of satellite glial cells toward a Schwann cell-like phenotype.

Key words: Schwann cell, satellite cell, myelin, *Krox-20*, gene disruption, mouse, PNS development, transcription control, NDF, CNTF, FGF

### INTRODUCTION

In vertebrates, the peripheral nervous system (PNS) is mainly derived from the neural crest (Le Douarin et al., 1991; Fraser and Bronner-Fraser, 1991; Frank and Sanes, 1991; Anderson, 1993). Its glial component can be classified into three categories: the Schwann cells surrounding the peripheral nerves, the satellite cells associated with neuronal somata in peripheral ganglia and the enteric glia. Schwann cell development appears to involve at least three steps: the generation of Schwann cell precursors from migrating neural crest cells, the transition of these precursors to Schwann cells and finally the maturation of the latter into myelinating or non-myelinating cells. In the rat, Schwann cell precursors have been characterised as early as embryonic day 14 (E14) as a population of peripheral nerve glial cells that differ from migrating crest cells as well as from differentiated Schwann cells in their morphology, migration behaviour, death regulation and expression of specific molecular markers (Jessen and Mirsky, 1991; Jessen et al., 1994). Between E15 and E17, Schwann cells are irreversibly generated from their precursors and  $\beta$  forms of Neu differentiation factor (NDF), also known as glial growth factor (GGF), Heregulin and ARIA (Raff et al., 1978; Holmes et al., 1992; Wen et al., 1992; Falls et al., 1993; Marchionni et al., 1993),

have been implicated in the regulation of this conversion in culture (Dong et al., 1995).

Besides dramatic morphological changes, multiple molecular markers are available to characterise the further development of Schwann cells toward the myelinating phenotype, including in particular early myelin proteins, such as myelin-associated glycoprotein (MAG; Owens and Bunge, 1989) and late peripheral myelin proteins, like protein P0 (Brookes et al., 1980). This ultimate differentiation of Schwann cells is thought to be regulated mainly by axon-derived signals (Aguayo et al., 1976; Lemke and Chao, 1988; Trapp et al., 1988; Brunden et al., 1990; Monuki et al., 1990), although the identity of the molecules involved has not yet been determined. In contrast to the transition of precursor to Schwann cell, the maturation of the Schwann cell into a myelin-forming cell is fully reversible as shown following nerve sectioning or in vitro culture in the absence of neurons (Mirsky et al., 1980; Neuberger and Cornbrooks, 1989; Jessen et al., 1990; Jessen and Mirsky, 1991).

We have recently demonstrated that the *Krox-20* gene, which encodes a zinc finger transcription factor (Chavrier et al., 1988, 1990), is involved in the control of Schwann cell myelination (Topilko et al., 1994). We have produced a null allele of *Krox-20* by in frame insertion of the *Escherichia coli*

*lacZ* gene (Schneider-Maunoury et al., 1993) and have shown that, in the homozygous state, the mutation appears to block Schwann cells at a very precise and early step of their differentiation toward myelin-forming cells; although Schwann cells express MAG, late myelin gene products are absent (Topilko et al., 1994). Mice carrying the *lacZ* insertion in the *Krox-20* locus present no phenotype in the heterozygous state. Since the *lacZ* expression pattern faithfully reflects that of *Krox-20* (Topilko et al., 1994 and data not shown), these mice provide a sensitive and simple system for studying *Krox-20* regulation. In heterozygous embryos,  $\beta$ -galactosidase is first detected in the PNS around 10.5 days post coitum (dpc) in boundary cap cells (Wilkinson et al., 1989; Schneider-Maunoury et al., 1993), which are glial cells found in the sensory and motor roots of both cranial and spinal nerves and are thought to be largely of neural crest origin (D'Amico-Martel and Noden, 1983; Le Douarin, 1986; Le Douarin et al., 1991). Up to 14.5 dpc, *Krox-20* expression in the PNS is confined to the nerve roots, with no labelling of the ganglia or of more distal parts of the fibres, with the exception of the XIth cranial nerve (Schneider-Maunoury et al., 1993; Topilko et al., 1994). A major transition occurs around 15.5 dpc, when *Krox-20/lacZ* is activated along the entire length of peripheral nerves. This expression is confined to Schwann cells and activation along the nerves coincides with the transition from precursors to Schwann cells (15.5 dpc in the mouse, which corresponds approximately to E16.5 in the rat). No  $\beta$ -galactosidase is detected in sensory ganglia or in the sympathetic system.

We have now extended our analysis of the expression of *Krox-20/lacZ* in Schwann cells. By studying cultures of Schwann cells performed in the presence or absence of neurons and cultures of dorsal root ganglion (DRG) glial cells, we have identified three levels of regulation of *Krox-20* expression, which appear to reflect important steps in peripheral glial cell development.

## MATERIALS AND METHODS

### Culture conditions

Standard culture medium for primary new-born Schwann cells, unless otherwise stated, was Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 IU/ml), sodium pyruvate (1 mM) and 10% fetal calf serum (FCS) (all from Gibco BRL). Serum-free defined medium (DM) consisted of a 1:1 mixture of DMEM and Ham's F12 (Gibco BRL) supplemented with selenium ( $3 \times 10^{-7}$  M), transferrin (100  $\mu$ g/ml), putrescine (10  $\mu$ g/ml), progesterone (60 ng/ml), bovine serum albumin (0.3%), insulin (5  $\mu$ g/ml), glucose (0.2%) (all from Sigma), and glutamine, penicillin, streptomycin and sodium pyruvate as above. DRG were cultured as explants either in DMEM, 5% FCS or DM both supplemented with 1 ng/ml nerve growth factor (NGF, Sigma). FUDR and forskolin (Sigma) were added to final concentrations of 10 and 5  $\mu$ M respectively. Human NDF $\alpha$ -2 and  $\beta$ -2 recombinant proteins and the polypeptide corresponding to the EGF-like domain of NDF $\beta$ -2 were kindly provided by Amgen, CNTF was purchased from Promega, bFGF and IGF-II were from Sigma and LIF from Gibco BRL.

All cultures, except 3-dimensional collagen gels, were performed on glass coverslips treated either with poly-DL-ornithine (3  $\mu$ g/ml) and laminin (2  $\mu$ g/ml) (Sigma), or poly-DL-ornithine alone. The presence of laminin changed only the density of axonal outgrowth achieved in DRG explants, *Krox-20* induction being equivalent in its presence or absence.

Neural tube cultures required a 3-dimensional collagen support. These cultures were carried out largely following the method of Placzek et al. (1993). Collagen was prepared from rat tail tendons. Explants were assembled as required on a dry collagen-treated culture dish in a drop of liquid collagen in DMEM. The collagen was allowed to set at room temperature for 40 minutes and the cultures were then flooded with medium and incubated at 37°C. At the end of the required culture period, the explants could be stained for  $\beta$ -galactosidase activity in situ.

### Primary Schwann cell culture and DRG explants

Schwann cells were prepared from new-born sciatic nerves following the method of Brockes et al. (1979). DRG were dissected from embryos between 12.5 dpc and 17.5 dpc and from new-born mice following removal of the spinal cord from the dorsal aspect. The DRG were then either cut into dorsal and ventral halves or placed whole in culture. If a pure neuronal culture was required, the explants were placed immediately in medium containing FUDR which was alternated with standard medium every 2-3 days. If a culture containing endogenous glia was required, only short pulses of FUDR (12 hours) treatment were administered every 3-4 days.

### Revelation of $\beta$ -galactosidase activity and immunochemistry

Cultures were fixed for 5 minutes in 0.2% glutaraldehyde/PBS, washed twice for 5 minutes in PBS containing 2 mM MgCl<sub>2</sub>, 0.02% NP40 and 0.1% sodium deoxycholate and stained in the same solution containing in addition 5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 5 mM K<sub>4</sub>Fe(CN)<sub>6</sub> and 0.4 mg/ml X-gal. Staining was carried out at 30°C for at least 1 hour but typically overnight. All cultures from the same experiment were stained for the same length of time. Whole embryos were stained as described in Schneider-Maunoury et al. (1993).

The S100 and GalC antibodies were purchased from Sigma. The P0 and MAG antibodies were gifts from B. Trapp. The MBP antibody was a gift from F. Lachapelle. The 2H3 monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank. The *Krox-20* antibody was derived against a *Krox-20* bacterial fusion protein (Vesque and Charnay, 1992). Antibodies were applied in PBS/5% FCS/0.2% gelatin/0.1% NP40 usually at a 1:200 dilution (1:50 for *Krox-20*; 1:500 for S100; 1:5000 for 2H3) for 1.5 hour. The goat anti-rabbit secondary antibody coupled with FITC was purchased from Biosys. Goat anti-rabbit and goat anti-mouse coupled with peroxidase were purchased from Sigma. All secondary antibodies were used diluted 1:200. Slides were either mounted in Mowiol for observation of fluorescence or DAB stained to reveal peroxidase activity. Immunofluorescence of paraffin sections of whole embryos was carried out largely as above with the exception that incubation was overnight with the primary antibody and 4 hours with the secondary antibody.

## RESULTS

### *Krox-20* is activated in Schwann cells co-cultured with neurons

In a first series of experiments to analyse the expression of *Krox-20* in Schwann cells in vitro, we prepared primary cultures of Schwann cells from sciatic nerves of new-born mice heterozygous for the *Krox-20* mutation. When placed in standard culture conditions, these Schwann cells quickly lost *Krox-20/lacZ* expression, with  $\beta$ -galactosidase activity no longer detectable after 24 hours (Fig. 1A,B and data not shown). Since the axon is known to regulate a number of important steps in Schwann cell differentiation, new-born *Krox-20*<sup>+/-</sup> Schwann cells were co-cultured with wild-type

sensory neurons. The neuron cultures were derived from 17.5 dpc DRG explants, which were maintained in culture for 3 weeks in the cyclical presence of Fluorodeoxyuridine (FUDR) to eliminate the majority of non-neuronal cells and to obtain an extensive network of naked axons. When primary Schwann cells were seeded onto these neuron cultures, *Krox-20/lacZ* expression was reactivated within 36 hours (Fig. 1C,D and data not shown).

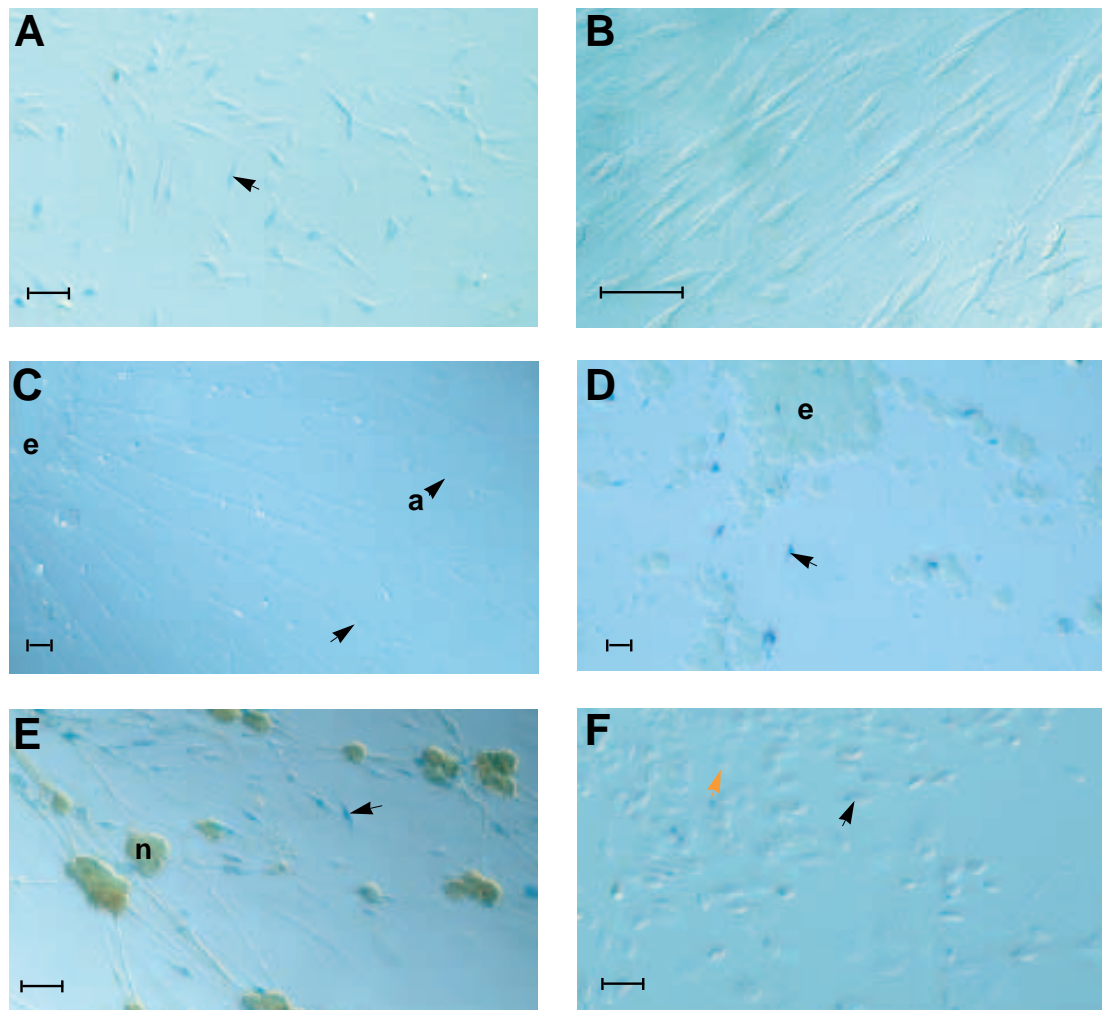
Co-culture with neurons can lead Schwann cells to undergo the myelination programme (Eldridge et al., 1987, 1989; Morrison et al., 1991). We have looked for the expression of several myelin markers (using antibodies against P0, MBP, MAG, and galactocerebroside (GalC)) in these short-term co-cultures but failed to reveal a detectable level of these antigens (data not shown). Therefore the rapid induction of *Krox-20* takes place within a time period that does not allow Schwann cells to fully differentiate and commence myelination of the axons. This is consistent with the requirement for *Krox-20* for myelination in vivo (Topilko et al., 1994).

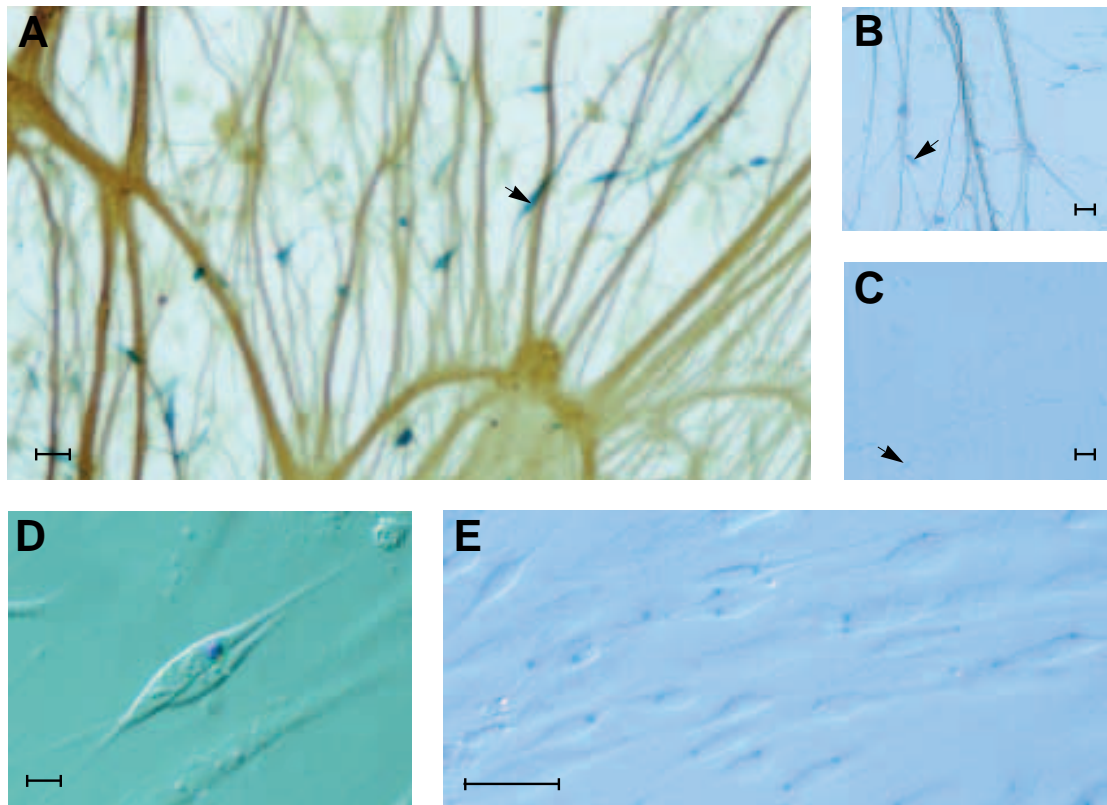
### ***Krox-20* induction requires axonal contact**

Not all of the Schwann cells in a co-culture are induced and, in order to better visualise the relationship between induced Schwann cells and axons, an anti-neurofilament antibody staining was carried out. This showed that strong *Krox-20/lacZ* induction was only seen in Schwann cells that achieved direct contact with an axon (Fig. 2A,B). The majority of Schwann cells that did not achieve axonal contact were negative for  $\beta$ -galactosidase activity, while a small minority showed a low level of induction with a surprisingly very localised staining (Fig. 2C,D). The site and origin of this localisation were not investigated further.

To further test the diffusibility of the axonal signal, conditioned medium was prepared from DRG explant cultures. This medium did not induce *Krox-20/lacZ* when added to primary Schwann cells (data not shown). We also used a 'millicell' system (Millipore) where the DRG explants were grown on a membrane suspended 1 mm above the floor of the culture dish where the Schwann cells were seeded. In this system, axons do not cross the filter (Bolin and Shooter, 1993), while secreted

**Fig. 1.** Expression of *Krox-20/lacZ* in cultured Schwann cells. (A,B) Primary Schwann cells prepared from *Krox-20*<sup>+/-</sup> new-born pups after 12 (A) or 48 hours (B) of culture; a low level of activity is still detectable in some of the cells after 12 hours (arrow), while at 48 hours all Schwann cells appear negative. (C) DRG explant prepared from a 17.5 dpc wild-type embryo and cultured in the cyclical presence of FUDR for 3 weeks; the axons are largely free of associated glia but a small number of endogenous Schwann-like cells persist (arrow). (D) A parallel DRG explant co-cultured for 48 hours with *Krox-20*<sup>+/-</sup> new-born primary Schwann cells; note the induction of *Krox-20/lacZ* expression (arrow). (E) 48 hour co-culture of *Krox-20*<sup>+/-</sup> new-born Schwann cells (arrow) with 3 week DRG explants prepared from a 12.5 dpc embryo. The neurons and axons are stained with an antibody directed against neurofilaments (2H3). (F) 48 hour co-culture where the 12.5 dpc wild-type DRG explant and the *Krox-20*<sup>+/-</sup> new-born Schwann cells were simultaneously placed in culture; the axons are relatively inaccessible due to the rich outgrowth of non-neuronal cells from the explant (orange arrow), which can be distinguished morphologically from the added Schwann cells (black arrow) in which *Krox-20/lacZ* is induced. e, DRG explant; n, neuron; a, axon; scale bars, 30  $\mu$ m.





**Fig. 2.** Efficient induction of *Krox-20* in new-born Schwann cells requires axonal contact. (A) 17.5 dpc wild-type DRG explant co-cultured with *Krox-20*<sup>+/-</sup> new-born Schwann cells for 48 hours, stained with X-gal and treated with an anti-neurofilament antibody (2H3) to visualise the axons; *Krox-20/lacZ* expression is observed in the added Schwann cells (arrow). (B-D) Schwann cells (arrows) in the same co-culture either in contact with (B) or distant from (C,D) an axon; in the absence of axonal contact a minority of cells show a low-level, localised staining (arrow in C and high magnification in D). (E) Co-culture of Schwann cells from *Krox-20*<sup>+/-</sup> new-born pups with a 17.5 dpc wild-type DRG explant in the Millicell system for 48 hours. Scale bar, 5 µm in D and 30 µm otherwise.

macromolecules are free to diffuse. Under these experimental conditions, even after 5 days of co-culture, only a low level of  $\beta$ -galactosidase activity was detected and the staining was again highly localised (Fig. 2E and data not shown). Altogether, these findings suggest that efficient *Krox-20* induction in Schwann cells requires direct axonal contact.

Several Schwann cell markers, including myelin proteins and the POU-domain gene *SCIP*, that are regulated by axonal signals also respond to changes in intracellular cAMP levels (Lemke and Chao, 1988; Monuki et al., 1989; Weinmaster and Lemke, 1990; Morgan et al., 1991). It has therefore been suggested that increasing cAMP levels mimic axonal signalling. The addition of forskolin (5–10 µM) to the culture medium, which effectively increases intracellular cAMP levels, led to a limited induction of *Krox-20/lacZ* expression within 30 hours, lower than that seen with axonal contact (data not shown). We also tested the ability of a number of growth factors and neurotrophic factors to mimic *Krox-20* axonal induction. These factors included brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), transforming growth factor  $\beta$ 3 (TGF $\beta$ 3), glial cell line-derived neurotrophic factor (GDNF), CNTF (all at 1–10 ng/ml), bFGF (3 ng/ml), NDF $\beta$ -2 (10 ng/ml) and bovine pituitary extract (100 µg/ml). None of these factors stimulated *Krox-20/lacZ* expression in primary Schwann cells (data not shown).

#### Acquisition of *Krox-20* inducibility at 15.5 dpc coincides with precursor to Schwann cell transition

Since the expression of *Krox-20* in the PNS involves axonal signalling, the activation of the gene in vivo at 15.5 dpc raises an important question: is this the consequence of a change in the neurons, which acquire the capacity to provide the signal, or a change in the Schwann cells, which become responsive to a pre-existing axonal signal? To differentiate between these two possibilities, we investigated the capacity of neurons from wild-type DRG collected at different stages of development (i.e. 12.5, 13.5, 14.5, 15.5, 17.5 dpc and new-born DRG) to induce *Krox-20/lacZ* expression in Schwann cells from new-born *Krox-20*<sup>+/-</sup> mice. The explants were maintained in culture for 3 weeks, as described above. Under these co-culture conditions, explants from all stages were capable of inducing *Krox-20/lacZ* to a similar level (Fig. 1D,E and data not shown), although a greater percentage of Schwann cells seemed to be induced with explants from more advanced stages (17.5 dpc and new born). This may be due to the larger calibre axons that are produced by developmentally older neurons. To ensure that the capacity of the developmentally younger axons to induce *Krox-20* was not due to continued neuronal maturation in culture, 12.5 dpc DRG explants were placed immediately in co-culture with new-born *Krox-20*<sup>+/-</sup> Schwann cells and the culture was stained for  $\beta$ -galactosidase activity after 48

hours. The axon growth in these cultures was less extensive and the axons were thin and densely packed. There was also a rich growth of endogenous fibroblasts and glial precursor cells, which migrated from the explant covering the axons, thus making contact difficult between the added Schwann cells and the axons. However, where contact occurred, the Schwann cells expressed *Krox-20/lacZ*, although at a reduced level (Fig. 1F), demonstrating that sensory axons from 12.5 dpc embryos have the capacity to activate *Krox-20* in new-born Schwann cells and suggesting that in vivo an axonal signal is present from 12.5 dpc. Given that Schwann cell precursors are in place along the nerve, presumably from at least 13.5 dpc based on comparison with the rat (Jessen et al., 1994), the appearance of *Krox-20* expression along the peripheral nerve only at 15.5 dpc suggests that Schwann cell precursors are not capable of responding to the axonal signal before that time. Acquisition of this capacity appears to be approximately coincidental with the precursor to Schwann cell transition (Jessen et al., 1994).

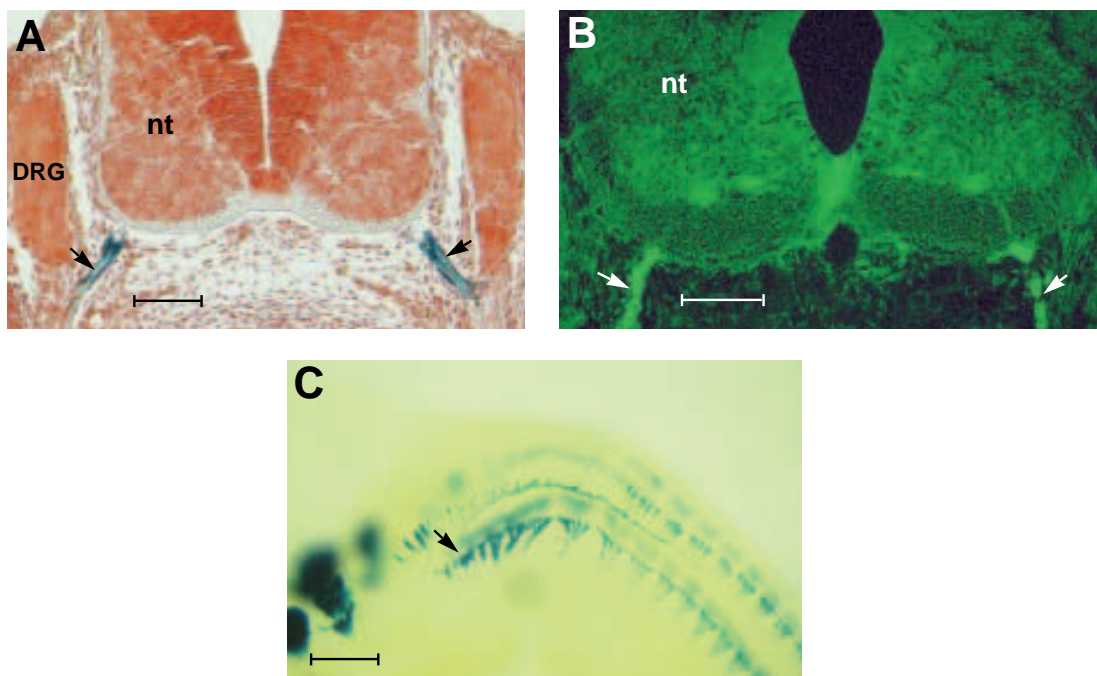
#### Precursor to Schwann cell transition occurs earlier within nerve roots

The observation of early (from 10.5 dpc) *Krox-20* expression in boundary cap and nerve root glial cells (Topilko et al., 1994; Fig. 3A) led us to investigate whether these cells may have reached an advanced stage in their differentiation. To address this question, we examined the expression of S100 within the nerve root, since this marker allows discrimination between Schwann cells and their precursors along the peripheral nerve (Jessen et al., 1994). Immunofluorescence analysis of 12.5 dpc wild-type

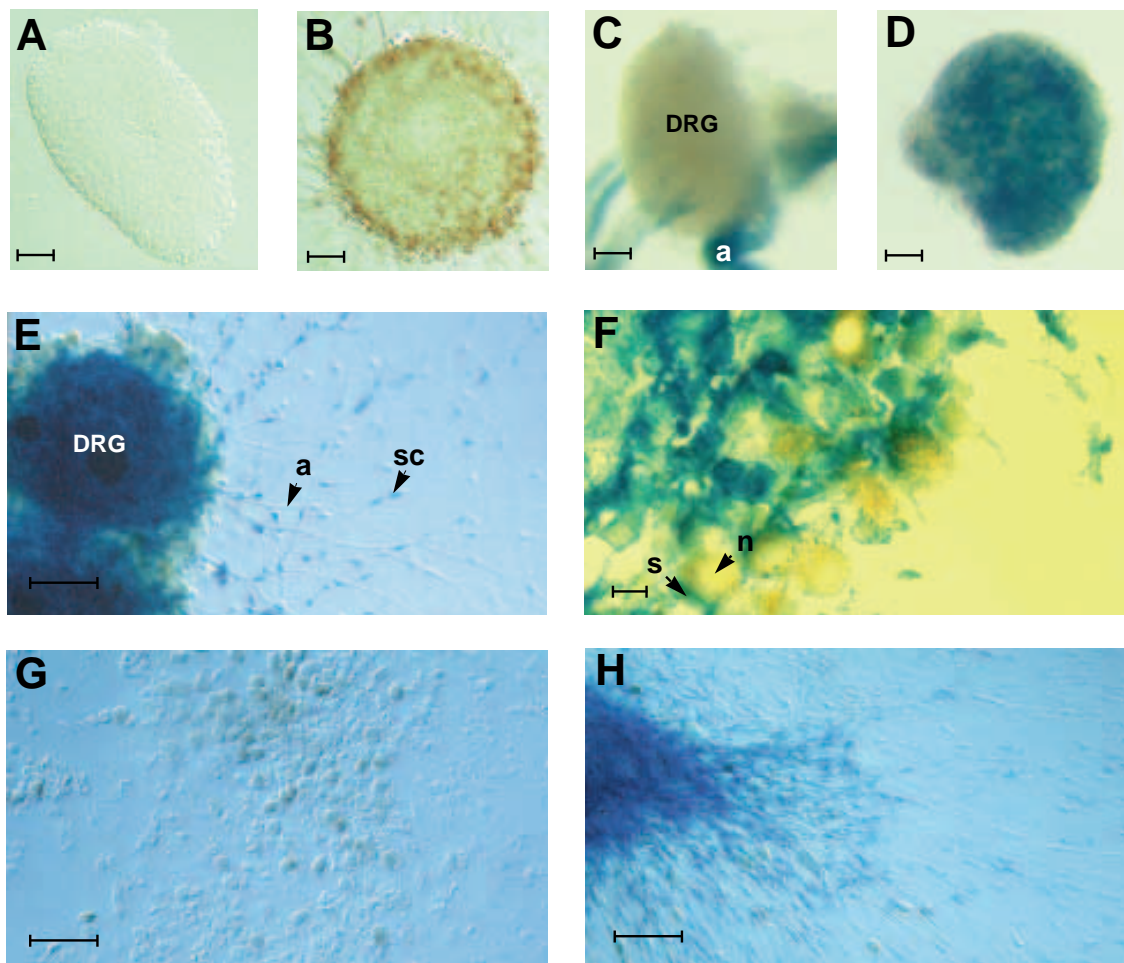
nerve root sections indicated that S100 was indeed present (Fig. 3B). These data suggest that the glial cells within the nerve roots convert to Schwann cells much earlier than elsewhere.

#### *Krox-20* is rapidly activated in cultured DRG glia

As indicated above, *Krox-20* is never expressed in DRG in vivo, although the glial cells that contact the neuronal cell bodies (satellite cells) constitute a lineage closely related to Schwann cells (Frank and Sanes, 1991; Fraser and Bronner-Fraser, 1991). In vitro culture of DRG has been shown to result in the acquisition by satellite cells of Schwann cell characteristics (Cameron-Curry et al., 1993). To examine the possibility that satellite cells could also be induced to express *Krox-20* in vitro, we cultured DRG explants from 17.5 dpc wild-type and *Krox-20*<sup>+/-</sup> embryos and examined *Krox-20* and *Krox-20/lacZ* expression respectively (the *Krox-20* protein was detected by immunocytochemistry using a rabbit polyclonal antibody raised against a *Krox-20* fusion protein (Vesque and Charnay, 1992)). The explants were treated with brief (12 hours) pulses of FUDR in order to restrict fibroblast growth but to allow survival of glial cells. While both *Krox-20* and *Krox-20/β-gal* could not be detected in freshly explanted DRG, after culture for 48 hours expression was observed throughout the ganglionic glia (Fig. 4A-E) and *Krox-20/lacZ* expression was detected within the explant 6 hours after initiation of the culture (data not shown). After about 36 hours of culture, β-galactosidase-positive glial cells migrated out from the explant to populate the growing axons (Fig. 4E). Within the explant, expression was not restricted to cells positioned along the axons but was also detected in satellite cells in contact with neuronal cell bodies,



**Fig. 3.** Expression of mature Schwann cell markers occurs in vivo in specific populations of peripheral glia cells before 15.5 dpc. (A) Transverse section of an 11.5 dpc *Krox-20*<sup>+/-</sup> embryo showing *Krox-20/lacZ* expression in the ventral nerve roots close to the neural tube (arrows). (B) Immunofluorescence analysis of a transverse section of a 12.5 dpc wild-type embryo showing that S100 is also expressed in the nerve roots (arrows). (C) Whole-mount X-gal staining of an 11.5 dpc *Krox-20*<sup>+/-</sup> embryo showing *Krox-20/lacZ* expression in a portion of the XIth nerve, which runs parallel and close to the neural tube up to the level indicated by the arrow. Rostral is to the left, dorsal to the top. nt, neural tube; scale bars, 200 μm (A,B) and 500 μm (C).



**Fig. 4.** *Krox-20* expression in cultured DRG glia. (A,B) DRG explants from a 17.5 dpc wild-type embryo stained with an antibody directed against *Krox-20*, immediately after dissection (A) or cultured for 48 hours (B). (C-E) DRG explants from a 17.5 dpc *Krox-20*<sup>+/-</sup> embryo stained for  $\beta$ -galactosidase activity immediately after dissection (C; note that X-gal staining is restricted to the attached bundles of axons) or cultured for 18 (D) or 48 hours (E); the 48 hours culture shows that *Krox-20/lacZ* is expressed by Schwann-like cells that have migrated along the axons as well as by the glia in the explant itself. (F) Section of a 17.5 dpc DRG explant cultured for 48 hours showing that expression is not detected in the anti-neurofilament positive neuronal cell bodies but is observed in the closely associated glia. (G,H) Comparison of *Krox-20/lacZ* expression in DRG explants prepared from *Krox-20*<sup>+/-</sup> embryos at 12.5 dpc (G) or 15.5 dpc (H) and cultured for 48 hours. a, axon; sc, Schwann-like cell; DRG, dorsal root ganglion; n, neuronal cell body; s, satellite cell; scale bar, 10  $\mu$ m in F and 100  $\mu$ m otherwise.

while the neurons themselves were negative (Fig. 4F). In conclusion, these data indicate that DRG culture leads to massive induction of *Krox-20* in the glial component. In addition, the detection of *Krox-20* in wild-type DRG cultures confirms that the  $\beta$ -galactosidase activity in heterozygous explants faithfully reflects the *Krox-20* expression pattern.

#### A transition occurs within DRG glia around 15.5 dpc

To investigate when DRG glial cells acquire the capacity to activate *Krox-20* in vitro, we compared DRG explant cultures from *Krox-20*<sup>+/-</sup> 12.5, 13.5, 14.5, 15.5, 17.5 dpc embryos and new-born mice. These cultures differed dramatically in both their capacity to express *Krox-20/lacZ* and in the morphology of their migrating glial cells depending on whether they were prepared before or after 15.5 dpc. With DRG collected before 15.5 dpc, the glial cells did not express *Krox-20/lacZ* for at least 48 hours in culture, even when in contact with axons, and the migrating cells had a flattened morphology (Fig. 4G and

data not shown). From 15.5 dpc,  $\beta$ -galactosidase activity was detected within the explant from 6 hours of culture (data not shown). The migrating cells expressed *Krox-20/lacZ*, showed the typical elongated, bipolar, Schwann cell morphology and extended themselves along the axons (Fig. 4H). This suggests that an important transition event in the development of DRG glial cells takes place around 15.5 dpc in the mouse. Such a modification appears similar to the transition of precursor to Schwann cell and may be required for *Krox-20* activation in response to axonal signalling.

#### Diffusible factor(s) produced by the neural tube can induce *Krox-20* expression in 12.5 dpc satellite cells

As indicated above, a restricted population of glial cells within the nerve roots express *Krox-20* from 10.5 dpc (Fig. 3A). In addition, the gene is also expressed in the portion of the XIth nerve lying close to the neural tube (Fig. 3C). These observations raise the possibility that the neural tube may exert an

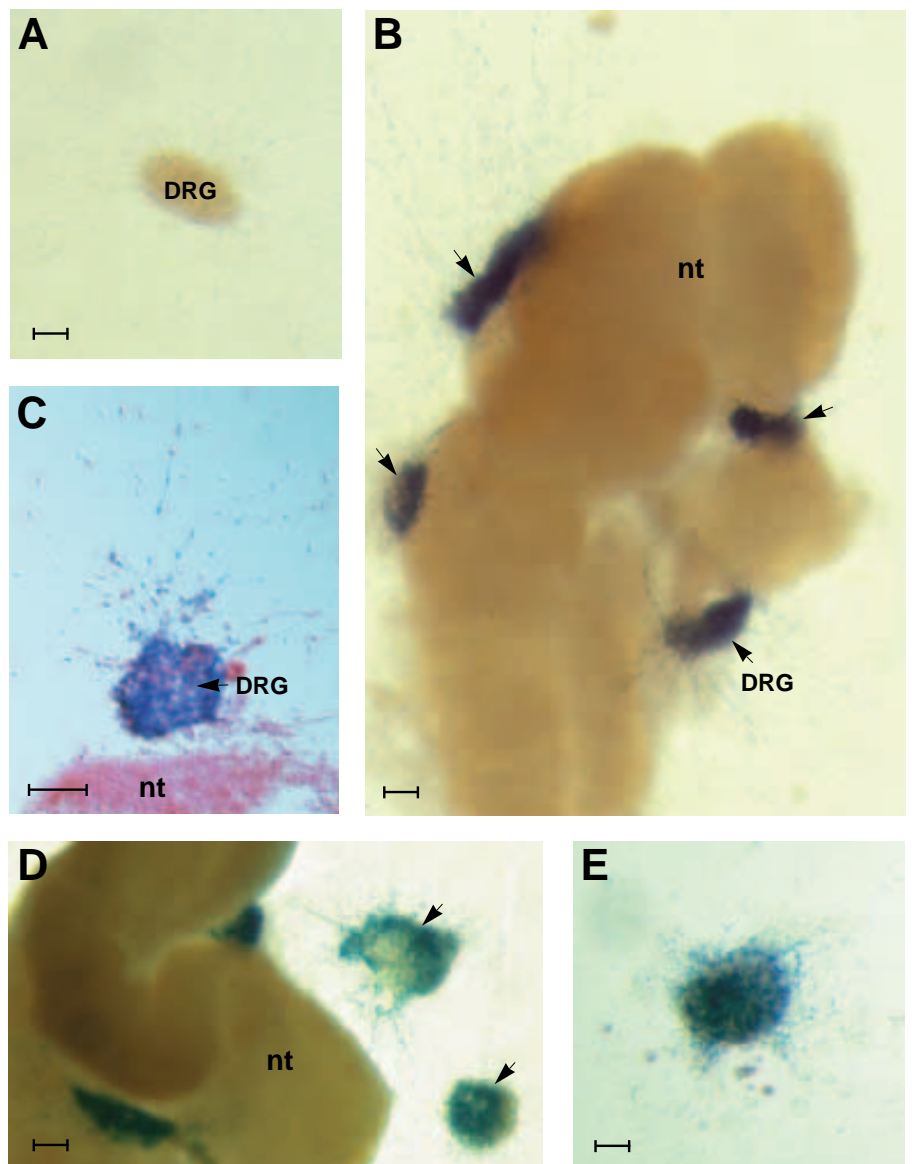
influence on Schwann cell precursors in its vicinity and induce the expression of *Krox-20*. To explore such a possibility, given the technical difficulties involved in isolating Schwann cell precursors in the mouse embryo, we examined the effect of the neural tube on glial cells in DRG explants, which we had previously established behave like Schwann cell precursors in culture. Co-culture experiments were performed in 3-dimensional collagen gels with 12.5 dpc DRG, which do not express *Krox-20* in vitro, using the ventral half of the DRG to eliminate any spontaneously blue cells possibly originating from the nerve root. The neural tube explants were prepared from 10.5 dpc wild-type embryos and consisted of the region from rhombomere 5 to the level of the anterior limb bud, opened at the dorsal midline. Under these circumstances, after a culture period of 48 hours, the presence of the neural tube led to a dramatic activation of *Krox-20/lacZ* in the DRG explants (Fig. 5A-C).

To test whether the signal provided by the neural tube is a freely diffusible factor, we: (i) placed the DRG explants at a distance from the neural tube, (ii) inserted a 'biopore' filter (pore

size, 0.2  $\mu\text{m}$ ) between the neural tube and the DRG explant, and (iii) grew the DRG explants in the presence of neural tube conditioned medium. In all three circumstances *Krox-20* induction was achieved within 48 hours (Fig. 5D,E and data not shown). The extent of induction was quantitatively affected by the distance from the neural tube explant: whereas a distance of 200  $\mu\text{m}$  or less led to a strong induction, a separation of 500  $\mu\text{m}$  gave a visibly weaker effect. These data demonstrate that the neural tube produces diffusible factor(s) that can activate *Krox-20* expression in 12.5 dpc glial cells in culture.

#### NDF $\beta$ is a major component of the neural-tube-inducing activity

A number of factors were tested for their ability to reproduce the effect of the neural tube factor(s) on 12.5 dpc DRG explants. These included bFGF (3 ng/ml), NGF, BDNF, NT-3, NT-4, CNTF, GDNF, NDF $\alpha$ -2 and NDF $\beta$ -2 (each at 10 ng/ml), leukemia inhibitory factor (LIF, 1000 U/ml) and bovine pituitary extract (100  $\mu\text{g/ml}$ ). While various effects were



**Fig. 5.** *Krox-20* is induced in 12.5 dpc DRG glia by diffusible factor(s) from the neural tube. (A) Explant prepared from the ventral half of a 12.5 dpc *Krox-20*<sup>+/−</sup> DRG cultured in a 3-dimensional collagen gel for 48 hours. (B) 4 sister explants to that shown in A (arrows) co-cultured with a 10.5 dpc wild-type neural tube. (C) Section of the co-culture shown in B, counter-stained with saphranine, showing expression of *Krox-20/lacZ* in the ganglionic glia and the migrating glial cells. (D) Co-culture similar to that shown in B where DRG explants (arrows) have been separated from the neural tube by a biopore filter (0.2  $\mu\text{m}$  pore size) and a distance of approximately 150  $\mu\text{m}$ . (E) DRG explant similar to that shown in A which has been cultured in neural tube-conditioned medium for 48 hours. nt, neural tube; scale bars, 100  $\mu\text{m}$ .

observed on the rate and density of axonal outgrowth with these different factors (data not shown), only CNTF, LIF, bFGF and NDF $\beta$ -2 had an influence on *Krox-20/lacZ* expression. CNTF and LIF treatment for 48 hours consistently induced expression to a clearly detectable level and varying the concentration of CNTF between 10 and 100 ng/ml did not significantly change the level of response (Fig. 6B and data not shown). The level of induction obtained with each of these factors alone was lower than the level achieved in co-cultures with the neural tube. bFGF alone had little or no effect on *Krox-20* expression. However, when bFGF (3 ng/ml) was added in the presence of CNTF (10 ng/ml) or of LIF, a strong synergistic effect was observed (Fig. 6B,C and data not shown). NDF $\beta$ -2 alone was also able to strongly activate *Krox-20/lacZ* expression and the effect was dose-dependant (Fig. 6D and data not shown). A polypeptide corresponding to the epidermal growth factor (EGF)-like domain of NDF $\beta$ -2 (residues 177-228) led to significant induction at a concentration of 1 ng/ml and to strong activation at 10 ng/ml (data not shown). Therefore the effect of the neural tube factor(s) on 12.5 dpc DRG explants can be reproduced with NDF $\beta$ -2 or a combination of CNTF and bFGF.

To directly investigate the possibility that NDF $\beta$  might be the inducing factor produced by the neural tube, we performed blocking experiments with a soluble hybrid protein containing the extracellular domain of the ErbB4 NDF receptor, which was shown to specifically block the action of NDF (Dong et al., 1995). Addition of this protein at a concentration of 2  $\mu$ g/ml resulted in a strong inhibition of *Krox-20/lacZ* activation by either purified NDF $\beta$  or neural tube-conditioned medium (Fig. 6E,F and data not shown). However, a low level of activation was achieved with both

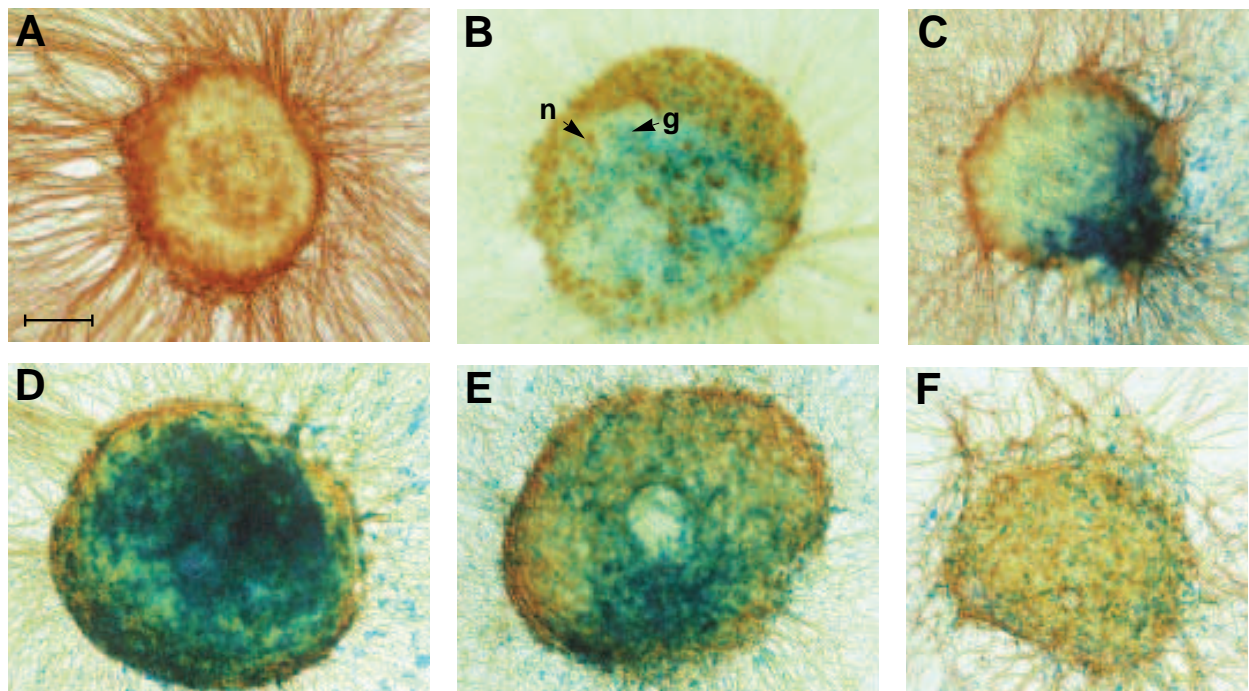
inducers even in the presence of the soluble receptor (Fig. 6F and data not shown). We conclude that NDF constitutes a major component of the inducing activity present in the neural tube conditioned medium.

## DISCUSSION

### *Krox-20* mediates axonal signalling to the Schwann cell nucleus

The differentiation of Schwann cells into myelin-forming cells depends on continuous signalling from the axon: myelin-forming Schwann cells, when removed from an axonal influence, continue to express S100, but the myelination process is reversed and the expression of corresponding markers is lost. Here we show that *Krox-20* also depends on axonal contact for continued high expression in Schwann cells. As in the case of the peripheral myelin protein P0 gene (Bolin and Shooter, 1993), a weak activation of *Krox-20* expression is nevertheless observed in the presence of neurons in the Millicell culture system, which prevents direct axonal contact. These data suggest that at least one of the neuronal factors required for *Krox-20* induction is membrane-bound, has a very limited diffusibility or is highly unstable.

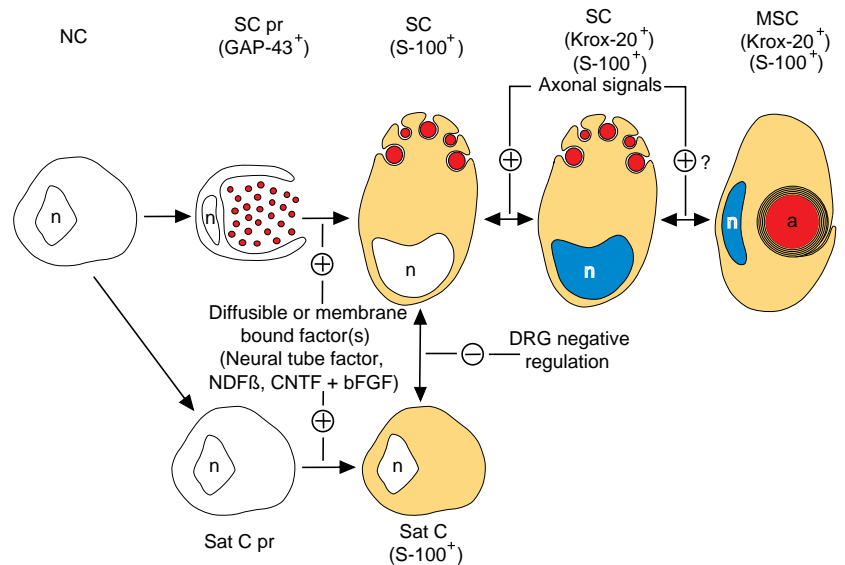
Analysis of *Krox-20*<sup>-/-</sup> mutant mice revealed that *Krox-20* is required for the completion of the myelination process in the PNS and that its absence prevents the activation of late myelin markers, including P0 (Topilko et al., 1994). The combination of these data with the present study suggests that one function of *Krox-20* in Schwann cells is to transmit an axonal signal to the nucleus, which is continuously required to express part of the genetic programme necessary for myelination. This pre-



**Fig. 6.** *Krox-20* is induced by NDF $\beta$ -2 alone and synergistically by CNTF and bFGF in 12.5 dpc DRG explants. (A-F) 12.5 dpc *Krox-20*<sup>+/-</sup> DRG explants stained for  $\beta$ -galactosidase activity and with an anti-neurofilament antibody (2H3), after 48 hours culture in serum-free defined medium supplemented with NGF alone (A), or NGF and the following additives: CNTF (10 ng/ml) (B), CNTF (10 ng/ml) plus bFGF (3 ng/ml) (C), NDF $\beta$ -2 protein (100 ng/ml) (D), neural-tube-conditioned medium (E), neural-tube-conditioned-medium plus a hybrid protein containing the extracellular domain of the ErbB4 NDF receptor (2  $\mu$ g/ml) (F). n, neuronal cell body; g, glia; scale bar, 60  $\mu$ m.



**Fig. 7.** Schematic representation of the development and differentiation of Schwann and satellite cells from their neural crest precursors. The three levels of regulation acting on *Krox-20* expression are indicated. See the discussion section for details of the model. Although S100 expression does not require *Krox-20* activation, the relative timing of their expression in vivo is not known. The existence of the satellite to Schwann cell conversion is hypothetical and the possible generation of a cell type distinct from Schwann cells upon culture of satellite cell is not represented for reasons of simplicity. Single arrows indicate irreversible processes, while double arrows indicate reversible transitions or conversions. Presence of *Krox-20* in the nucleus and of S100 in the cytoplasm are indicated in blue and yellow respectively. Axons are represented in red. NC, neural crest cell; SCpr, Schwann cell precursor; SC, Schwann cell; MSC, myelinating Schwann cell; Sat pr, satellite cell precursor; Sat, satellite cell; n, nucleus; a, axon.



sumably involves the direct transcriptional activation of a set of genes by *Krox-20*. Whether the late myelination markers are among these direct targets is not yet known.

#### Acquisition of *Krox-20* inducibility is part of the precursor to Schwann cell transition

Our co-culture experiments suggest that the induction of *Krox-20* along peripheral nerves at around 15.5 dpc is not directly due to a change in signalling from the neurons, but rather to a modification of the Schwann cells themselves, which allows them to respond to an axonal signal that pre-exists. However, our experiments do not address the possibility that this modification of the Schwann cells may actually be induced by the neurons. Several arguments issued from the present work suggest that the acquisition of *Krox-20* inducibility is part of the precursor to Schwann cell transition. (i) This event occurs along peripheral nerves at around the same time as the transition, i.e. 15.5 dpc, as estimated from the work in rat (Jessen et al., 1994). (ii) In nerve root glial cells, *Krox-20* activation occurs at 10.5 dpc, raising the possibility that in this microenvironment, the precursor to Schwann cell transition might occur much earlier than elsewhere. In the PNS, the S100 protein appears to mark this transition (Jessen et al., 1994). We have found that early nerve roots express S100, suggesting that the transition has indeed occurred. The possible functional significance of this localised early transition is so far unknown. (iii) The capacity of glia from sensory ganglia to activate *Krox-20* in culture is also acquired at around 15.5 dpc. This might again involve a transition, from precursor to mature satellite cells, with the same timing and possibly under the control of the same regulators as the precursor to Schwann cell transition. Indeed we have shown that NDF $\beta$ -2, which has been found to promote the precursor to Schwann cell transition in vitro (Dong et al., 1995), also activates *Krox-20* expression in early ganglia in culture. The concentration of NDF $\beta$ -2 effective for *Krox-20* activation in 12.5 dpc DRG explants (in the range of 200 pM) is similar to that required for the full modification of Schwann cell precursor properties in vitro (Dong et al., 1995). Furthermore, the EGF-like domain (residues 177-228) is sufficient for the two types of induction phenomena.

In conclusion, we propose that the capacity to activate *Krox-*

20 in response to axonal signalling is characteristic of the Schwann cell as opposed to its precursor. One possible mechanism for this would be that the transition to the mature Schwann cell involves the expression of a molecule required for the reception or transduction of the axonal signal. In any case, the expression of *Krox-20* itself does not appear to be required for the acquisition of the characteristic survival and proliferation capacities of mature Schwann cells, since these are observed in the absence of axonal signalling (Dong et al., 1995). Furthermore, Schwann cells from homozygous *Krox-20* mutant mice express S100 (Topilko et al., 1994).

#### Factors regulating *Krox-20* expression and glial cell maturation

The results of our experiments demonstrate that diffusible factor(s) from the 10.5 dpc neural tube induce *Krox-20* expression in the glial cells of 12.5 dpc DRG explants. We propose that this activation is the result of the combination of two modifications occurring in these cells, followed by axonal signalling: (1) a precursor to satellite cell transition, induced by the neural tube factor, and (2) a modification of the satellite cell phenotype, which is allowed by in vitro culture and involves a release of *Krox-20* repression.

At present, we do not know whether the factors responsible for the neural tube activity measured in our in vitro system, for the early expression of *Krox-20* in boundary cap cells and for its late activation in Schwann cells are identical. Nevertheless NDF $\beta$  appears to be a good candidate for mediating the activation of *Krox-20* in these different situations. (i) We have established that NDF $\beta$  is the major component of the inducing activity originating from the neural tube measured in our assay. (ii) NDF $\beta$  has been shown recently to regulate survival, proliferation and lineage progression of rat Schwann cell precursors (Dong et al., 1995) and inactivation of its gene was shown to prevent normal development of Schwann cell precursors (Meyer and Birchmeier, 1995). (iii) The known expression sites of the NDF gene, including in particular the spinal motor neurons and DRG neurons (Marchionni et al., 1993; Orr-Urtreger et al., 1993; Meyer and Birchmeier, 1994), are consistent with a role in the activation of *Krox-20* in the PNS. We propose the following hypothesis for the activation of *Krox-20*

in two phases during PNS development: at early stages (i.e. 10.5 to 15.5 dpc), due to specific splicing, NDF $\beta$  might be expressed as a secreted molecule and released by the soma of the neurons from the neural tube and possibly from the DRG, therefore acting only locally on the Schwann cell precursors in the nerve roots. From 15.5 dpc, a modification of the splicing pattern might occur, resulting in the synthesis of a membrane-bound form of NDF $\beta$  which would reach the axons and therefore be able to stimulate Schwann cell precursors along the nerves. Analysis of the specific expression of the different isoforms of NDF should allow this hypothesis to be tested.

Although the case of NDF $\beta$  is strong, an involvement of other factors in the maturation of Schwann cells is also worth consideration. It has been shown that bFGF alone can promote precursor to Schwann cell transition as assayed by cell survival in vitro and morphological modifications (Jessen et al., 1994; Gavrilovic et al., 1995). However, recent studies have shown that this effect is only transient and that long-term survival requires co-operation between bFGF and other serum factors (Dong et al., 1995). In our assay, bFGF alone is only very weakly active, but it can act synergistically with other factors to induce *Krox-20* expression. This observation again reinforces the parallel between our assay involving 12.5 dpc ganglia and the maturation of rat Schwann cell precursors. In this respect, it will be interesting to determine whether the combination of bFGF with CNTF or LIF allows long-term survival of Schwann cell precursors.

#### ***Krox-20* negative regulation in peripheral ganglia may reflect the specification of the glial cell phenotype by the microenvironment**

The observation of a massive and very rapid induction of *Krox-20* expression in the glial component of explanted DRG, which cannot simply be due to an expansion of a subpopulation of Schwann cells, indicates that *Krox-20* activation occurs in the satellite cells. This regulation of *Krox-20* expression in satellite cells could be explained in two ways. (i) The explantation of the DRG and/or the exposure of the cells to culture medium could result in the activation of *Krox-20* via a positive regulatory mechanism. It is in fact known that axotomy induces morphological and biochemical changes in the neuronal cell body and that these changes are reflected in the behaviour of the satellite cells (Wen et al., 1994). (ii) An alternative hypothesis is that, in the in vivo environment of the DRG, *Krox-20* is subject to negative regulation in satellite cells and that disruption of DRG structure in culture results in derepression. We favour the second hypothesis since *Krox-20* activation occurs in very short-term culture and is observed in culture with serum-free defined medium.

The observation that *Krox-20* activation, which is restricted to Schwann cells in the PNS in vivo, can occur in DRG satellite cells in culture raises the possibility that this phenomenon may reflect a more profound modification of satellite cells, corresponding to a conversion to the Schwann cell-like phenotype. As mentioned previously, satellite cells are closely related to Schwann cells in term of lineage. Furthermore, our observation that in vitro culture cannot activate *Krox-20* in DRG explanted before 15.5 dpc suggests that satellite cells presumably undergo a transition from precursors to mature cells in a similar way to Schwann cells. These transitions occur approximately at the same time for the two glial cell types and can be

mediated by the same factor (NDF $\beta$ -2), indicating that the precursor cells present in the peripheral nerves and sensory ganglia share similar properties. DRG explants constitute rich sources of Schwann cells in culture and these may be partly generated from the satellite cells (Wood, 1976). In addition, in the avian PNS, the Schwann cell myelin protein (SMP) gene, which is expressed exclusively by Schwann cells in vivo (Dulac et al., 1988), can be activated in DRG satellite cells in vitro (Cameron-Curry et al., 1993). These data support the idea that different types of glia are derived from a common lineage and differentiate according to microenvironmental influences and that conversion between the alternative cell types is possible when environmental signalling is modified. In conclusion, our results are consistent with the occurrence of a satellite to Schwann cell conversion upon DRG culture, involving the release of *Krox-20* repression.

#### **Conclusion: three levels of *Krox-20* regulation in peripheral glia reflect important developmental steps**

Despite the availability of several molecular markers, our knowledge of the molecular and cellular mechanisms controlling the development of peripheral glia is very limited. In the present paper, we have provided evidence for the existence of three levels of control of *Krox-20* gene expression in PNS glial cells, which are schematically depicted in Fig. 7. We have shown that the regulation of *Krox-20* expression is linked to three fundamental developmental steps: (i) the maturation of glial cells from their precursors, (ii) a modification of satellite cells, which may consist in a conversion into Schwann cells, and (iii) axonal signalling, which mediates the activation of the myelination programme. It is interesting to note that the activation of *Krox-20* in Schwann cell precursors might actually require two ordered signals from the axon: the first one may correspond to NDF $\beta$  and leads to an irreversible transition toward mature Schwann cells and the second one is presumably membrane-bound and engages the cells into a reversible differentiation process. The availability of a simple and sensitive assay for *Krox-20* expression has allowed us to initiate the analysis of some aspects of Schwann cell development. Future exploitation of this system may provide access to additional molecular regulators of the Schwann cell developmental pathway.

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