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# *Pax3*: A Paired Domain Gene as a Regulator in PNS Myelination

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## Summary

***Pax3* RNA is expressed in neural crest when Schwann cell (SC) precursors migrate to the PNS. *Pax3* RNA and SC markers were monitored in sciatic nerves of mice during development and nerve repair. An inverse correlation was observed between expression of *Pax3* RNA and myelin basic protein (MBP). Inverse correlation was also observed in SC primary cultures. Treating cultures with forskolin, an adenylate cyclase agonist, repressed *Pax3* RNA, GFAP, NGFR, N-CAM, and L1 and elevated MBP. Subsequent microinjection with *Pax3* expression vector elevated *Pax3* RNA, GFAP, NGFR, N-CAM, and L1 and repressed MBP. Thus, *Pax3* is likely involved in the differentiation pathway to myelinating SCs. *Pax3* repressed a 1.3 kb MBP promoter fragment in cotransfection assays, suggesting that it represses MBP transcription.**

## Introduction

Many events in neural development are mediated through cell–cell interactions. In vertebrates, a particularly striking example is provided by the interaction of neurons and glia, which leads to the formation of myelin, the insulating sheath that surrounds all rapidly conducting axons (Ritchie, 1984). This specialized cell type is elaborated postnatally, by oligodendrocytes in the CNS and by Schwann cells (SCs) in the PNS. Neurons and SCs exchange signals during development, maintenance, degeneration, and regeneration processes. SC development can be considered in two stages. First, S-100-positive, embryonic SCs (eSCs) are generated from S-100-negative precursor SCs of the neural crest (stage 1 differentiation) by a process involving migration and proliferation. Second, eSCs are terminally differentiated (stage 2 differentiation) into the nonproliferative myelinating SCs (mSCs) found in adult nerves. The nonmyelinating SCs (nmSCs) found in adult nerves closely resemble eSCs in morphology and molecular markers (Webster and Favilla, 1984; Bunge et al., 1986; Mirsky and Jessen, 1990; Jessen and Mirsky, 1991).

Understanding of peripheral nerve maturation has been aided by analysis of prenatal SC development with panels of molecular markers (Jessen et al., 1990). A well-characterized set of differentiation markers is used to monitor this development closely (Mirsky et al., 1990; Mirsky and Jessen, 1990; Martini and Schachner, 1986; Snipes et al., 1992). Induction and maintenance of myelin-specific gene expression is dependent upon axonal contact. Axons must provide some signal(s) that activates second messenger cascades in SCs. As a consequence, SCs withdraw from the cell cycle and express myelin-specific genes (Lemke and Axel, 1985; Lemke and Chao, 1988; Trapp et al., 1988).

When mSCs are withdrawn from axonal contact, either by peripheral nerve transection in vivo (Trapp et al., 1988) or through culture in vitro (Lemke and Chao, 1988), the levels of myelin-specific mRNAs are dramatically reduced. In vitro, the molecular phenotype (expression levels of SC marker proteins such as $P_0$, neural cell adhesion molecule [N-CAM], L1, nerve growth factor receptor [NGFR], glial fibrillary acidic protein [GFAP], myelin basic protein [MBP], and S-100) associated with axonal contact can largely be simulated by agents that elevate the intracellular concentration of cAMP (e.g., forskolin; Lemke and Chao, 1988; Lemke, 1988). The myelin proteins $P_0$ and MBP are each up-regulated by forskolin treatment. At both the mRNA and protein levels, the ratio of MBP to $P_0$ is similar in cAMP-treated cultures (in vitro) (Morgan et al., 1991) and in mSCs in contact with axons (in vivo) (Lemke and Chao, 1988; Lemke, 1988). In both systems, cAMP-mediated up-regulation of the myelin proteins is followed by down-regulation of NGFR, GFAP, and some members of the immunoglobulin gene superfamily, such as L1 and N-CAM (Morgan et al., 1991).

*Pax3*, a member of the murine paired box gene family, encodes a 479 amino acid protein containing both a paired domain and a paired-type homeodomain (Goulding et al., 1991). It acts as a sequence-specific DNA-binding protein (Goulding et al., 1991; Chalepakis et al., 1994a) and regulates gene expression (Chalepakis et al., 1994a, 1994b). The *splotch* allele has a deletion in the homeodomain (Epstein et al., 1991), whereas the *splotch delayed* (*spd*) allele has a point mutation in the paired domain of the *Pax3* gene (Moase and Trasler, 1990). Both mutations likely affect the DNA binding properties of the protein in different ways yet still produce *Pax3* RNA. *Pax3* RNA is expressed in the dorsal part of the neuroepithelium, in neural crest derivatives, and in somitic mesoderm (Goulding et al., 1991).

Neural crest cells that migrate along the axons of the PNS become precursor SCs and, upon expression of S-100, are named eSCs (Jessen et al., 1987). In wild-type mice, S-100-positive cells are detected between embryonic day (E) 13.5 and birth. Homozygous *splotch* mice die at E13.5. No S-100-positive cells were detected at this stage (data not shown). However, in homozygotes of the *spd* allele, which survive until E18.5 (Moase and Trasler, 1990), a very small number of S-100-positive glial cells could occasionally be detected along the sciatic nerve at E13.5; 2 days later, S-100-positive cells were no longer detected (data not shown), and eSCs are no longer observed in these mutants (Franz, 1990). Thus, dysfunctional *Pax3* is associated with variable reduction of the SC lineage in the sciatic nerve. S-100 could occasionally be

turned on in the absence of fully functional *Pax3*, but levels were not maintained before *spd* homozygotes died, suggesting that *Pax3* functions both in maintaining and establishing the S-100-positive phenotype.

In this report, we describe *Pax3* RNA expression in the SCs of developing and regenerating mouse sciatic nerves and establish an inverse correlation between MBP and *Pax3* RNA expression. Immunofluorescent double labeling for *Pax3* RNA and cell-specific protein markers in degenerating and regenerating sciatic nerve suggests that *Pax3* expression depends on axon–SC interaction. In cultured SCs, *Pax3* RNA and nmSC marker proteins are expressed, but MBP is not. Forskolin inverts this molecular phenotype. Subsequent injection of *Pax3* expression vectors causes a rereversal of MBP and nmSC marker protein expression. Cotransfection experiments with *Pax3* expression vectors and a reporter plasmid containing 1.3 kb of the MBP promoter upstream of lacZ (MBP–lacZ) demonstrated that the MBP promoter can be down-regulated by *Pax3* RNA expression.

## Results

### *Pax3* RNA Expression in Schwann Cells during PNS Development

Closure of the neural fold gives rise to neural crest cells that migrate into the PNS. In E8.5 mouse embryos, *Pax3* RNA is expressed in those regions of the neural tube from which the neural crest cells will migrate (Goulding et al., 1991). The axons of the embryonic sciatic nerve are accompanied by SC precursors (S-100-negative) derived from neural crest cells. As the embryos approach birth, eSCs (S-100-positive) differentiate from the precursors.

Teased nerve preparations of embryonic, early postnatal, and adult mouse sciatic nerve were examined to determine which cells of the sciatic nerve express *Pax3* RNA. This method separates the individual fibers of the nerve, thereby exposing SCs. Immunochemical characterization of the glia in these preparations was performed with antibodies against S-100 (Brockes et al., 1979), GFAP, and MBP proteins, typical SC markers.

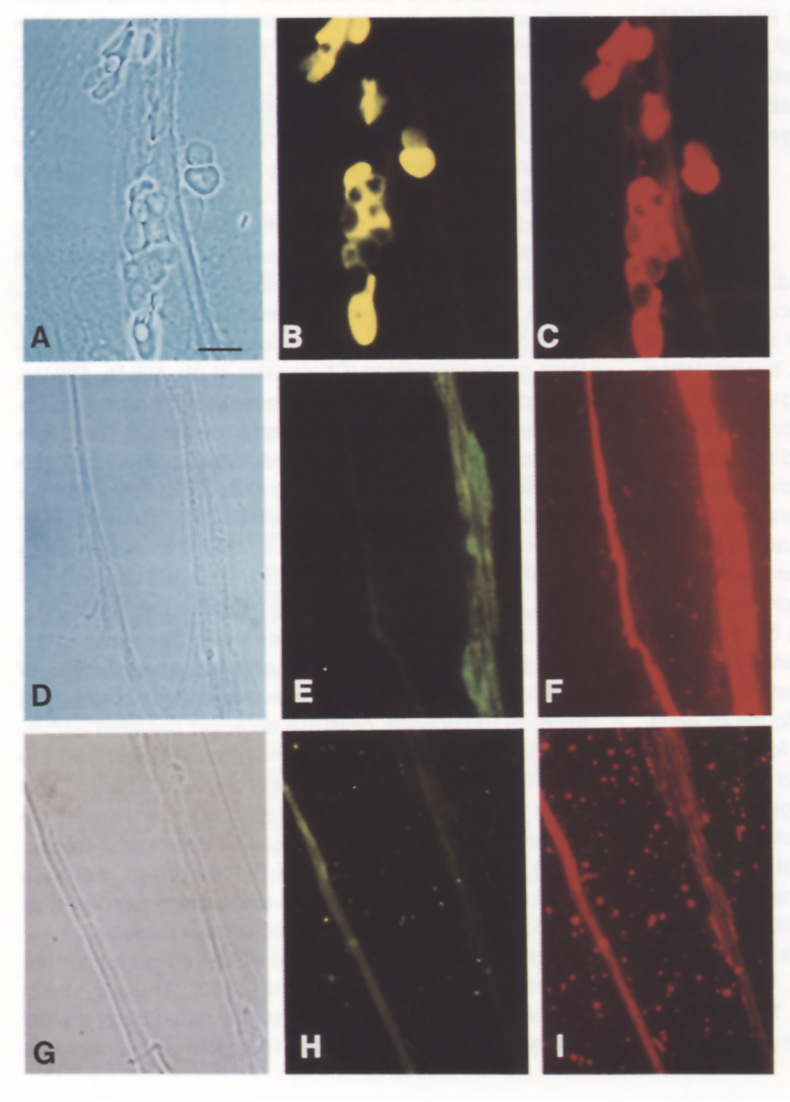


Figure 1. *Pax3* RNA and Schwann Cell Marker Proteins at Various Developmental Stages

Double-labeling in situ hybridization was performed with a DIG–*Pax3* RNA probe (green) and antibodies against S-100 (C) or GFAP (F and I) proteins (red) in teased nerve preparations from the sciatic nerve of E14.5 mouse embryos (A–C) and P5 mice (D–F) and from the cervical sympathetic trunk of young adult mice (P30; G–I). Left panels (A, D, and G) are phase-contrast images. Fluorescein-labeled anti-DIG antibody was used to detect the DIG–*Pax3* RNA probe (B, E, and H), and rhodamine-labeled secondary antibody was used to visualize anti-S-100 and anti-GFAP antibodies (C, F, and I). All SCs in the sciatic nerve of E14.5 embryos (S-100-positive) were positive for *Pax3* RNA. Later in development, only nmSCs (GFAP-positive) expressed *Pax3* RNA. GFAP-negative cells are also *Pax3* negative. All mSCs were *Pax3* negative (data not shown). Bar, 20 μm.

In the mouse, S-100 protein was first expressed in cells along the axons at E14, indicating the emergence of eSCs from precursor neural crest derivatives (rat E16; Jessen and Mirsky, 1991). Immunofluorescent double labeling for *Pax3* RNA and S-100 protein in teased sciatic nerve preparations of E14.5 embryos showed that *Pax3* RNA was expressed in all eSCs (Figures 1A–1C).

At later time points, GFAP was used as a specific marker for nmSCs (Figure 2B), and MBP or Gal-c were used as specific markers for mSCs. Teased sciatic nerves of postnatal day (P) 5 mice showed *Pax3* RNA in GFAP-positive SCs (see Figures 1D–1F). No *Pax3* RNA signal was observed in mSCs at this stage (data not shown).

In the postnatal period, between P5 and P30, SCs undergo radical changes in morphology and molecular expression that result in either the formation of a myelin sheath or the enclosure of axons to form unmyelinated fibers. In situ fluorescence of teased nerve preparations at P30 (adult), when myelination is largely completed, showed that *Pax3* RNA was associated with nmSCs (Figures 2A–2C) and not with mSCs (Figures 3G–3I). More than 70% of the axons in the adult sciatic nerve were myelinated and showed no significant *Pax3* RNA signal (Figures 3G–3I). In contrast, SCs of the adult cervical sympathetic trunk (see Figures 1G–1I), in which 99% of the axons are nonmyelinated, were *Pax3* RNA positive (see Figure 1H). These SCs are also GFAP positive (see Figure 1I). In summary, *Pax3* RNA was present in eSCs and mature nmSCs, but was down-regulated in mature mSCs during development.

### *Pax3* RNA Down-Regulation Is Axon Dependent and Reversible

To test whether down-regulation of *Pax3* RNA during myelination depends on continuous contact with axons, degeneration and regeneration experiments were used (Trapp et al., 1988). The sciatic nerves of P30 mice were cut or crushed. Teased axons of the distal side were analyzed from 1 to 60 days by immunostaining for *Pax3* RNA and cell type–specific protein markers. After 1 month, no more myelin fibers remained, and the nerve was completely degenerated. In nerve crush experiments, the nerve was completely regenerated by 60 days. In the proximal nerve stump, in which myelin remains intact, *Pax3* RNA expression was observed only in nmSCs throughout the time course (data not shown).

In the adult sciatic nerve, *Pax3* RNA was always detected in nmSCs (see Figures 2A–2C) but was absent in mSCs. *Pax3* RNA expression in mSCs was up-regulated between 3 and 4 days after lesion (see Figures 2D–2G), with the highest expression levels near the onset of up-regulation. Demyelination was already apparent at this time. In a time course analysis, with time points at 3, 4, 5, 7, 15, 30, and 60 days, the induction of *Pax3* RNA in

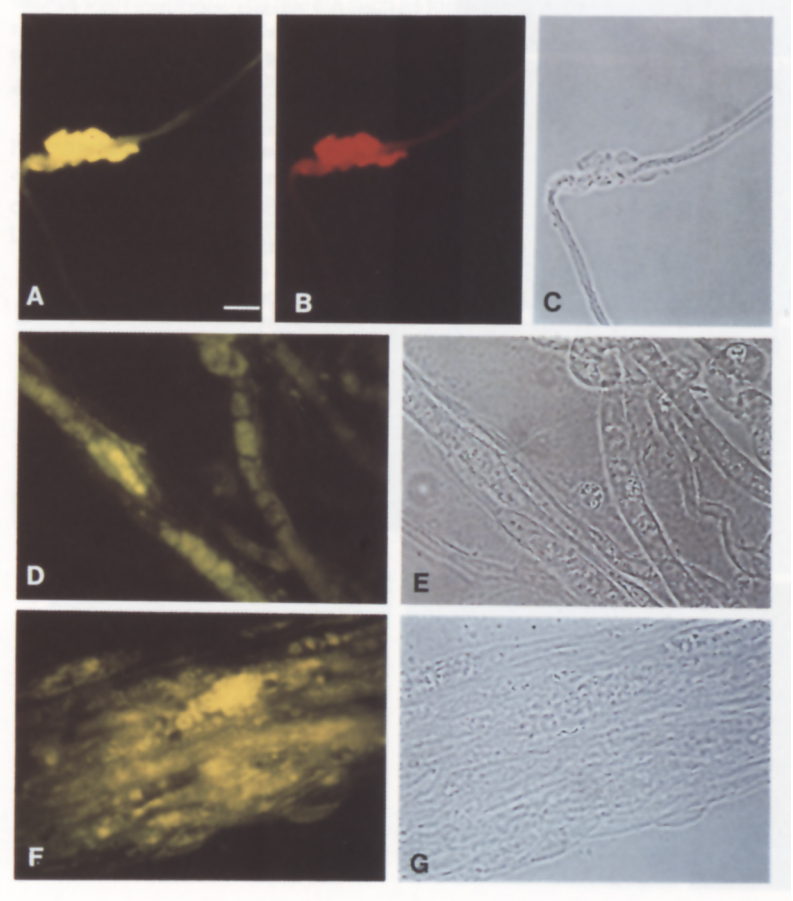


Figure 2. *Pax3* RNA Induction in mSCs 4 Days after Sciatic Nerve Transection

Double-labeling in situ hybridization was performed with a DIG–*Pax3* RNA probe (green) and an antibody against GFAP protein (red) in transected sciatic nerve preparations of P30 mice (A–C, uncut control; D and E, distal stump 4 days postcut; F and G, distal stump 7 days postcut). Right panels (C, E, and G) are phase-contrast images. Fluorescein-labeled anti-DIG antibody was used to detect the DIG–*Pax3* RNA probe (A, D, and F), and rhodamine-labeled secondary antibody was used to visualize anti-GFAP antibody (B). *Pax3* RNA is expressed in untreated, GFAP-positive nmSCs (A–C). mSCs of this preparation showed no signal (data not shown). Bar, 20 μm.

mSCs was followed by induction of eSC markers such as GFAP, N-CAM, L1, and NGFR and by down-regulation of MBP and $P_0$ (data not shown).

To determine whether *Pax3* RNA expression in SCs would be repressed again as a result of axonal regeneration, the sciatic nerve was crushed (Scherer et al., 1994). From the previous results obtained by nerve transection, it was clear that the myelinated fibers undergoing Wallerian degeneration lost MBP and gained *Pax3* RNA expression between days 3 and 4 (see Figure 2D) after lesion. This was also the case with crushed nerves (Figures 3A and 3B). Crushing results in degeneration of nerve axons distal to the crush point, with subsequent regeneration. In rat, degeneration takes 7–12 days, and regeneration starts after 15 days (Scherer et al., 1994). In mouse, remyelination of regenerating axons started after 7 days. At this stage, the SCs just begin to produce and store myelin (Lemke, 1988) and showed high levels of *Pax3* RNA (Figures 3C and 3D). After an additional week, such myelinating SCs on regenerating axons (Figures 3E and 3F) showed very low *Pax3* RNA signals, which then disappeared completely as myelination was completed. Suppression of *Pax3* RNA expression in the reinnervating distal nerve segment following nerve crush injury suggested that *Pax3* RNA was down-regulated in SCs by axonal contact. A parallel immunocytochemical study with antibodies recognizing NGFR, L1, N-CAM and GFAP showed that these markers also undergo transient increases between crushing and regeneration (data not shown). In particular, L1 was induced at the same time as *Pax3* RNA (3 days). For comparison, NGFR, N-CAM, and GFAP were induced at 5, 5, and 4 days, respectively. Transient induction of *Pax3* RNA after lesion of the adult sciatic nerve suggested that this gene plays a role in nerve repair. The inverse correlation between MBP and *Pax3* RNA expression suggested that *Pax3* may suppress MBP gene expression.

### *Pax3* RNA Expression in Cultured Schwann Cells

To test whether the expression patterns observed in vivo could be recapitulated in vitro, *Pax3* RNA expression was

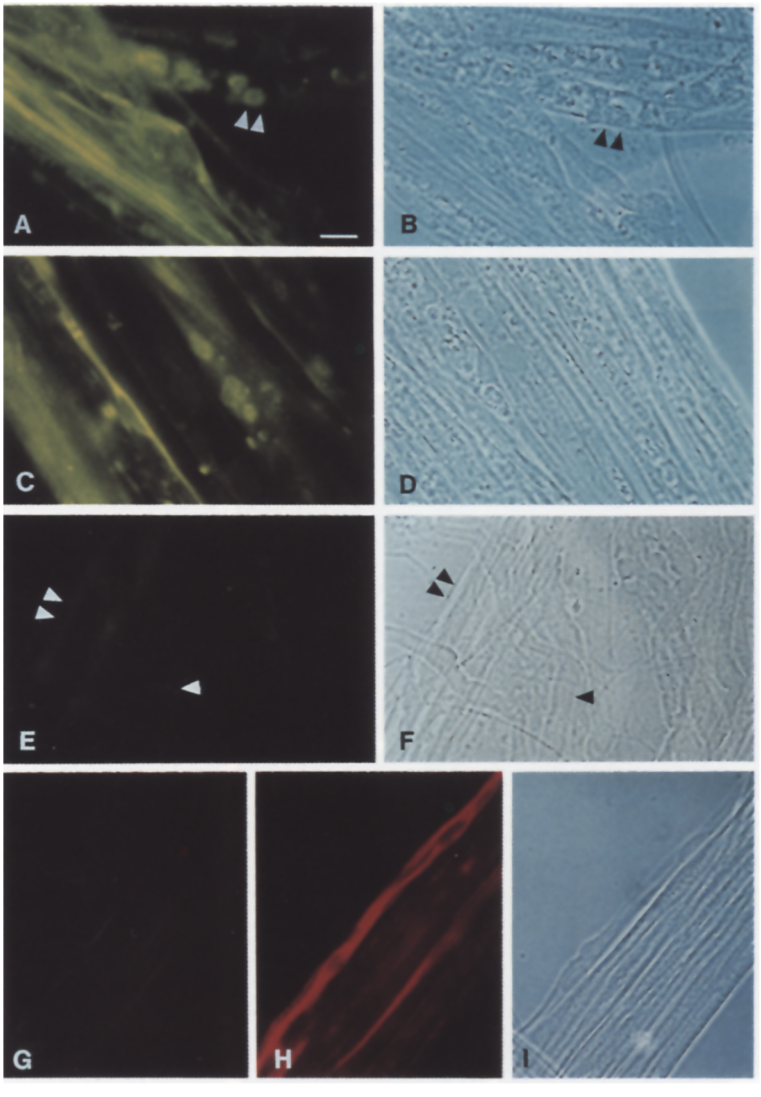


Figure 3. Regeneration Reverts the *Pax3* RNA Induction Caused by Nerve Crushing

Double-labeling in situ hybridization was performed with a DIG–*Pax3* RNA probe (green) and an antibody against GFAP protein (red) in the distal stump of crushed sciatic nerves of P30 mice (A and B, 4 days postcrush; C and D, 7 days postcrush; E and F, 15 days postcrush; G–I, 60 days postcrush). Right panels (B, D, F, and I) are phase-contrast images. Fluorescein-labeled anti-DIG antibody was used to detect the DIG–*Pax3* RNA probe (A, C, E, and G), and rhodamine-labeled secondary antibody was used to visualize anti-GFAP antibody (H). *Pax3* RNA was always expressed in GFAP-positive nmSCs (data not shown). At 4 (A and B) and 7 (C and D) days after crushing, *Pax3* RNA was expressed by degenerating mSCs (arrowheads). At 15 days postcrush (E and F), *Pax3* RNA was expressed only by the nmSCs (two arrowheads) and not by the regenerated mSCs (single arrowhead). At 60 days postcrush (G–I), the nerve was regenerated, and *Pax3* RNA was expressed only in GFAP-positive SCs (nmSCs), as in untreated sciatic nerves. Bar, 10 μm.

studied in primary SC cultures from dissociated sciatic nerves. SC cultures were prepared from P5 mouse sciatic nerves. Shortly after plating, cultured SCs have a short, condensed morphology (Figure 4A) and express protein markers characteristic of eSCs (S-100; Figure 4C) or adult nmSCs (GFAP and NGFR; data not shown). After a further 4 days in serum-free defined medium, SCs lost their condensed morphology and had long bipolar protrusions extending from the cell body (Figure 4D). The same markers were still expressed (S-100, GFAP, and NGFR; data not shown). *Pax3* RNA was expressed at both early (Figure 4B) and late (Figure 4E) times after plating. MBP or $P_0$ was not detected at any time (data not shown).

Thus, with respect to the SC marker proteins, cultured SCs have the molecular phenotype of eSCs or adult nmSCs of the normal animal. This molecular phenotype predominates in SC populations during the Wallerian degeneration–regeneration transition. At later stages in regeneration, the second molecular phenotype, characterized by MBP expression and *Pax3* RNA suppression, predominates.

### *Pax3* Represses MBP and Activates Four nmSC Protein Markers

Forskolin treatment was used to mimic the second molecular phenotype (axon-dependent, MBP-positive) in a cultured system. Cultured rat neonatal SCs do not express myelin genes (Lemke and Chao, 1988), presumably because of the withdrawal from axonal contact. MBP gene expression is turned on by agents such as forskolin, which raise intracellular levels of cAMP (Lemke and Chao, 1988). In culture, the effect of axonal presence is mimicked by elevation of intracellular cAMP levels.

Dissociated SCs from P5 mouse sciatic nerve, after 48 hr in vitro, were treated with 2 μM forskolin for a further 48 hr. Forskolin treatment resulted in a marked increase of MBP (Figure 5C) and a down-regulation of GFAP, N-CAM, L1, and NGFR (data not shown). As expected, *Pax3* RNA was not or very weakly detectable in MBP-positive SCs (Figure 5B). Thus, as cAMP levels were elevated by forskolin, the levels of *Pax3* RNA (compare Figures 4B and 4E with Figure 5B) and four nmSC marker proteins declined. In contrast, MBP levels increased (Figure 5C), and S-100 levels were unaffected (data not shown). Therefore, cAMP elevation in culture mimicked qualitatively the "axonal presence" of regeneration experiments, insofar as *Pax3* RNA, MBP, GFAP, N-CAM, L1, NGFR, and S-100 molecular phenotypes are concerned. It is not surprising that so many genes alter their expression (in opposite directions) in response to an agent such as forskolin, which is known to interfere at the level of second messenger metabolism.

Microinjection experiments were performed to test whether ectopic *Pax3* DNA production could suppress forskolin-induced MBP expression. Cultured SCs were microinjected after 48 hr in forskolin (Table 1). Plasmids or buffer was microinjected into SC nuclei. After 24 hr, ~50% of the injected cells had survived and had characteristic enlarged nuclei due to injection (Figures 5D, 5G, 5J, 5M, and 5P). Cells were fixed and analyzed by double-labeling in situ hybridization with a *Pax3* RNA probe (green; Figures 5E, 5H, 5K, 5N, and 5Q) and one of six antibodies to marker proteins (red; Figures 5C, 5F, 5I, 5L, 5O, 5R). For example, 30 cells injected with SV40–*Pax3* survived 24 hr (Table 1). All were analyzed and found to be positive for *Pax3* RNA (Figures 5E, 5H, 5K, and 5N); 27 were also analyzed for one of the six marker proteins; 12 were analyzed for MBP (Figure 5F); and 3 were analyzed for each of the other five protein markers (Table 1; Figures 5I [GFAP], 5L [NGFR], and 5O [N-CAM]; data not shown for L1 and S100). Mock injections with control plasmid (SV40-only; Figures 5P–5R) or buffer (data not shown) were performed to control for artifacts due to the expression vector or injection procedure, respectively. To ensure that effects observed with SV40–*Pax3* were not due merely to the overexpression of any transcription factor,

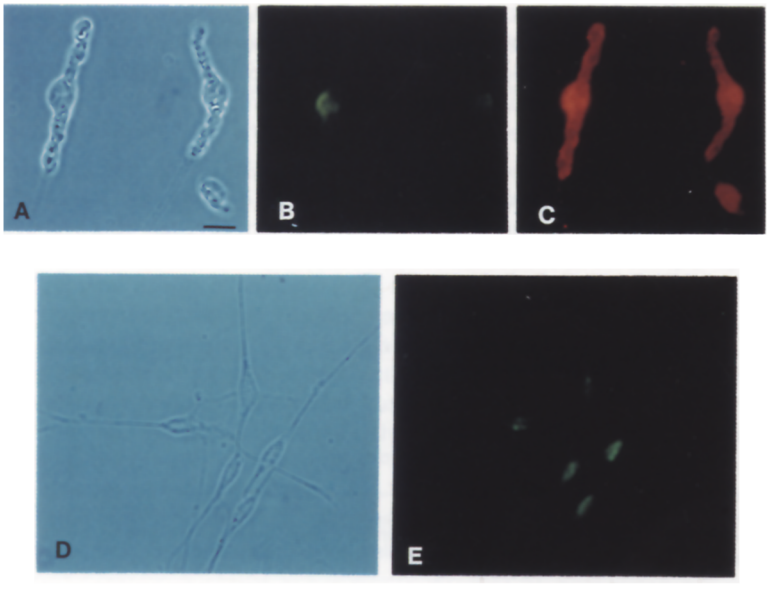


Figure 4. Cultured Schwann Cells Express *Pax3* RNA and S-100

Cell cultures were prepared from dissociated sciatic nerves of P5 mice. After 3 hr (A–C) or 4 days (D and E) in culture, double-labeling in situ hybridization was performed with a DIG–*Pax3* RNA probe (green) and an antibody against S-100 protein (red). Fluorescein-labeled anti-DIG antibody was used to detect the DIG–*Pax3* RNA probe (B and E), and rhodamine-labeled secondary antibody was used to visualize anti-S-100 antibody (C). Phase-contrast micrographs depict the morphology in short- (A) and long- (D) term cultures. *Pax3* RNA was expressed by SCs in short- (B) and long- (E) term cultures. Bar, 20 μm.

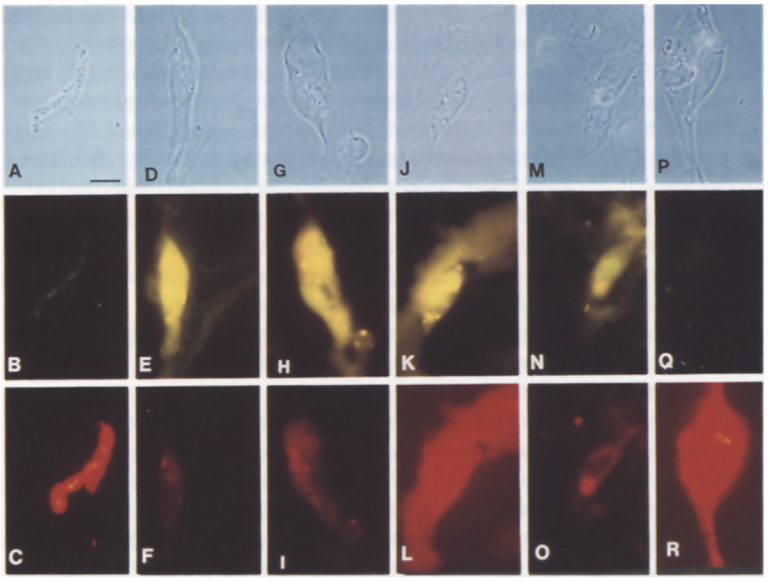


Figure 5. Microinjection of *Pax3* Expression Vectors Inverts Expression of Schwann Cell Marker Proteins

Cultured SCs from the sciatic nerves of P5 mice were treated with forskolin, which makes them *Pax3* RNA negative (B) and MBP positive (C). Cells were microinjected with SV40–*Pax3* (D–O) or SV40-only (P–R). After 24 hr, cells were fixed and analyzed by double-labeling in situ hybridization to detect *Pax3* RNA (green) and one of four SC marker proteins (red). Primary antibodies against MBP (C, F, and R), GFAP (I), NGFR (L), and N-CAM (O) were visualized by a rhodamine-labeled secondary antibody. The DIG–*Pax3* RNA probe was detected by a fluorescein-labeled anti-DIG antibody. Upper panels show phase-contrast micrographs before (A) and after (D, G, J, M, and P) microinjection. Injection of SV40–*Pax3* results in *Pax3* RNA expression (E–N), whereas mock injection leads to no detectable *Pax3* RNA (Q). Expression of *Pax3* RNA results in reduction of MBP protein (F) and elevation of GFAP (I), NGFR (L), and N-CAM (O) protein levels. Bar, 10 μm.

mock injections with SV40–Hoxa7 and CMV–Hoxa7 expression vectors were performed. The functionality of these plasmids was demonstrated previously (Maulbecker and Gruss, 1993). Forskolin-treated, SV40–*Pax3*-injected SCs gave a higher *Pax3* RNA signal than untreated SCs, suggesting that greater than physiological doses of *Pax3* RNA were present in these experiments.

The injection data (Figure 5; Table 1) clearly demonstrate that MBP expression was down-regulated by SV40–*Pax3* injection (all 12 were negative; Figure 5F) but was unaffected by all mock injections (all 50 were positive; Figure 5R). In addition, they suggest that NGFR (Figure 5L), GFAP (Figure 5I), L1 (data not shown), and N-CAM (Figure 5O) responded in an opposite manner to *Pax3* DNA injection, being activated rather than repressed. Only 3 SV40–*Pax3*-injected cells were analyzed for each of these proteins, and all were positive. Levels detected were similar to those seen prior to forskolin treatment. For each of these four protein markers, all 7 mock-injected cells (11 for NGFR) were negative. The S-100 protein marker behaved the same in SV40–*Pax3* and mock injections, indicating that not all genes are affected by SV40–*Pax3* injection. Together, these results demonstrate that ectopic *Pax3* DNA expression causes MBP down-regulation and also induces the four marker proteins NGFR, GFAP, L1, and N-CAM. It remains to be seen whether regulation of all five markers by *Pax3* occurs by direct mechanisms, such as binding of *Pax3* to all five promoters, or by an indirect mechanism, such as interference with second messenger metabolism.

### *Pax3* Inhibits MBP Promoter Activity

Cotransfection assays were used to investigate whether the inverse correlation between *Pax3* and MBP expression was due to inhibition of MBP promoter function by *Pax3*. N-2A (neuroblastoma) cells were cultured in DMEM supplemented with 10% fetal calf serum. The reporter plasmid, pBG1b (Miura et al., 1989; Okano et al., 1988), contains 1.3 kb of MBP promoter fused to trpS/lacZ. A control reporter contains the RSV-LTR upstream of lacZ. Reporters were cotransfected with CMV–*Pax3*, CMV–*Pax6*, and CMV-only effector plasmids. After 48 hr, cells were harvested and assayed for reporter activity.

The weight ratio of effector to reporter plasmid was systematically altered in a range from 0.02 to 0.4 to minimize the effector to reporter ratio. The CMV–*Pax3* effector sig-

Table 1. Microinjection Summary

| | SV40–*Pax3* | | SV40-only | | Buffer | | SV40–Hoxa7 | | CMV–Hoxa7 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Molecular Markers | Inj | + | Inj | + | Inj | + | Inj | + | Inj | + |
| *Pax3* RNA | 30 | 30 | 40 | 0 | 34 | 1 | 24 | 0 | 17 | 0 |
| MBP | 12 | 0 | 20 | 20 | 10 | 10 | 10 | 10 | 10 | 10 |
| NGFR | 3 | 3 | 5 | 0 | 3 | 1 | 2 | 0 | 1 | 0 |
| GFAP | 3 | 3 | 2 | 0 | 2 | 0 | 2 | 0 | 1 | 0 |
| L1 | 3 | 3 | 2 | 0 | 2 | 0 | 2 | 0 | 1 | 0 |
| N-CAM | 3 | 3 | 2 | 0 | 2 | 0 | 2 | 0 | 1 | 0 |
| S-100 | 3 | 3 | 4 | 4 | 2 | 2 | 2 | 2 | 2 | 2 |

Molecular markers were detected by a DIG–*Pax3* RNA probe or by antibodies against the proteins. For injected material, 3–6 pg of circular plasmid was injected per cell. For each injection, the number of cells that survived for 24 hr (Inj) and the number that gave a positive signal for the indicated molecular marker (+) (at 24 hr after injection) are shown.

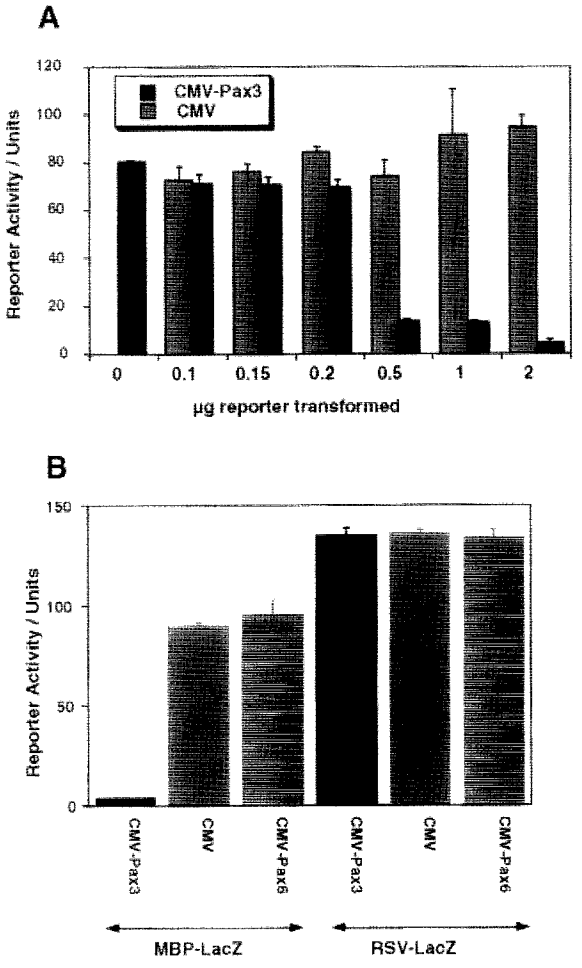


Figure 6. *Pax3*-Dependent Transrepression of the MBP Promoter

(A) Minimizing the effector to reporter ratio. N-2A neuroblastoma cells ($2 \times 10^4$ cells in 6 cm dishes) were transiently cotransfected with 5 µg of effector plasmid (CMV–*Pax3* or CMV-only) and increasing amounts of reporter plasmid (MBP–lacZ). A 1:10 ratio was the lowest ratio that gave significant transrepression.

(B) *Pax3* transrepresses the MBP promoter, but *Pax6* does not. N-2A neuroblastoma cells were cotransfected at a 1:10 ratio (0.5 µg:5 µg) of effector (CMV–*Pax3*, CMV, and CMV–*Pax6*) to reporter (MBP–lacZ and RSV–lacZ) as indicated. Each dish was harvested 48 hr after transfection and assayed for β-galactosidase activity. Each bar shows the average and SD of reporter activity from three parallel transfections (Unit = $A_{420} \times 10^5/240$ min × cell number).

nificantly reduced the MBP reporter activity at ratios as low as 0.1 (Figure 6A). The CMV–Pax6 effector was unable to reduce MBP–lacZ reporter activity even at ratios as high as 0.4 (data not shown). No effectors could reduce RSV–lacZ reporter activity at any ratio (data not shown).

A 1:10 ratio of CMV–*Pax3* to MBP–lacZ cotransfected into neuroblastoma cells (N-2A) gave a 23-fold lower reporter signal than parallel cotransfections utilizing the CMV-only or CMV–Pax6 effectors (Figure 6B). In parallel, the RSV–lacZ signal was the same with all three effector plasmids (Figure 6B), indicating that *Pax3* does not act as a general repressor of all reporters. These results are consistent with the model that *Pax3* represses the MBP promoter. Furthermore, the cis-acting elements within the 1.3 kb MBP promoter fragment examined can mediate at least some of the *Pax3*-dependent repression of endogenous MBP observed in microinjection experiments.

## Discussion

### *Pax3* during Myelination

A number of observations implicate *Pax3* in the differentiation of eSCs into mSCs and nmSCs. *Pax3* RNA was down-regulated when eSCs differentiated to the myelin type but continued to be expressed in the nonmyelin type. Similarly, N-CAM, L1, GFAP, and NGFR are also turned off in mSCs and remain on in nmSCs. Thus, both *Pax3* and these markers of eSCs or nmSCs are down-regulated during myelination, both in development and during regeneration. The majority of eSCs terminally differentiate to mSCs in a prolonged process that begins several days after birth. The first MBP-positive cells were detected at P5. *Pax3* may be involved in maintaining the molecular phenotype of eSCs in some glial cells of the adult PNS. In sciatic nerve crushing, *Pax3* RNA expression is induced in the demyelinating distal nerve stump in a new "eSC-like" population of cells. It is not clear whether these eSC-like cells are derived from nmSCs transiently proliferating to replace dying mSCs or from mSCs that have lost their myelin. In late stages of nerve regeneration, the eSC-like cell population terminally differentiates to mSCs in a process apparently recapitulating development.

The ability of undifferentiated glial cells to adhere to neurons may depend on expression of adhesion molecules such as N-CAM. Homeobox-containing proteins are directly involved in the control of transcription of these molecules (Edelman and Jones, 1992). If *Pax3* were involved in maintaining the expression of adhesion molecules, cells with dysfunctional *Pax3* could no longer adhere to the proper surfaces (e.g., axons). Thus, they could not receive the normal inductive signals (e.g., from the axons) required for further proliferation, and eventually differentiation. N-CAM and NGFR were both expressed 2 days after *Pax3* in nerve regeneration, suggesting that these genes may be positively regulated targets of *Pax3*. MBP would provide an example of a negatively regulated target.

*Pax3* binding could be demonstrated for sequences of the N-CAM promoter (Chalepakis et al., 1994b). Similarly, *Pax3* specifically binds in vitro to a region 1.1 kb upstream of the MBP cap site (data not shown). However, we found it surprising that five out of the six marker proteins we assayed specifically responded to *Pax3* in microinjection experiments. Although direct gene regulation by *Pax3* binding to all five promoters is possible, other models should not be excluded at this stage.

*Pax3* RNA is detected in astrocytes but not in oligodendrocytes of the CNS (Kioussi and Gruss, 1994). These two cell types are nonmyelinating and myelinating, respectively. Thus, *Pax3* RNA expression follows a similar pattern during genesis of myelinating cell types in both the CNS and PNS. In both systems, *Pax3* may help to maintain cells in a nonmyelinating state.

### A Gap in *Pax3* RNA Expression

The expression pattern of *Pax3* RNA is biphasic with respect to the SC lineage. In the first phase (until E13.5), *Pax3* may play a role in the establishment and maintenance of eSCs. The patterning of limb and body is largely complete by E13.5. Cartilaginous precursors of the skeleton can be identified at this stage, and the innervation of the PNS is laid out. The second phase (E18.5 to P5) begins in eSCs with weak *Pax3* RNA expression 1 day before birth, followed by a dramatic *Pax3* RNA induction at birth. In the 5 days between the two phases of *Pax3* RNA expression, eSCs rapidly proliferate as the embryo enlarges quickly. Although organogenesis occurs in this time span, the basic layout of the remaining body does not change drastically.

Although *Pax3* RNA and MBP expression are inversely correlated during terminal differentiation (myelination), MBP is not always on when *Pax3* RNA is absent. MBP is absent in eSCs between E13.5 and E18.5, the time between the two *Pax3* RNA expression phases. Thus, MBP promoter activity either is repressed by another factor that is absent during terminal differentiation or requires an activating factor during myelination that is missing between E13.5 and E18.5.

### Three Switches in *Pax3* RNA Expression

The expression analysis demonstrated that *Pax3* RNA expression was switched on or off at least three times in the maturation of SCs of the PNS. The eSC population at E13.5 was small relative to the number of axons. A few round S-100-positive, *Pax3* RNA–positive cells accumulated on axons bundles, and some had short cytoplasmic projections. Between E13.5 and birth, the projections lengthened, and the ratio of eSCs to axons increased owing to rapid proliferation. *Pax3* RNA was turned off, but S-100 remained on. Near birth, *Pax3* RNA was turned on again in the S-100-positive eSC population just before the onset of terminal differentiation (myelination). The first mSCs were detected ~5 days later. Thus, the signal that induced *Pax3* RNA expression around birth likely preceded the signal for myelinating. The third switch in *Pax3* RNA expression occurs only in mSCs during terminal differentiation, when *Pax3* RNA is turned off. If an eSC receives no signal for myelinating, *Pax3* RNA remains on as that cell becomes an adult nmSC.

The first two switches in *Pax3* RNA expression correlate with changes in the proliferative rate of eSCs during development. It is unclear whether reduced *Pax3* RNA expression is caused by, is an effect of, or is unrelated to increased proliferation. However, paired domain proteins may have roles in controlling cell proliferation. Overexpression of Pax genes results in deregulation of growth control (Maulbecker and Gruss, 1993; Stuart et al., 1995).

The third switch in *Pax3* RNA expression occurred during terminal differentiation of mSCs. The "signal" for myelinating (not yet identified) comes from the axon and can be delivered only if the axon has continuity with the SC perikaryon. Those SCs whose perikarya make close contact with axons transmitting the signal begin terminal differentiation. Some eSC or eSC-like cells do not receive the signal, and they remain in the nonmyelinating state.

Prior to terminal differentiation, axon-associated SCs proliferate and express NGF and NGFR (Taniuchi et al., 1986). Similarly, the rate of synthesis of NGF and NGFR by eSC-like cells is high during regeneration, before axonal contact with the target tissue is reestablished (Taniuchi et al., 1988). Thus, NGF and its receptor are present in SCs until nerve innervation, in development or repair, is complete. The signal for myelinating from the axons would be sent at this time.

In the neuron-like PC12 cell line, NGF binding to NGFR can initiate a signal transduction cascade, one outcome of which is the elevation of intracellular cAMP levels (Knipper et al., 1993). During the NGF-dependent transdifferentiation of chromaffin cells to sympathetic neurons, artificial elevation of intracellular cAMP levels antagonizes the effects of NGF during an initial proliferative phase, but promotes the neuronal phenotype after cells withdraw from the cell cycle (Herman et al., 1993). NGF treatment of neuronal cell cultures also leads to a transient elevation of *Pax3* RNA (Kioussi and Gruss, 1994) and c-Jun (Matsushima and Bogenmann, 1993). It will be of interest to determine whether SCs also respond to NGF in a proliferation-dependent manner and in which situations this leads to an elevation of intracellular cAMP and the expression of *Pax3* RNA and c-Jun.

Forskolin treatment artificially raises intracellular cAMP levels. Treatment of cultured SCs with forskolin suppresses nmSC marker proteins such as NGFR, N-CAM, L1, GFAP, Ran-1, and O4 and induces myelin-specific proteins such as MBP, $P_0$, and proteolipid protein (PLP) (Jessen and Mirsky, 1991). c-Jun (Bohman et al., 1987; Bharucha et al., 1994) and *Pax3* RNA are also down-regulated by forskolin in this system. Thus, elevating intracellular cAMP in cultured SCs leads to repression of certain immediate-early and nonmyelin genes and leads to induction of myelin-specific genes. It will be of interest to determine whether cAMP elevation actually occurs in SCs as a result of the signal for myelinating in vivo.

## Experimental Procedures

### Antibodies

Rabbit polyclonal antibodies against S-100 (Sigma), a SC marker; GFAP (Sigma), a nmSC marker; and MBP (Dakopatts, Denmark), a mSC marker, were used at 1:100 dilution. Detection antibodies such as 5(6)-carboxy-fluorescein-N-hydroxysuccinimide ester (FITC) conjugated to anti-digoxigenin sheep F(ab') fragments (Boehringer Mannheim Corp.) and tetramethylrhodamine B isothiocyanate (TRITC) conjugated to goat antibody against rabbit IgG (Sigma) were used at 1:500 and 1:100 dilution, respectively.

### Other Materials

Transferrin, selenium, putrescine, triiodothyronine, thyroxine, dexamethasone, insulin, cytosine arabinoside, forskolin, laminin, poly-L-lysine, fetal calf serum, DMEM F-12 Ham, and o-Nitrophenyl-β-D-galactopyranoside (ONPG) were obtained from Sigma; tribromoethyl alcohol was obtained from Aldrich; and tertiary amyl alcohol was obtained from Merck.

### Defined Medium

Defined medium consisted of DMEM F-12 Ham with (final concentration in parentheses) transferrin (100 mg/ml), progesterone (60 ng/ml),

putrescine (16 μg/ml), insulin (5 μg/ml), thyroxine (0.4 μg/ml), selenium (160 ng/ml), triiodothyronine (10 ng/ml), dexamethasone (40 ng/ml), penicillin (100 IU/ml), streptomycin (100 IU/ml), glutamine (2 mM), and 10% heat-inactivated fetal calf serum.

### Schwann Cell Cultures

SC cultures were prepared essentially as described previously (Kioussi et al., 1992). Briefly, sciatic nerve from E14.5 and P5 mice was dissected, chopped, and incubated in 400 μl of enzyme solution containing collagenase (0.2%) and trypsin (0.125%) for 45 min at 37°C. After centrifugation, the cells were resuspended in medium, counted, and plated at a density of 3000 cells per coverslip in a 20 μl drop on poly-L-lysine/laminin-coated coverslips. After 3 hr, cultures were covered with 180 μl of medium. For long-term dissociated SC cultures, 24 hr after plating the cells were treated with 10 μM cytosine arabinoside for 24 hr to kill dividing cells, and the cells were grown in serum-free medium. In some experiments, forskolin (2 μM; Lemke and Chao, 1988), a reversible activator of adenyl cyclase, was added to cells cultured in media for 48 hr. Cells were washed, cultured in serum-free defined medium for 24–48 hr, and microinjected. After a further 24 hr, injected cells were fixed and analyzed.

### Microinjections

After treatment with forskolin, SCs were microinjected with SV40 promoter *Pax3*, SV40 promoter control plasmid (150 ng/ml), or Tris–EDTA buffer. Microinjections were performed in a Zeiss Axiophot reverse microscope using the Eppendorf microinjector 5242 (manual pressure [P2] 400–500 hPa and constant pressure [P3] 90–100 hPa). Glass capillaries (GC 120F-10, CLARK Electronic Instruments) were filled with 0.5–1 μl of DNA solution, and 20–30 cells were microinjected per capillary.

### Sciatic Nerve Transection and Crush

NMRI mice (3-week-old) were anesthetized with 0.015 ml per gram of body weight avertin (8% tribromoethyl alcohol, 4% tertiary amyl alcohol), and the left sciatic nerve was aseptically transected 1–3 mm below the sciatic notch. To prevent regrowth into the distal stump, 3 mm of the proximal side was removed. The contralateral sciatic nerve served as control. Nerve crush was performed by tightly compressing the sciatic nerve at the sciatic notch with flattened forceps twice, each time for 10 s. This technique causes all axons to degenerate, but allows axonal regeneration. At 4, 7, 15, and 30 days after injury, the animals were killed by cervical dislocation, and both sciatic nerves were immediately removed for in situ analysis.

### Teased Nerve Preparations

Sciatic nerves from mice of various ages were excised, placed in ice-cold PBS, and partially teased into bundles and individual fibers using fine needles on gelatin-coated microscope slides. Samples were allowed to dry before double-labeling in situ hybridization.

### Double-Labeling In Situ Hybridization

Cells grown on coverslips and teased nerve preparations were prepared for immunofluorescent double labeling for *Pax3* RNA and different protein markers as described by Kioussi and Gruss (1994). Briefly, cells and teased nerves were fixed with 4% paraformaldehyde in PBS, rinsed in PBS prehybridized for 3 hr in a humid 45°C chamber, and washed successively at room temperature for 2 min in 70%, 90%, and 100% ethanol. Digoxigenin-labeled *Pax3* RNA probe was synthesized using T7 polymerase. Preparations were hybridized for 16 hr. For immunofluorescent double labeling, preparations were incubated with primary antibodies for 2 hr and rinsed before applying both secondary antibodies for 2 hr at room temperature. Immunofluorescence-stained preparations were viewed with a Zeiss Axiophot photomicroscope. Unless otherwise stated, photographic prints referring to comparative immunostaining were prepared under identical conditions.

### DNA Transfections and β-Galactosidase Assays

N-2A (neuroblastoma) cells were cultured in DMEM supplemented with 10% fetal calf serum. Cells ($2 \times 10^4$) were transfected with DNA using the calcium phosphate precipitation technique (Graham and van der Eb, 1973). At 12 hr after addition of the precipitate, the medium was removed, cells were washed 3 times with PBS, and fresh medium was added. Cells were harvested at 48 hr after addition of the precipitate and suspended in 100 μl of TEN buffer (40 mM Tris–HCl [pH 7.5], 1 mM EDTA, 150 mM NaCl). Cells were disrupted by freeze-thaw (3 times at −100°C and 37°C) and microfuged to remove debris. Extract (30 μl) was added to 1 ml of β-galactosidase assay buffer (60 mM $Na_2HPO_4$, 40 mM $NaH_2PO_4$, 10 mM KCl, 1 mM $MgCl_2$, 50 mM β-mercaptoethanol). The enzymatic reaction was started with 200 μl of ONPG (4 mg/ml in 60 mM $Na_2HPO_4$, 40 mM $NaH_2PO_4$ [pH 7.0]), incubated at 37°C for 4 hr, and stopped with 500 μl of 1 M $Na_2CO_3$. Absorbance was measured at 420 nm, and activity was normalized by the number of cells per transfected dish (Unit = $A_{420} \times 10^6$/240 min × cell number).

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