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Mitogen-expanded Schwann cells retain the capacity to myelinate regenerating axons after transplantation into rat sciatic nerve

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ABSTRACT **We have developed a method for genetically modifying Schwann cells (SCs) *in vitro* and then assessed whether these SCs could interact normally with axons *in vivo*. Rat SCs were transduced *in vitro* with the *lacZ* gene by using a retroviral vector and then expanded with the SC mitogens forskolin and glial growth factor. These mitogen-expanded SCs had an abnormal phenotype as compared to both SCs *in vivo* and primary SCs *in vitro*, yet when they were introduced into a regenerating rat sciatic nerve, they formed morphologically normal myelin sheaths around the axons. These results demonstrate that SCs can be genetically altered, their numbers expanded in culture, and yet respond appropriately to axonal signals in the peripheral nervous system. This approach offers a plausible way to manipulate genes involved in axon–SC interactions, including genes that may be defective in some inherited peripheral neuropathies.**

Schwann cells (SCs) are the major glial cell of the peripheral nervous system and are associated with either myelinated or unmyelinated axons. During development, SCs proliferate and migrate along and ensheathe axons (1). Whether a SC simply ensheathes an axon or goes on to form a myelin sheath around it appears to be under axonal control (2, 3). Prior to myelination, SCs express a specific set of proteins, including the low-affinity nerve growth factor receptor (NGFR), the neural cell adhesion molecule N-CAM, and the adhesion cell molecule L1 (4). As SCs myelinate axons, the expression of these proteins ceases as SCs begin expressing myelin-specific proteins such as peripheral myelin protein zero (P_0), myelin basic protein, and myelin-associated glycoprotein (5–8). If axonal contact is lost, as in Wallerian degeneration after nerve transection, the myelinating SCs dramatically decrease their expression of the myelin-specific genes and reexpress NGFR, N-CAM, and L1 (9).

The cellular events involved in SC–axonal interactions, including myelination, have been extensively studied in tissue culture. Axons and agents that elevate cAMP, such as forskolin (Fsk) (10), induce SCs freshly dissociated from nerve (primary SCs) to increase their expression of P_0 and myelin basic protein and dramatically decrease the expression of NGFR (11). In primary SCs, the induction of P_0 by Fsk appears to occur only if the proliferative response of SCs to cAMP is prevented (12). However, as SCs are passaged in culture (so-called "secondary SCs"), they progressively lose these expression characteristics. Fsk now induces P_0 expression and proliferation concomitantly (13). Fsk also induces less of a decrease in NGFR mRNA than it did in primary SCs (see Fig. 2). Eventually, secondary SCs proliferate autonomously without requiring growth factors in culture and lose their ability to myelinate axons *in vitro* (14). These data thus suggest that secondary SCs progressively become independent of the signals to which they previously responded as their phenotype diverges from that of primary SCs.

Whether secondary SCs can respond appropriately to axons in peripheral nerve *in vivo* is unknown. To address this issue, we examined the ability of transplanted secondary SCs to myelinate rat sciatic nerve. To identify transplanted SCs in nerve, we first transduced cultured SCs with the *lacZ* gene contained in the BAG retroviral vector (15), since the *lacZ* gene product, the bacterial enzyme β-galactosidase, can be detected histochemically. Myelination is the culmination of a series of coordinated cellular responses to axonal signals (2). Therefore, it is an appropriate system for the study of SC interactions with axons. Moreover, myelin internodes can be unambiguously identified histochemically.

MATERIAL AND METHODS

SC Cultures. SCs were isolated from the sciatic nerve from a 3-day-old Sprague–Dawley rat and cultured by the method of Brockes *et al.* (16). These secondary SCs were plated onto 100-mm² poly(L-lysine)-coated tissue culture plates at 8×10^5 cells per plate and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum, 2 μM Fsk, and glial growth factor. Glial growth factor was prepared by partial purification of bovine pituitary extracts as described by Brockes *et al.* (17).

Retroviral Vector and SC Infection. SCs were transduced by the BAG retroviral vector (15) contained in the Ψ2 packaging line (American Type Culture Collection). The BAG vector is constructed by cloning the *lacZ* gene into the pDOL vector derived from the Maloney murine leukemia virus. The simian virus 40 early promoter and the Tn5 neomycin-resistance (neo) gene, transmitting G418 resistance, are present downstream from *lacZ* to permit selection of infected colonies. Supernatants from packaging cells grown to confluence were used, in the presence of Polybrene (8 μg/ml), to infect cultured SCs, which were rapidly proliferating under the influence of Fsk and glial growth factor. Transduced SCs were then selected by incubation with G418 (1 mg/ml) and resistant cells were assayed with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) for β-galactosidase expression, as described below. The presence of helper virus in the tissue culture medium was assayed according to Cepko (18).

X-Gal Staining of SCs in Culture. β-Galactosidase-expressing cells were detected by incubating the cells with X-Gal to form a blue color within the cell. Cells were fixed in 0.5% glutaraldehyde, washed, and then incubated with X-Gal to a final concentration of 1 mg/ml in X-Gal developer [35

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Abbreviations: SC, Schwann cell; NGFR, nerve growth factor receptor; P_0, peripheral myelin protein zero; Fsk, forskolin; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

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mM $K_3Fe(CN)_6$/35 mM $K_4Fe(CN)_6 \cdot 3H_2O$/2 mM $MgCl_2$ in phosphate-buffered saline]. Cells were then incubated at 37°C overnight and examined by light microscopy for the presence of blue color.

Northern Blot Analysis. Total RNA was isolated from SC cultures by the method of Chomczynski and Sacchi (19). RNA (10 μg) from either transduced or nontransduced SCs was electrophoresed in a 2.2 M formaldehyde/1% agarose gel, transferred to a nylon membrane, and UV-cross-linked. The membrane was prehybridized for several hours in 50% (vol/vol) formamide/5× SSPE (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA)/5× Denhardt's solution/0.1% SDS/salmon sperm DNA (100 mg/ml) and then hybridized overnight at 42°C with randomly primed cDNA probes prepared using [α-^{32}P]dCTP according to the manufacturer's instructions (Promega).

SC Transplantation. After anesthesia, left and right sciatic nerves of 8-week-old Sprague–Dawley rats were exposed and transected at a midthigh level. Proximal and distal stumps were inserted into 1-cm silicone tubes, leaving a gap of 0.5 cm between the stumps. The tube on one side was filled with 10^6 *lacZ*-positive cells suspended in 20 μl of DMEM; the contralateral tube was filled with either 10^6 nontransduced SCs or sham Hanks' balanced salt solution. The nerve was allowed to regenerate through the tube for various lengths of time.

Nerve Processing and X-Gal Staining of Myelinating SCs *in Vivo*. Seven to 18 weeks after transection, regenerated nerves were removed, fixed in phosphate-buffered 0.5% glutaraldehyde for 2 h, and then placed in the X-Gal solution overnight according to the method of Dannenberg and Suga (20) with the exception that the molarity of the phosphate buffer was doubled to reduce nonspecific staining (see *Results*). The nerves were fixed for an additional 3 h in phosphate-buffered 3.6% (vol/vol) glutaraldehyde and then placed either in glycerol for teasing apart individual nerve fibers or in 1% osmium tetroxide/0.37% ferrocyanide for light and electron microscopy. Prior to embedding in epon, the osmicated nerves were dehydrated in a graded series of ethanol and infiltrated briefly with propylene oxide. Thick sections (1 μm) were counterstained with *p*-phenylenediamine; thin sections were stained with lead citrate.

RESULTS

Transduction of Cultured SCs with *lacZ*. To identify transplanted SCs in nerve, we first transduced cultured SCs with the *lacZ* gene contained in the BAG retroviral vector (15) and selected for transduced cells with the antibiotic G418. Within 7 days of selection, colonies resistant to G418 were clearly evident and by 10 days many blue cells could be identified after X-Gal staining (Fig. 1). About 60% of the G418-resistant cells were blue, most likely reflecting the fact that there were different insertional sites of the vector into the SC genomes and the transduced cells were not clonal. Supernatants from transduced SCs were not able to infect NIH 3T3 cells as outlined by Cepko (18).

Transduction Does Not Alter SC Behavior *in Vitro*. To examine the possible effect of transduction on the cells, we compared proliferation rates and gene expression of transduced and nontransduced secondary SCs in culture. Both groups of SCs were induced to proliferate more rapidly in the presence of serum and 4 μM Fsk, as evaluated by [^{3}H]thymidine incorporation (data not shown). As shown (13), Fsk increased and decreased the steady-state levels of P_0 and NGFR mRNA, respectively, in both transduced and nontransduced SCs (Fig. 2). Thus, consistent with previous reports (23), transduction with the *lacZ* gene did not appear to alter the behavior of the SCs.

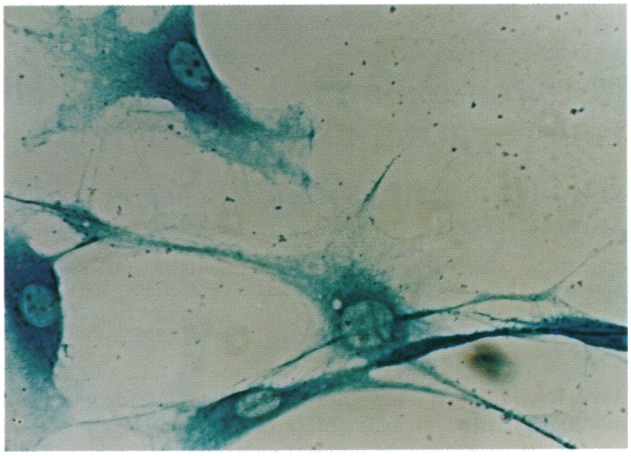

FIG. 1. X-Gal staining of transduced secondary SCs. Rat SCs were prepared by the method of Brockes *et al.* (16) and identified by immunostaining with S-100. SCs were plated on polylysine-coated plates at 8 × 10^5 cells per plate in DMEM supplemented with 10% fetal calf serum, 4 μM Fsk, and glial growth factor. Supernatants from BAG-producing φ2 packaging cells plus Polybrene (8 μg/ml) were used to infect the SCs (18). Transduced SCs were selected and maintained with G418 (1 mg/ml). LacZ expression was detected by incubating the SCs in X-Gal solution, which stains transduced cells blue (15). (×400.)

Transduced Secondary SCs Myelinate Regenerating Axons *in Vivo*. To examine the behavior of transduced SCs *in vivo*, they were transplanted into syngeneic rat sciatic nerves in a manner designed to foster axonal–SC contact. Sciatic nerves were transected and the proximal and distal nerve stumps were placed in a silicone tube containing either transduced or control rat SCs. After 7 and 18 weeks, whole mounts of regenerated nerves were dissected and subjected to X-Gal histochemistry to reveal the cells that had stained blue. Regenerated nerve fibers were gently teased apart into small groups. Only nerves that received transduced SCs contained blue cylindrical-shaped cells that appeared to surround regenerated axons (Fig. 3). These blue cylinders looked like myelin internodes, as they tapered at either end at a node of

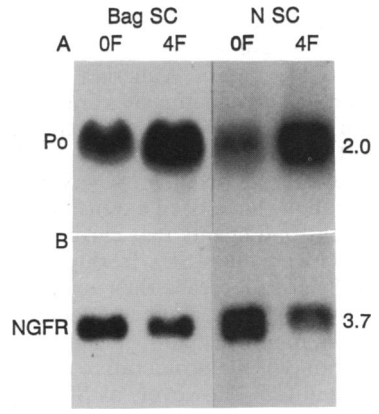

FIG. 2. Northern blot analysis of transduced and control secondary SCs. Fsk increases the steady-state mRNA level of P_0 (*A*) and decreases the steady-state mRNA level of NGFR (*B*) in transduced and control SCs. The transduced SCs have been passed nine times and express more P_0 mRNA in the absence of Fsk than do the control SCs passed five times. Total RNA (10 μg) isolated from SC cultures (19) was electrophoresed, transferred to nylon membrane, and then hybridized overnight at 42°C with the following cDNA probes: a full-length cDNA (2.0 kilobases) of rat P_0 (21) and a 0.7-kilobase *Bam*HI fragment of rat NGFR (22). BAG SC, SCs transduced with the BAG vector; NSC, nontransduced SCs; 0F, 0 μM Fsk; 4F, 4 μM Fsk. Molecular sizes are expressed in kilobases.

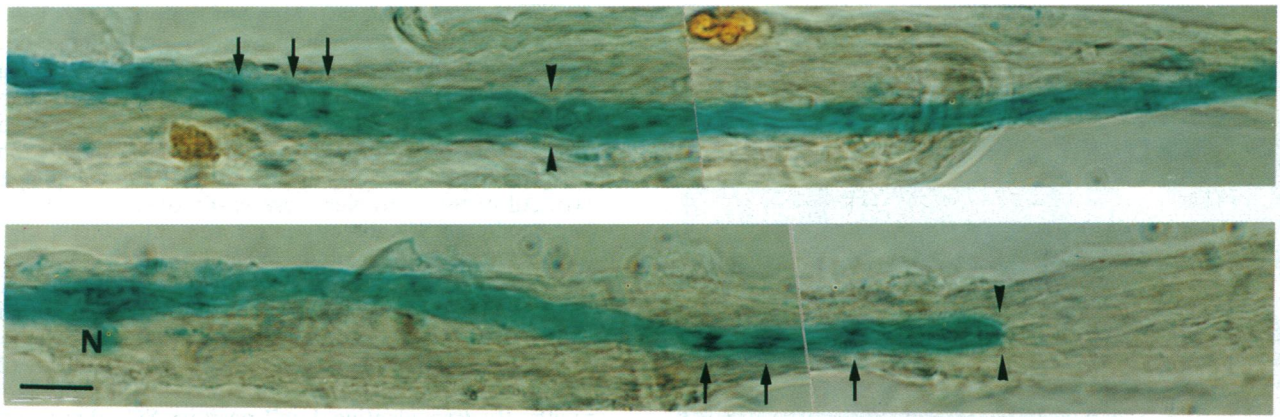

FIG. 3. X-Gal staining of myelinating SCs *in vivo*. A photomosaic of light micrographs of a bundle of teased nerve fibers from a 7-week regenerated nerve. The photomosaic is separated into two rows of micrographs and shows one complete blue internode between the pairs of arrowheads (nodes of Ranvier), which separate the complete internode from another blue internode (*Upper*), and an unstained internode (*Lower*). The arrows mark incisures of Schmidt–Lantermann, and the SC nucleus of the complete myelin internode is labeled (N). (Bar = 10 μm.) One million transduced or control SCs were transplanted into a silicone tube (1 cm in length) that connected the proximal and distal stumps of a transected sciatic nerve in syngeneic rats. After 7–18 weeks, regenerating nerves were removed, fixed, and stained in the X-gal solution overnight according to Dannenberg and Suga (20). The nerves were fixed again and then placed in glycerol for teasing apart.

Ranvier and even had Schmidt–Lantermann incisures. The blue internodes tended to occur in clusters, often near the proximal nerve stump and many blue internodes abutted other blue internodes at nodes of Ranvier. As expected for remyelinated internodes (24), they were on average 281 mm (range, 216–334 mm; $n = 13$) in length. Since transduced SCs did not produce helper virus (18), they could not have infected preexisting host SCs.

To further analyze the anatomical details of the transduced SCs, nerves were examined by light and electron microscopy. With light microscopy, we found many SCs with cuffs of blue-staining cytoplasm that surrounded the myelin sheaths (Fig. 4). As in the teased fibers, these tended to occur in groups. We did not see any such blue-stained SCs in animals that received nontransduced SCs, nor in the proximal or distal nerve stumps of any regenerated nerve. With electron microscopy, these blue SCs contained rectangular crystals that were associated with membranous organelles, including the smooth endoplasmic reticulum, nuclear envelope, and lysosomes (Fig. 5); such crystals have been reported to represent products of the X-Gal reaction (21). The transduced SCs formed a normal-appearing multilamellar myelin sheath around an axon and were surrounded by a basal lamina.

In nerves that had received either transduced or nontransduced SCs, large round blue cells were scattered throughout the endoneurium, particularly adjacent to the silicone tube and suture material. By electron microscopy, these cells did not have a basal lamina and contained crystals that were associated with membranous organelles, particularly lysosomes. Thus, these cells had the features of macrophages, which are present in damaged nerve (25) and stain blue with X-Gal histochemistry (18).

DISCUSSION

The significance of these results lies in several areas. To genetically alter SCs, large numbers of cells must be obtained by serial passage in culture. However, as the SCs are passaged in culture, their phenotype progressively diverges from that of primary SCs (14, 26). Thus it is significant that serially passaged secondary SCs can enter into normal SC–axonal relationships in peripheral nerve, as shown by the normal morphology of their regenerated internodes and ultrastructure of the myelinating SCs. The ability of secondary SCs to myelinate axons, however, is not maintained indefinitely. As is the case *in vitro* (14), we found that secondary SCs were not able to myelinate axons *in vivo* after >25 passages (M.L.F. and M.E.S., unpublished observations). Although early-passage secondary SCs have a number of physiological differences compared to primary SCs, our transplantation data suggest that these cells can, at this stage,

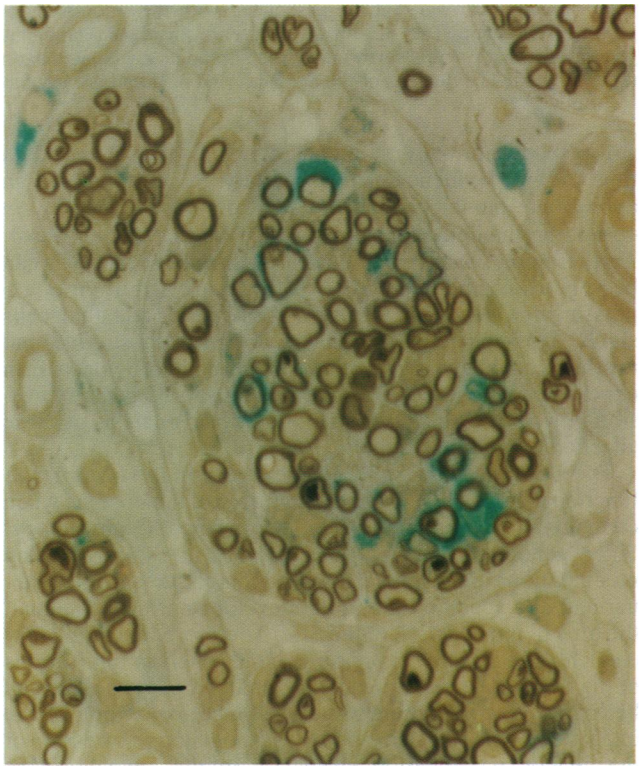

FIG. 4. Light micrograph of a fascicle of regenerating axons in cross-section. β-Galactosidase expression is detected by the blue color in many SCs in the central fascicle. Note that the blue staining is confined to the cytoplasm and is not in the circular myelin sheaths. The adjacent fascicle in the upper right has no blue SCs, indicating that the transplanted SCs appear to myelinate focal areas of the regenerating axons. (Bar = 10 μm.) After fixation and X-Gal staining, nerves were osmicated, dehydrated in a graded series of ethanol, infiltrated briefly with propylene oxide, and then embedded in epon. Sections (1 μm thick) were counterstained with *p*-phenylenediamine.

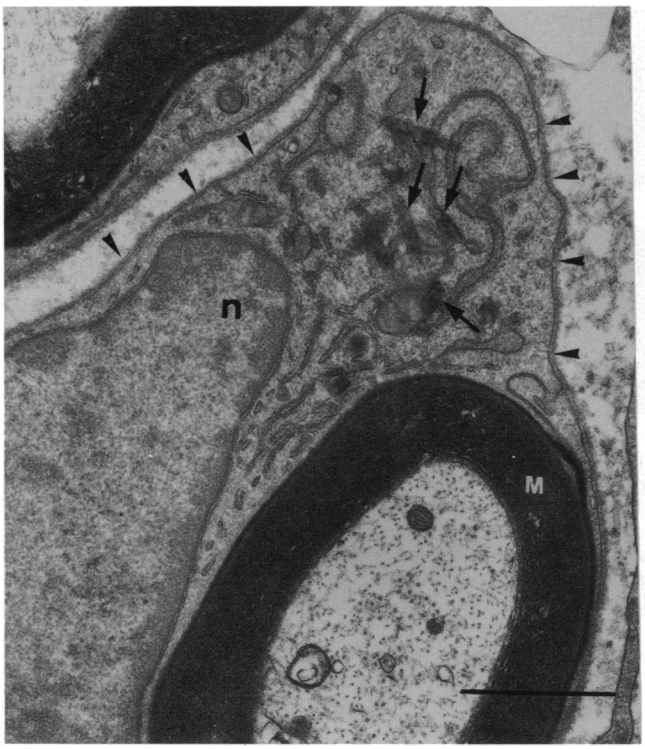

FIG. 5. Electron micrograph of a transduced myelinating SC. The transduced SCs form a normal-appearing myelin sheath when examined by electron microscopy. The X-Gal product forms rectangular crystals (arrows) that are associated with the smooth endoplasmic reticulum. The SC forms a myelin sheath (M) and has generated a basal lamina surrounding the SC and its myelinated axon (arrowheads). The nucleus of the SC is labeled n. (Lead citrate; bar = 10 μm.)

still respond normally to axonal signals *in vivo*. These data thus provide evidence that secondary SC cultures are an appropriate model system in which to study SC activity *in vivo* after manipulation and passage of the cells *in vitro*. Furthermore, we have demonstrated that secondary SCs can be genetically modified *in vitro*, by using a retrovirus expressing the bacterial β-galactosidase gene, and that cells stably expressing this foreign gene can both survive and myelinate axons *in vivo*. Thus these data provide a firm experimental basis for the modification of SC gene expression *in vitro* and the subsequent functional analysis of these genetically modified cells *in vivo*.

The ability to modify SC gene expression *in vitro* prior to functional analysis *in vivo* has a number of advantages when compared to other methods of perturbing SC physiology. Since gene expression is altered only in SCs, any such modifications are always expressed in a tissue-specific manner and at a specific time in the development of the animal, the time of introduction of the SCs. Therefore, the regulatory elements required for expression of the genetic modifications of the SC are less restricted. Also, analysis of the animal can be limited to the peripheral nerve. Since expression of the introduced gene is restricted to SCs, anomalous expression of the genetic modification elsewhere in the animal cannot indirectly affect the resulting phenotype. In contrast, other *in vivo* methods, such as the production of transgenic animals, may require tissue-specific and developmentally regulated expression of a transgene in SCs, which may be technically difficult to accomplish. For example, previous studies of sea urchin CyIIIa cytoskeletal actin gene expression have identified distinct cis-acting elements responsible for regulating the amplitude of expression, tissue-specific expression, or developmental expression, all within 2.3 kilobases of 5′ regulatory domain (27, 28). Many of the promoters used to manipulate gene expression in SCs, primarily those of the myelin structural proteins (e.g., P_0), are only preliminarily characterized either *in vitro* or *in vivo*. Therefore, guaranteeing tissue specificity and developmental expression of a transgene in a SC is difficult. Furthermore, dysregulation of transgene expression may be lethal to the developing embryo. All of these problems are easily overcome by transplanting altered SCs directly into regenerating nerve.

Two potential applications exemplify the advantages of this system. (*i*) SCs express growth factors, such as NGF, which are likely to play important roles in peripheral nerve development and regeneration. NGF is expressed by at least two cell types in peripheral nerve, SCs and fibroblasts (29), making it difficult to isolate the effects of SCs on axonal growth. By transplanting LacZ-positive SCs that overexpress NGF, the role of NGF derived from SCs on axonal regeneration can be investigated. (*ii*) This model may prove useful in confirming proposed etiologic gene defects in inherited disorders of peripheral nerve, confirming their pathogenesis, and ultimately evaluating their treatment. The autosomal dominant trembler mutation (*Tr*) in mouse causes a hypomyelinating peripheral neuropathy clinically and pathologically similar to the demyelinating form of Charcot-Marie-Tooth disease in humans (30). The trembler mutation has recently been identified as a point mutation in the gene encoding the myelin protein PMP-22 (31). The identified G→A transition mutation results in the substitution of an aspartic acid residue for a glycine residue in a putative transmembrane domain of PMP-22. Charcot–Marie–Tooth disease patients have a duplication in a portion of chromosome 17 that is syntenic to the region of mouse chromosome 11 containing the PMP-22 gene (32). Therefore, it appears that either a mutated form of PMP-22 or overexpression of PMP-22 may result in similar phenotypes. By expressing the dominant trembler allele of PMP-22 or overexpressing PMP-22 in LacZ-expressing SCs and transplanting the cells into regenerating nerve, the phenotype resulting from these SC-specific alterations may be evaluated and compared.

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1. Webster, H. deF. & Favilla, J. T. (1984) in *Peripheral Neuropathy*, eds. Dyck, P. J., Thomas, P. K., Lambert, E. H. & Bunge, R. (Saunders, Philadelphia), pp. 329–359.
2. Bray, G. M., Rasminsky, M. & Aguayo, A. J. (1981) *Annu. Rev. Neurosci.* **4**, 127–162.
3. Weinberg, H. J. & Spencer, P. S. (1976) *Brain Res.* **113**, 363–378.
4. Jessen, K. R. & Mirsky, R. (1991) *Glia* **4**, 185–194.
5. Wood, J. G. & Engel, E. L. (1976) *J. Neurocytol.* **5**, 605–615.
6. Trapp, B. D., Quarles, R. H. & Suzuki, K. (1984) *J. Cell Biol.* **99**, 594–606.
7. Owens, G. B. & Bunge, R. P. (1989) *Glia* **2**, 119–128.
8. Owens, G. B. & Bunge, R. P. (1990) *Glia* **3**, 118–124.
9. Scherer, S. S. & Asbury, A. K. *Axonal Peripheral Neuropathies* (Wiley, Philadelphia), in press.
10. Seamon, K. B. & Daly, J. W. (1981) *J. Cyclic Nucleotide Res.* **7**, 201–224.
11. Mokuno, K., Kambolz, J., Behrman, T., Black, C., Sessa, M., Feinstein, D., Lee, V. & Pleasure, D. (1989) *J. Neurosci. Res.* **23**, 396–405.
12. Morgan, L., Jessen, K. R. & Mirsky, R. (1991) *J. Cell Biol.* **112**, 457–467.
13. Lemke, G. & Chao, M. (1988) *Development* **102**, 499–504.

14. Porter, S., Glaser, L. & Bunge, R. P. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 7768–7772.
15. Price, J., Turner, D. & Cepko, C. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 156–160.
16. Brockes, J. P., Fields, K. P. & Raff, M. C. (1979) *Brain Res.* **165,** 105–118.
17. Brockes, J. P., Jeremy, P., Lemke, G., Balizer, S. R. & David, R. (1980) *J. Biochem. (Tokyo)* **255,** 8374–8377.
18. Cepko, C. (1989) in *Neuromethod: Molecular Neurobiological Techniques*, eds. Boulton, A. A., Baker, G. B. & Campagnoni, A. T. (Humana, Clifton, NJ), Vol. 16, pp. 177–219.
19. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
20. Dannenberg, A. M. & Suga, M. (1981) in *Method for Studying Mononuclear Phagocytes*, eds. Adams, D. O., Edelson, P. J. & Koren, M. S. (Academic, New York), pp. 375–396.
21. Langford, L. A. & Owens, G. C. (1990) *Acta Neuropathol.* **80,** 514–520.
22. Lemke, G. & Axel, R. (1985) *Cell* **40,** 501–508.
23. Radeke, M. J., Misko, T. P., Hsu, C., Herzeaberg, L. A. & Shooter, E. M. (1987) *Nature (London)* **325,** 593–597.
24. Vizoso, A. D. & Young, J. Z. (1948) *J. Anat.* **82,** 110–134.
25. Stoll, G., Griffin, J. W., Li, C. Y. & Trapp, B. D. (1989) *J. Neurocytol.* **18,** 671–683.
26. Eccleston, P. M., Mirski, R. & Jessen, K. R. (1991) *Development* **112,** 33–42.
27. Franks, R. A., Anderson, R., Moore, J. G., Hough-Evans, B. R., Britten, R. J. & Davidson, E. H. (1990) *Development* **110,** 31–40.
28. Hough-Evans, B. F., Franks, R. R., Zeller, R. W., Britten, R. J. & Davidson, E. H. (1990) *Development* **110,** 41–50.
29. Matsuoka, I. M., Meyer, M. & Thoenen, H. (1991) *J. Neurosci.* **11,** 3165–3177.
30. Lupski, J. R., Montes de Oca-Luna, R., Slaugenhaupt, S., Pentao, L., Guzzetta, V., Trask, B. J., Saucedo-Cardenas, O., Barker, D. F., Killian, J. M., García, C. A., Chakravart, A. & Patel, P. I. (1991) *Cell* **66,** 219–232.
31. Suter, U., Welcher, A. A., Ozcelik, K. T., Snipes, G. J., Kosaras, B., Francke, V., Billings-Gagliardi, Sidman, R. L. & Shooter, E. M. *Nature (London)* **356,** 241–244.
32. Buckburg, A. M., Moscow, J., Buchwalter, S. M. & Camper, S. B. (1990) *Mamm. Genome* **1,** S158–S191.