Immunoreactive Myelin Basic Proteins Are Not Detected When Shiverer Mutant Schwann Cells and Fibroblasts Are Co-cultured with Normal Neurons

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ABSTRACT Shiverer (*shi*) is an autosomal recessive mutation in mice that results in hypomyelination in the central nervous system (CNS) but normal myelination in the peripheral nervous system (PNS). Myelin basic proteins (MBPs) are virtually absent in both PNS and CNS. It is not known whether the cellular target in the PNS is the myelin-forming Schwann cell or another cell type which secondarily affects the Schwann cell. To determine the cellular target of the *shi* gene, we have adapted tissue culture techniques that allow co-culture of pure populations of mouse sensory neurons of one genotype with Schwann cells and fibroblasts of another genotype under conditions that permit myelin formation. These cultures were stained immunocytochemically as whole mounts to determine whether MBPs were expressed under various in vitro conditions. In single-genotype cultures, presence or absence of MBPs was consistent with earlier in vivo results: +/+ cultures were MBP-positive and *shi/shi* cultures were MBP-negative. In mixed-genotype cultures, visualization of MBPs in myelin accorded with the genotype of the non-neuronal Schwann cells and fibroblasts and not with the neurons—those cultures that contained +/+ non-neuronal cells were MBP-positive and those with *shi/shi* non-neuronal cells were MBP-negative, independent of the neuronal genotype. These results rule out neurons or circulating substances as mediators of the influence of the *shi* genetic locus on MBP synthesis and deposition in peripheral myelin.

Shiverer is an autosomal recessive mutation (4) affecting formation of myelin in mouse peripheral nervous system (PNS)[1] and central nervous system (CNS). Histological and biochemical analysis of animals homozygous for the mutation (*shi/shi*) show that the amount of myelin in the CNS is greatly reduced whereas a normal amount of myelin is present in the PNS (5, 11, 15). The feature that distinguishes this myelination disorder from all others is that concentrations of myelin basic proteins (MBPs) are disproportionately reduced in relation to other myelin constituents. Four immunologically related MBPs with molecular weights of 14,000, 17,000, 18,500, and 21,500 are present in the PNS and CNS of mice and rats (2, 10). Radioimmunochemical and quantitative electrophoretic measurements of MBPs in PNS myelin show that MBPs are virtually absent in *shi/shi* animals (9) and are in reduced amounts in *shi/+* animals (7).

The primary cellular target of the shiverer mutation has not been determined. The deficit in the PNS may be intrinsic to Schwann cells, whose wrapped surface membranes constitute the myelin sheath, or it may be extrinsic, initially affecting contiguous or distant cells which in turn affect Schwann cells. To explore the intrinsic versus extrinsic target issue, we have adapted tissue culture techniques so as to be able to culture pure populations of mouse sensory neurons of one genotype with Schwann cells and fibroblasts of another genotype under conditions that permit Schwann cells to differentiate and form myelin around axons. These mixed-genotype cultures were then assessed immunocytochemically for the presence of MBPs in myelin.

MATERIALS AND METHODS

Culture Conditions: Inbred normal mice (+/+) of the C57BL/6J strain and mice homozygous for shiverer on a noninbred genetic background

[1] *Abbreviations used in this paper:* 5-BUdR, 5-bromodeoxyuridine; BSA, bovine serum albumin; CMF-HBSS, Ca^{2+}-Mg^{2+}-free Hank's balanced salt solution; CNS, central nervous system; DRG, dorsal root ganglia; FUdR, fluorodeoxyuridine; MBP, myelin basic protein; PNS, peripheral nervous system.

THE JOURNAL OF CELL BIOLOGY · VOLUME 98 APRIL 1984 1291-1295

provided tissue for culture. Dorsal root ganglia (DRG) were dissected aseptically from lumbar regions of embryos removed at 17 d of gestation and were dissociated by incubating 10–60 ganglia in 2 ml of 0.25% trypsin (Gibco Laboratories, Inc., Grand Island, NY) in Ca^{2+}-Mg^{2+}-free Hank's balanced salt solution (CMF-HBSS; Gibco Laboratories, Inc.) at 37°C for 45 min. After the incubation ganglia were washed with CMF-HBSS, suspended in culture medium, triturated with a Pasteur pipette, and filtered through a nylon screen with a 15-μm pore size. Cells were grown on air-dried collagen in Aclar (Allied Chemical Corp., Morristown, NJ) dishes (6) in a culture medium consisting of 15% human placental serum, 5% chick embryo extract, 600 mg% glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, and nerve growth factor (a crude preparation at an empirically determined concentration found to support test cultures of mouse superior cervical ganglia), in minimal essential medium supplemented with 2 mM glutamine (Gibco). The cultures were incubated at 36°C in a 5% CO_2 humidified atmosphere and medium was changed three times a week. Discarded out-of-date blood samples routinely taken from umbilical veins at birth were used as the source of human placental serum.

Photo-induced Killing of Schwann Cells and Fibroblasts in Dispersed DRG Cultures: Several treatment protocols were tested to determine the optimal conditions for introducing 5-bromodeoxyuridine (5-BUdR; Sigma Chemical Co., St. Louis, MO) and Hoechst dye 33258 (Aldrich Chemical Co., Inc., Milwaukee, WI) into newly synthesized DNA of dividing non-neuronal cells. Complete removal of non-neuronal cells from cultures of dissociated DRG was accomplished when the ganglia were dissociated in standard medium supplemented with 5×10^{-5} M 5-BUdR and grown in this medium for 3 d. The medium was then replaced with one supplemented with 5 μg/ml Hoechst dye 33258 (Aldrich Chemical Co., Inc.) for 3 h. Finally, the cultures were fed with medium lacking dye, but containing 1×10^{-5} M fluorodeoxyuridine (FUdR; Sigma Chemical Co.) and were exposed to light for 1 h by being placed 6 cm below a 60-W fluorescent bulb in the culture incubator. The medium containing FUdR was removed after 3 d and thereafter the cultures were fed with normal medium. Living cultures were observed microscopically for 1–2 wk after this treatment to confirm that all non-neuronal cells had been removed before co-culture experiments were performed.

Addition of Postnatal Sciatic Nerve to Sensory Neuron Cultures: We introduced Schwann cells and fibroblasts into cultures of sensory neurons by placing explants of sciatic nerve from neonatal mice in the culture dish. 1-d-old mice were killed by decapitation and a segment of sciatic nerve was removed aseptically. The tissue was cleaned of epineural fat and connective tissue in CMF-HBSS and teased apart into fascicles of nerve fibers with associated non-neuronal cells. Such fascicles were then placed on the collagen substrate in the vicinity of sensory neurons and their neurites.

Immunocytochemical Localization of MBPs in Dissociated DRG Cultures: Once myelinated axons were identified by phase-contrast microscopy, cultures were fixed and stained immunocytochemically to detect MBP. The cultures were washed once in CMF-HBSS and fixed in $HgCl_2$-formalin (2.0 ml 40% formalin and 7.6 ml saturated aqueous $HgCl_2$) at 4°C for 1 h. The cultures were then washed for 1 min with H_2O and overnight with 0.5 M Tris-HCl, pH 7.6, at 4°C. The next day cultures were washed with H_2O for 1 min, treated with 90% ethanol for 20 min at 25°C, washed with H_2O for 1 min, and treated with 0.25% Triton X-100 in 0.5 M Tris-HCl, pH 7.6, for 10 min at 25°C. The cultures were then treated with 3% normal goat serum and 0.025% Triton X-100 in 0.5 M Tris-HCl, pH 7.6, for 20 min at 25°C and then washed with the same solution containing 1% normal goat serum, which served as the diluent in subsequent steps. Cultures were then incubated with antiserum to mouse MBPs raised in rabbits (provided by J. Carson, University of Connecticut), diluted 1/500, for 3 h at 25°C and 20 h at 4°C. The cultures were washed with biotin-labeled goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) at a concentration of 3 μg/ml in 10 mM phosphate buffer, pH 7.8, containing 0.5% BSA (BSA-PB) for 1 h at 25°C. The cultures were then washed in BSA-PB for 1 h and were incubated with horseradish peroxidase–labeled avidin D (Vector Laboratories, Inc.) at a concentration of 10 μg/ml in BSA-PB at 25°C. The cultures were then washed in 0.1 M Tris-HCl, pH 7.6, reacted with diaminobenzidine for 8 min, postfixed with 1% OsO_4 in 0.1 M phosphate buffer, pH 7.4, for 4 min, counterstained with cresyl violet, and mounted in Permount.

RESULTS

Removal of Non-neuronal Cells from Cultures by Photo-induced Killing of 5-BUdR–substituted Cells

When cultures of trypsin-dissociated DRG were treated in the manner outlined in Materials and Methods, all non-neuronal cells were killed (Fig. 1). The effectiveness of this treatment depended on incorporation of 5-BUdR into all non-neuronal cells. If Schwann cells and fibroblasts did not divide, and therefore failed to incorporate 5-BUdR into newly synthesized DNA, they were not killed. This was evident if 5-BUdR was added several days after plating when presumably some cells had stopped dividing, or if cells were cultured at low density (data not shown). We detected no cell death after incorporation of Hoechst dye 33258 and light treatment alone. We observed no difference in the rate of cell division of non-neuronal cells or the survival of neuronal cells in the presence of 5-BUdR prior to light treatment. Sensory neurons appeared normal in the absence of non-neuronal cells while grown in serum- and nerve growth factor-supplemented medium. They extended and maintained numerous neurites and were capable of eliciting cell division and myelin formation on the part of Schwann cells introduced into the culture.

Immunocytochemical Localization of MBPs in Cultures of Dissociated DRG of One Genotype

When cells from trypsin-dissociated ganglia were grown in the presence of human placental serum and embryo extract, sensory neurons thrived and extended neurites. Schwann cells proliferated, in part in response to sensory axons (23), ensheathed individual axons, and formed myelin around a few of the axons. Cultures consisting of neurons, fibroblasts, and Schwann cells of a single genotype (*shi/shi* or +/+) formed myelin in comparable amounts, as judged by inspection of living or fixed and stained preparations. However, only the +/+ cultures contained immunologically detectable MBPs (Fig. 2). The reaction produced a discontinuous visible product within the boundaries of the myelin sheath, an observation that suggests that immunoreactive MBP was not uniformly accessible to the reagents possibly because of the necessarily disruptive fixation and staining techniques. The lack of MBP in cultured *shi/shi* myelin, comparable to the in vivo situation, indicates that simple isolation of the DRG cell system from the rest of the animal does not alter the mutant phenotype. Factors extrinsic to the DRG cell system itself are not responsible for the failure of *shi/shi* Schwann cells to express MBP in myelin.

Co-culture of shi/shi Neurons with +/+ Schwann Cells and Fibroblasts, or +/+ Neurons with shi/shi Schwann Cells and Fibroblasts

When neonatal sciatic nerve was added to cultured sensory neurons, Schwann cells and fibroblasts migrated from the explant, proliferated, and repopulated the culture. 3–4 wk after their introduction into the cultures Schwann cells had formed myelin. We examined four cross-genotype cultures in two separate experiments. Of the many myelinated axons per culture dish, 30–50 myelin sheaths in each living culture were photograpically documented and their positions were identified in phase-contrast micrographs. When the same areas were examined in the subsequent immunocytochemical preparations made from each culture, all myelin sheaths identified in the living cultures were now stained positively for MBP in the cases when Schwann cells and fibroblasts from +/+ sciatic nerve repopulated cultures of *shi/shi* sensory neurons (Fig. 3, *a* and *b*). Every myelin sheath was unstained in all areas when Schwann cells and fibroblasts from *shi/shi* nerve repopulated

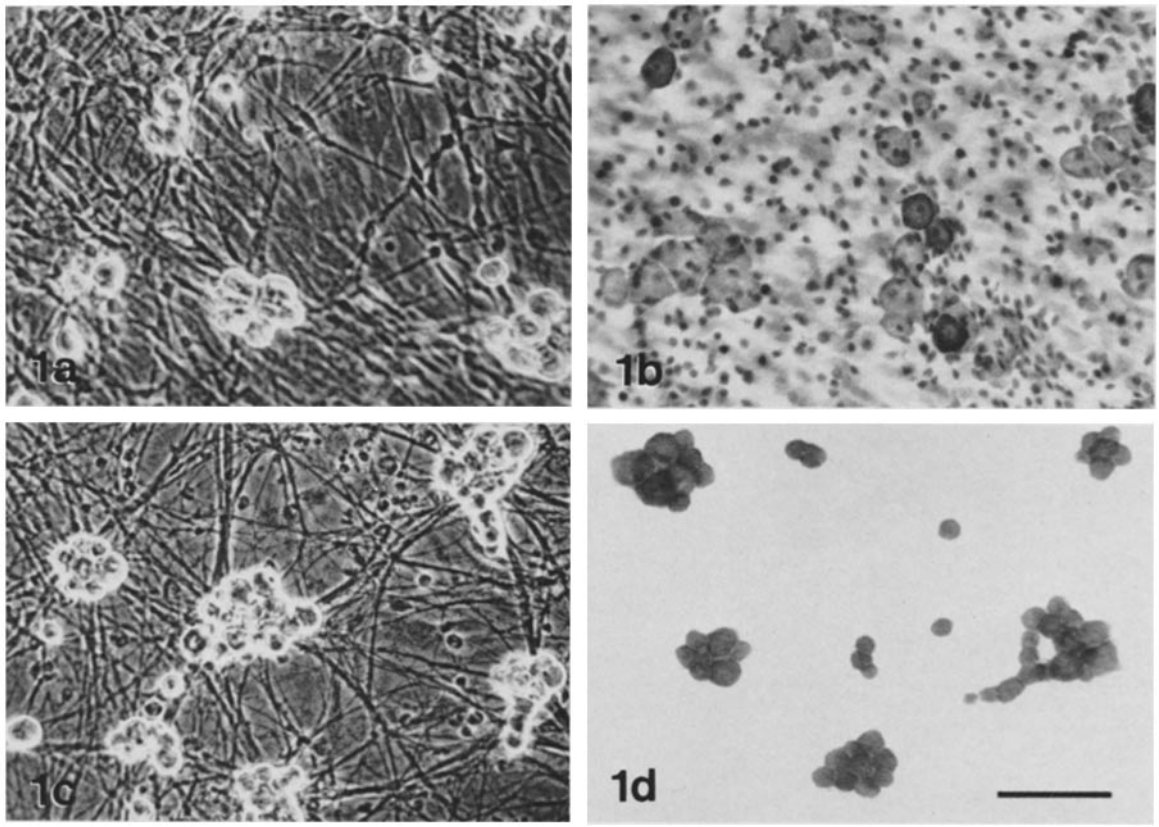

FIGURE 1 Dissociated DRG in culture demonstrating effects of photo-induced killing. (a) Phase-contrast photomicrograph of living culture 6 d in vitro showing phase-bright sensory neurons and interconnecting neurites on a background of fibroblasts and Schwann cells. (*b*) Photomicrograph of culture in *a* after fixation and staining with cresyl violet showing large neurons and smaller Schwann cells. (*c*) Phase-contrast photomicrograph of living culture after treatment to rid the culture of non-neuronal cells. The clumped sensory neurons are interconnected with numerous fine and course neurite bundles. Cellular debris from dead non-neuronal cells is still evident. (*d*) Photomicrograph of fixed and cresyl violet-stained culture of sensory neurons free of non-neuronal cells. Neurites are essentially unstained, and dead cell debris has washed away during fixation and staining procedures. Bar, 100 μm. × 150.

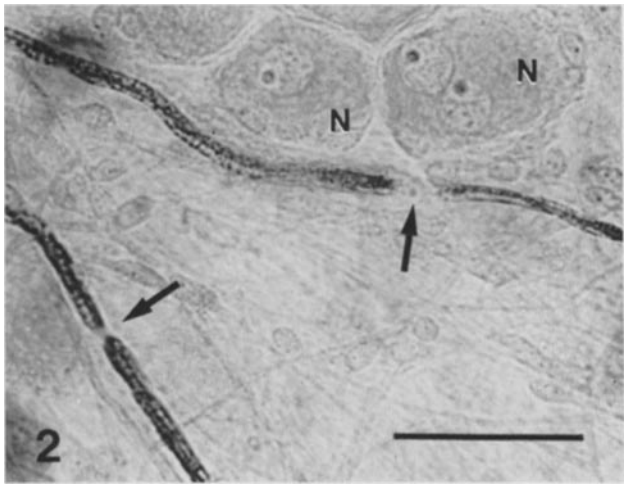

FIGURE 2 Immunocytochemical localization of MBPs in myelin sheaths in a culture of +/+ DRG cells. Two nodes of Ranvier (arrows) as well as several sensory neurons (*N*) are visible. Bar, 50 μm. × 500.

cultures of +/+ neurons (Fig. 3, *c* and *d*). The lack of MBP staining was associated with the presence of *shi/shi* Schwann cells and fibroblasts regardless of the genotype of the sensory neurons, and thus it is evident that one or both of these non-neuronal cell types is the primary target of the *shi* genetic locus.

DISCUSSION

Cellular development is governed not only by intrinsic genetic programs but also by extrinsic humoral, local environmental, and cell contact signals. A cell exhibiting a mutant phenotype may not be the initial target of the mutant gene but may receive inappropriate extrinsic influences from another cell which is the primary target. A general experimental approach to elucidate the primary cellular target of a mutant gene has been to introduce the cell of interest into different cellular environments and observe what mutant phenotype is expressed. This has been accomplished by producing chimeric animals with a mix of cells of two genotypes (12, 13), grafting tissue of one phenotype into immunotolerant animals of another phenotype (1), and by co-culturing cells of two genotypes (8). In the present study we have cultured pure populations of sensory neurons of one genotype and then added Schwann cells and fibroblasts of another genotype. The interpretability of the experiment depends on the effectiveness of the cell purification and on the specificity and sensitivity of the immunocytochemical assay for MBPs.

The use of 5-BUdR, Hoechst dye 33258, light, and FUdR on the recommended schedule allows complete removal of dividing cells from cultures of embryonic mouse dissociated

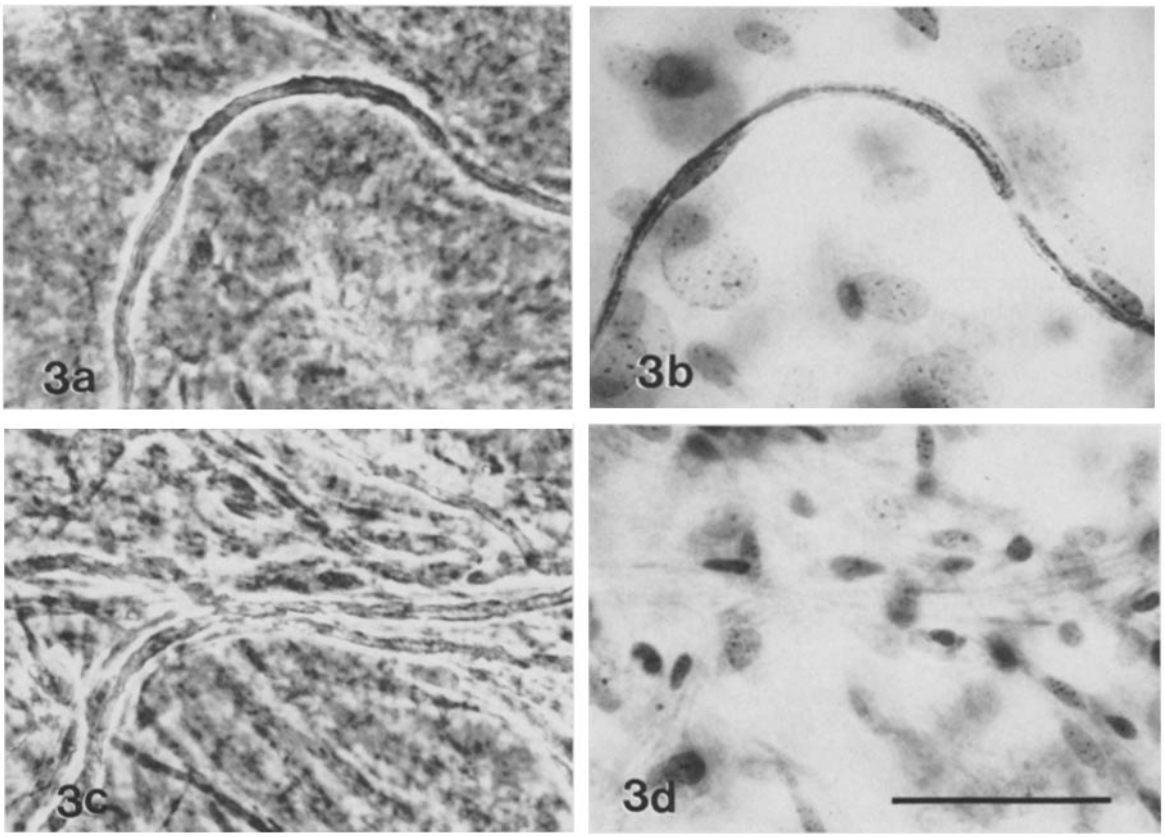

FIGURE 3 Representative photomicrographs of immunocytochemical localization of MBPs in *shi/shi* and +/+ co-cultures. (*a*) Phase-contrast photomicrograph of living culture consisting of *shi/shi* sensory neurons and +/+ fibroblasts and Schwann cells; a single myelinated axon loops across the field. (*b*) Same field as in *a*, after the culture was fixed and stained to show the MBP-positive myelin sheath. (*c*) Phase-contrast photomicrograph of living culture consisting of +/+ sensory neurons, *shi/shi* fibroblasts, and Schwann cells and numerous myelinated axons. (*d*) Same field as in *c*, fixed and stained for MBP, and showing no MBP-positive staining. Bar, 100 μm. × 290.

DRGs. Other methods that are successful with rat DRG cells, such as cytosine arabinoside and FUdR treatments (22), were unsuccessful in our hands when used on dissociated mouse cells. Non-neuronal cells, predominately Schwann cells and endoneurial fibroblasts, begin dividing immediately upon plating and to some extent continue dividing throughout the life of the culture so that in the presence of the thymidine analog 5-BUdR every non-neuronal cell in culture incorporates the drug within the time span of the treatment. Conversely, since sensory neurons are already postmitotic at the time of culture, they do not divide further and therefore do not incorporate 5-BUdR into their DNA. Although light exposure alone will kill some 5-BUdR–substituted cells (16), the addition of Hoechst dye 33258, which binds to DNA, improves the efficiency of the photo-killing 10,000-fold (19). The final addition of FUdR is used as a precautionary measure and was not proved to be needed.

The anti-MBP antiserum that we used has good specificity, giving a detectable reaction in gel preparations only with the four known rodent MBPs (2) and with a few poorly characterized high molecular weight protein bands (3). Regarding sensitivity, choice of fixative always requires a compromise between retention of antigen reactivity and preservation of tissue structure. We used the $HgCl_2$-formalin fixative of Sternberger et al. (18), which permits high sensitivity for detection of MBPs but severely distorts myelin architecture in tissue sections (20) as well as in whole-mount cultures, compared with a double aldehyde-OsO_4 fixation procedure followed by epoxy plastic embedding (17, 21). Our concern in the present study was less with the precise localization of MBPs and more with the threshold for detection, so that we accepted, for example, the distortions of myelin architecture evident in Fig. 3 (see also reference 20).

Our culture experiments partially answer the question as to the cellular target of the shiverer genetic locus. In cultures in which neurons, fibroblasts, and Schwann cells were all of one genotype, the phenotype expressed was the same as in vivo, i.e., +/+ cultures expressed MBP in myelin sheaths while *shi/shi* cultures did not. If circulating factors were solely responsible for the lack of MBP in shiverer myelin, one would predict that *shi* cultures would contain MBP. No such extrinsic humoral influence was indicated.

Co-culture of Schwann cells and fibroblasts of one genotype with sensory neurons of another genotype further localizes the primary target of the shiverer gene. If the neuron or its axon played a key role in expression of the shiverer phenotype, one would expect myelin formed around *shi/shi* axons by +/+ Schwann cells to lack MBP and myelin formed around +/+ axons by *shi/shi* Schwann cells to exhibit MBP staining. This was not the case. Myelin formed by *shi/shi* Schwann cells, whether in the presence of +/+ or *shi/shi* axons, lacked MBP by the immunocytochemical criterion. Conversely, the myelin always contained MBPs when +/+ Schwann cells and fibroblasts were co-cultured with +/+ or *shi/shi* neurons. Concordance was complete for about 200 myelin sheaths photographically documented in both living and immunocy-

tochemically stained preparations. Further, no exceptions were found in hundreds of additional myelin sheaths that were observed but not photographically documented. The implication of these experiments is that the site of *shi* gene action is in the Schwann cells and/or fibroblasts. It is not yet technically feasible to obtain consistent cultures of Schwann cells alone.

The results of the tissue culture analysis are consistent with those determined by chimera or graft analysis (14, 17). The strengths of the culture procedure are the ability to observe cell behavior closely in the living state and to manipulate cells more flexibly with reference to the parameters of cell numbers, stages of maturation, and genotypes. This procedure should prove applicable as well to analysis of the primary cellular target of other genes affecting the peripheral nervous system (see also reference 8).

We thank Paul G. Harcourt and Wolfgang Busse for their technical assistance and Dr. Mitchael Estridge for his helpful suggestions.

This research was supported by funds from the Jeane B. Kempner, Charles King, and Dysautonomia Foundations, National Institutes of Health grants NS14768 and NS11237, and Institutional Core grant HD06276.

A portion of the work reported here was presented at the 12th Annual Meeting of the Society for Neuroscience, Minneapolis, Minnesota, 31 October, 1982.

Received for publication 29 August 1983, and in revised form 19 December 1983.

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