Transfection of Neonatal Rat Schwann Cells with SV-40 Large T Antigen Gene under Control of the Metallothionein Promoter

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Abstract. Secondary cultures of Schwann cells were transfected with a plasmid containing the SV-40 T antigen gene expressed under the control of the mouse metallothionein-I promoter. We used the calcium phosphate method for transfection and obtained a transfection efficiency of 0.01%. The colonies were cloned by limited dilution, and these cloned cell lines were carried in medium containing zinc chloride (100 μM). One cloned cell line, which has now been carried for 180 doublings, appears to have a transformed phenotype with a doubling time of 20 h. These cells express SV-40 T antigen while maintaining established Schwann cell properties (positive staining for 217c, Ran-2, A5E3, glial fibrillary acidic protein, presence of 2',3'-cyclic nucleotide phosphohydrolase [CNPase] activity, and the ability to synthesize sulfogalactosylceramide and mRNA for the myelin protein, P_0). Removal of zinc chloride from the medium resulted in reduced expression of T antigen and a change in the appearance of the cells to a more bipolar shape, although they still did not exhibit contact inhibition and maintained a doubling time of 20 h. These cells now became Ran-2-negative and showed increases in CNPase activity and in their ability to synthesize sulfogalactosylceramide. The amount of P_0 mRNA remained unchanged. Transfected Schwann cells, however, stopped dividing when they contacted either basal lamina or neurites and became bipolar in appearance. The Schwann cells in contact with the neurites then extended processes to wrap around bundles of neurites. Transfection with the SV-40 T antigen gene therefore provides a method for obtaining Schwann cell lines that continue to express properties associated with untransfected cells in culture and may be used to study axon–Schwann cell interaction.

THE availability of Schwann cell lines from the peripheral nervous system would be an invaluable aid for analyzing normal biological events as well as for investigating mechanisms of human disease. Our interest in developing an established line of Schwann cells stems from studies of Schwann cell–axon interactions and myelinogenesis in the peripheral nervous system. Schwann cells arise from neural crest cells (24) and envelop both myelinated and unmyelinated nerves. Previous studies have shown that although all Schwann cells can produce myelin, only a certain proportion do, and this expression is regulated in an undetermined manner by the axon (1, 10, 61). The two types of Schwann cells, one group surrounding unmyelinated axons and a second group of cells surrounding myelinated axons, can be distinguished by immunological and biochemical markers (6, 8, 17, 19, 26, 35, 36, 45, 46).

Schwann cells can be isolated from neonatal rat sciatic nerves and maintained in culture. Primary cultures of Schwann cells express several markers (including galactocerebroside and P_0 protein) that are consistent with myelin-forming Schwann cells (6, 8, 35, 46). Maintenance of these cells in culture results in loss of P_0 and galactocerebroside expression, however, and the assumption of some new markers (A5E3, glial fibrillary acidic protein [GFAP],[1] and Ran-2) known to be present in Schwann cells surrounding unmyelinated axons (6, 8, 17, 18, 35, 36). Furthermore, Schwann cells maintained in culture undergo degenerative changes after ~60 doublings, and spontaneous immortalization events in these cells are extremely rare.

In an effort to derive continuous cell lines of rat Schwann cells, we attempted to immortalize these cells with the SV-40 T antigen gene. This gene can transform both primary and established lines in culture (9, 27, 34, 40, 53, 56; for a review see reference 57) as well as immortalize primary cells (2, 42). Since transformation of cells often results in de-differentiation, it is important to be able to regulate the expression of large T antigen. For this reason, the SV-40 T antigen gene was placed under the control of the heavy metal inducible mouse metallothionein-I (MT-I) promoter (5, 15, 51, 52).

1. *Abbreviations used in this paper*: CNPase, 2'-3'-cyclic nucleotide phosphohydrolase; GFAP, glial fibrillary acidic protein, MT-I, metallothionein-I.

The present study was designed to determine whether we could obtain stable transformants of Schwann cells and, if so, to compare the growth and differentiation properties of these cells with those of secondary (untransfected) Schwann cells, including the interaction with basal lamina and neurites. In addition, we wished to investigate the relationship between the amount of large T antigen, the growth of Schwann cells, and the expression of Schwann cell properties.

Materials and Methods

Preparation of Schwann Cells

Neonatal Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) were killed by decapitation. The sciatic nerves were dissected and pooled, the nerves were washed once, then incubated for 20 min at 37°C with 0.1% collagenase (Type CLS III; Worthington Biochemical Corp., Freehold, NJ). At the end of this incubation, 1.0 ml of 0.1% trypsin (Worthington Type TRL3), 0.005% DNase I (Worthington Type D) was added, and the nerves were further digested at 37°C for 30 min. Then 2.0 ml of 0.005% trypsin inhibitor (Worthington Type SI) and 0.004% DNase I (Worthington Type D) were added. The nerves were dissociated by three cycles of trituration through a 21-gauge needle and three further cycles of trituration through a 23-gauge needle. The resulting cell suspension was passed through 110-μm pore nylon mesh. The cells were suspended in DME (Microbiological Research Corp., Bountiful, UT) containing 10% FBS. Upon isolation, 95% of the cells excluded trypan blue, and the usual yield was 4×10^6 cells from 20 sciatic nerves. By immunofluorescence criterion (Ran-1 and anti-Thy 1.1 [6]), 90% of these cells were Schwann cells and the remaining 10% were fibroblasts. The rapidly dividing fibroblasts were removed by incubating with cytosine arabinoside (10^{-5} M) for 48 h, followed by treatment with anti-Thy 1.1 (monoclonal IgM from New England Nuclear, Boston, MA) at a 1:100 dilution in combination with fresh complement (Cappel Laboratories, Cochranville, PA). Since Thy 1.1 is a surface antigen of fibroblasts, this complement-mediated killing step resulted in removal of the remaining fibroblasts (7, 39).

Transfection of Secondary Schwann Cells

Before transfection of secondary Schwann cells, the ability of these cells to withstand zinc and cadmium chloride was tested. The medium in all cases contained 100 nM dexamethasone. The zinc concentration was varied from 2 to 200 μM, and the cadmium concentration from 0 to 40 μM. Toxicity was estimated by the morphological appearance of the cells as well as [^{3}H]thymidine (New England Nuclear) incorporation into DNA. Cadmium was toxic at levels as low as 0.5 μM, whereas zinc was well tolerated up to 100 μM; at 200 μM zinc also was toxic.

Transfection of pMtSV.neo, 0.5 μg/dish, was performed by using the calcium phosphate procedure (23, 25, 60) and incorporating a 15% glycerol shock (22). Transfections were carried out on 6- or 10-cm plastic dishes each containing 5×10^5 Schwann cells. 3 h before transfection the medium was changed from DME containing 10% FBS to DME containing 20% FBS. After the glycerol shock, the medium was changed to DME containing 10% FBS and 100 μM zinc chloride. The following day the cells were trypsinized and passed in the same medium. After 1 wk in culture, the cells were grown in medium that contained 200 μg/ml of G418 (Geneticin sulfate; Gibco, Grand Island, NY). The medium containing G418 was changed every 3 d and the cells were exposed to G418 for 3 wk. Routinely we obtained single plates having 50 colonies of cells, giving a transfection efficiency of 0.01%. Individual colonies were removed with a sterile cotton swab previously moistened with tissue culture medium. Cells were shaken off the swab into culture tubes containing 2 ml of medium (DME + 10% FBS with 100 μM $ZnCl_2$). The tubes were centrifuged, and the cell pellet was resuspended and placed into single wells of a 24-well plate (Costar, Cambridge, MA). When the cells reached confluence they were passaged and eventually expanded into T25 and then in T75 flasks. From these expanded cultures the cells were cloned by limited dilution. For the present study, clones growing in wells with a theoretical distribution to 0.5 cell/well were expanded and recloned by limited dilution before characterization.

The plasmid pMtSV.neo contains the SV-40 T antigen gene (nucleotides 5171 to 2533; reference 57) under the control of the mouse MT-I promoter derived from a plasmid designated pMK (5) and also carries resistance to the aminoglycoside antibiotic G418. Transfected Schwann cells thus could be selected by using G418 at a concentration of 200 μg/ml (14).

Indirect Immunofluorescence

Monoclonal antibody Thy 1.1. (New England Nuclear) was used at a dilution of 1:100. Monoclonal anti-217c (41) was obtained in the form of hybridoma supernatant fraction from Dr. K. L. Fields, Albert Einstein School of Medicine and used at a dilution of 1:50. Monoclonals anti-A5E3 and anti-Ran-2 were obtained in the form of hybridoma supernatant fractions from Dr. R. Mirsky, University College, London and used at dilutions of 1:5 and 1:1, respectively. Ran-1 antiserum was obtained from Dr. M. Raff, University College, London, and used at a dilution of 1:100. A rabbit polyclonal anti-P_0 antibody was obtained from Dr. B. Trapp, Johns Hopkins School of Medicine, and used at a dilution of 1:100. Monoclonal anti-S-100 was obtained in the form of hybridoma supernatant fraction from Dr. Y. Gillespie, University of North Carolina, Chapel Hill and was used at a dilution of 1:2. Polyclonal anti-glial fibrillary acidic protein (GFAP) was obtained from Dr. L. Eng, Stanford University School of Medicine and used at a dilution of 1:100. Anti-2',3'-cyclic nucleotide phosphohydrolase (CNPase) was obtained in the form of hybridoma supernatant fractions from Dr. T. J. Sprinkle, University of Georgia, Augusta and used at a dilution of 1:20. Polyclonal hamster anti-large T was obtained from the National Cancer Institute and used at a dilution of 1:50. Fluorescein-conjugated anti-immunoglobulins were obtained from Cappel Laboratories and used at dilutions of 1:20 and 1:40.

Cells were grown on 35-mm dishes and were processed with or without fixative. Cells stained with Thy 1.1 and 217c were fixed in 1% formaldehyde in PBS for 20 min at room temperature. Cells stained with Ran-2, A5E3, CNPase, and GFAP were treated with 1% paraformaldehyde in PBS at room temperature for 20 min, followed by treatment with 5% acetic acid in ethanol for 10 min at −20°C. Cells stained with S-100 were fixed in 3.5% formaldehyde for 20 min at room temperature. Cells stained with anti-large T antigen were treated for 10 min with 5% acetic acid in ethanol at −20°C for 10 min (39). Small circular areas were delineated by use of vacuum grease and these areas were stained in a two-step indirect immunofluorescence procedure as described by Raff et al. (45).

Growth Curves

After plating of cells in 35-mm dishes, at appropriate times the cells were trypsinized for determination of cell number. After completely dissociating the cells by gentle trituration, the cell suspensions were diluted to 10 ml with isotonic saline and counted on a Coulter counter (model 2B1; Coulter Electronics, Hialeah, FL). Cell densities are reported as cell number per 35-mm dish and represent mean values from triplicate plates.

DNA synthesis was measured by [^{3}H]thymidine incorporation into TCA-precipitable counts. Transfected Schwann cells (5×10^4) were plated on plastic or basal lamina (matrigel from Collaborative Research Inc., Waltham, MA) and 0.5 μCi/ml of thymidine was added for various times before harvesting cells. The cells were then lysed and the DNA was precipitated with TCA. After collecting the precipitate on glass fiber filters (GF/C; Whatman Inc., Clifton, NJ), the filters were washed and the radioactivity on the filters was counted by liquid scintillation spectrometry.

Isotope Labeling and Product Identification

Earlier studies demonstrated that the free sulfate concentration in DME had no effect on [^{35}S]sulfate (up to 150 μM) labeling into sulfatide by secondary Schwann cells. The FCS used, however, was dialyzed to remove amino acids and free sulfate. Control and transfected cells were grown to 90% confluence in 10-cm dishes. For each experiment three 10-cm dishes were used and the experiment was repeated on two separate occasions. The values from each experiment were averaged. The Schwann cells were incubated with 200 μCi of sodium [^{35}S]sulfate in DME + 10% FBS for 8 h. At the end of the incubation period the medium was removed, and the cells were washed twice with PBS. The cells were scraped into 1.0 ml of PBS and homogenized with use of a ground-glass homogenizer. The lipids were extracted with chloroform/methanol (2:1 vol/vol) and then partitioned according to the method of Folch et al. (21). The lipids were analyzed further by thin layer chromatography (54). In secondary Schwann cells 95% of the sulfate counts on the thin layer plate migrated as sulfatide, whereas with transfected Schwann cells grown with or without added zinc, 60% of the counts migrated as sulfatide and 30% migrated above the sulfatide band.

For determining protein synthesis (28, 30), the Schwann cells were changed to methionine-free DME medium containing 10% dialyzed FCS. After 1 h [^{35}S]methionine (50 μCi/ml) was added, and the mixture was incubated for 6 h. The cells were washed, harvested by scraping, and then homogenized in 1.0 ml of buffer (containing 0.05% SDS, 0.05% NP-40, and 2 mM methionine). To 20 μl of this homogenate, 40 μl of 10% protein A-Sepharose in NETS were added (NETS is composed of 150 mM sodium chloride, 5 mM EDTA, 20 mM Tris, pH 7.5, 2 mM methionine, 0.05% SDS, 0.05% NP-40, 0.1% mM phenylmethylsulfonyl fluoride, and 0.02% sodium azide), and the mixture was incubated at 4°C for 4 h and then centrifuged (microfuge). To the supernatant fraction 2 μl of anti-large T antibody plus an additional 50 μl of 10% protein A-Sepharose in NETS were added. The samples were incubated overnight at 4°C, centrifuged, and washed four times with NETS. To the protein A-Sepharose complex Fairbanks buffer (16) was added and the samples were placed in boiling water for 3 min. Samples containing equal numbers of counts were added to each lane of a 5–17% SDS-polyacrylamide gradient gel and subjected to electrophoresis. After the tracking dye had reached the bottom of the gel, the gels were stained, destained, and then soaked for 30 min in ENHANCE (New England Nuclear) before drying and autoradiography. The autoradiograms were scanned by use of a fiberoptic scanning densitometer (Kontes Co., Vineland, NJ), and the integrated area was determined by an integrater (model 3390 A; Hewlett-Packard Co., Palo Alto, CA).

Immunodetection of Myelin P_0 Protein

Cell extracts from transfected Schwann cells were analyzed by 10% SDS-polyacrylamide gels and the proteins then transferred to nitrocellulose paper (58). Myelin P_0 was detected by using a polyclonal rabbit anti-P_0 protein (1:100) followed by biotinylated goat anti-rabbit and horseradish-coupled avidin. Peroxidase activity was detected with 3,3′-diaminobenzidine tetrachloride (20).

Ganglioside Determination

The cells were homogenized, the total lipids were extracted with chloroform-methanol (1:4 vol/vol), and the different lipid classes were separated by use of a Unisil column (Clarkson Chemical Co., Williamsport, PA). The partially purified ganglioside fraction was treated with 2,4-dinitrophenylhydrazine and then analyzed by HPLC as described by Miyazaki et al. (37).

2′,3′-Cyclic Nucleotide Phosphohydrolase Activity

Schwann cells were collected by centrifugation after treatment with trypsin, homogenized in 10 mM Tris, pH 7.4. CNPase was assayed according to the method of Sogin (49), except that the final concentration of protein after activation was 400 μg/ml.

Preparation of Total Cellular RNA

Total cellular RNA was isolated from five confluent 10-cm dishes by the procedure described by Chirgwin et al. (13).

Northern and Dot Blot Analyses

The cDNA clone PSN63c (29) containing the coding sequence of P_0 mRNa was obtained from Dr. G. Lemke (Salk Institute). The coding sequences were transferred as an EcoRI fragment to pGEM-2 (Promega Biotec, Madison, WI). This plasmid and SV-40 DNA, and pMtSV.neo DNA plasmid were labeled with [^{32}P]dCTP by nick translation (47) to specific activities of 1×10^8 cpm/μg of DNA. DNA was separated from labeled dCTP by use of a Sephadex G-50 column or by ethanol precipitation of the DNA in 2.5 M ammonium acetate.

For Northern analysis (55), total cellular RNA (5–10 μg) was denatured with glyoxal by heating at 50°C for 1 h, subjected to electrophoresis on 1% agarose gel, and transferred in 20× SSC (3 M sodium chloride, 0.3 M sodium citrate) to Genescreen (New England Nuclear). The filters were air-dried and then baked at 80°C for 90 min. The remaining glyoxal was removed by boiling in 50 mM Tris-HCl, pH 8.0, for 5 min. The filters were prehybridized with 50% formamide, 10× Denhardt's (0.2% Ficoll, 0.2% polyvinylpyrrolidone, and 0.2% BSA) solution, 1 M NaCl, 1% SDS, 0.05 M Tris (pH 7.5), 10% dextran sulfate, and denatured salmon sperm DNA (10 μg/ml) at 42°C for 6 h. The denatured radioactive probes (10^6 cpm/ml) were added and hybridized at 42°C for 48 h. The filters were extensively washed according to a standard protocol (33), and the final washes were done at 50°C with 0.1× SSC and 0.1% SDS for 30 min.

For dot blot analysis, the method described by White and Bancroft (62) was used. Serial dilutions were made in 20× SSC, starting with 10 μg of RNA denatured with formaldehyde. The total amount of RNA was kept constant by adding calf thymus tRNA. The filters were handled as described for Northern analysis. All the autoradiograms were scanned using a Kontes Co. fiberoptic scanning densitometer and the integrated area was computed by a Hewlett-Packard Co. integrater.

Southern Analysis

DNA was isolated from secondary untransfected and transfected Schwann cells as described (32). The DNA (10 μg) from Schwann cells was digested with BamHI, EcoRI, HindIII, XbaI, and BglII, followed by electrophoresis on a 1% agarose gel. The DNA was photographed under ultraviolet light after staining with ethidium bromide, transferred to Genescreen according to Southern (50), and hybridized with ^{32}P-labeled SV-40 DNA and ^{32}P-labeled pMtSV.neo.

Dorsal Root Ganglion Cultures

Dorsal root ganglion cultures were prepared according to the method of Salzer et al. (48). The ganglia were removed from 15-d-old rat fetuses, dissociated by incubating with 0.25% trypsin for 45 min, and plated on ammoniated rat-tail collagen-coated 35-mm plastic dishes at a density of two ganglia per dish. Plating medium consisted of MEM containing 10% FBS with additional 0.3% glucose and 50 ng/ml of crude nerve growth factor. After 1 d, antimitotic medium (plating medium containing 10^{-5} M uridine and fluorodeoxyuridine) was added. Plating medium and antimitotic medium were alternated at 2-d intervals for 12 d at which time the cultures

Table I. Indirect Immunofluorescence Staining

Antibody	Site of staining	Untransfected secondary Schwann cells	Transfected Schwann cells	
			With 100 μM zinc chloride	No added zinc
217c*	Plasma membrane	+	+	+
Ran-1*	Plasma membrane	+	+	+
Anti-S-100	Cytoplasmic	+	+	+
Anti-GFAP	Cytoplasmic	+	+	+
Anti-CNPase	Cytoplasmic	+	+	+
Ran-2	Plasma membrane	−	+	−
A5E3	Plasma membrane	+	+	+
Anti-SV-40 large T	Nuclear	−	+	−
Anti-Thy 1.1	Plasma membrane	−	−	−
Anti-P_0	Plasma membrane	+	+	+
Anti-galacto-cerebroside	Plasma membrane	−	−	−

*Study by Fields and Dammerman (17) demonstrated that Ran-1 and 217c antisera had identical staining patterns, suggesting that they recognized the same antigen.

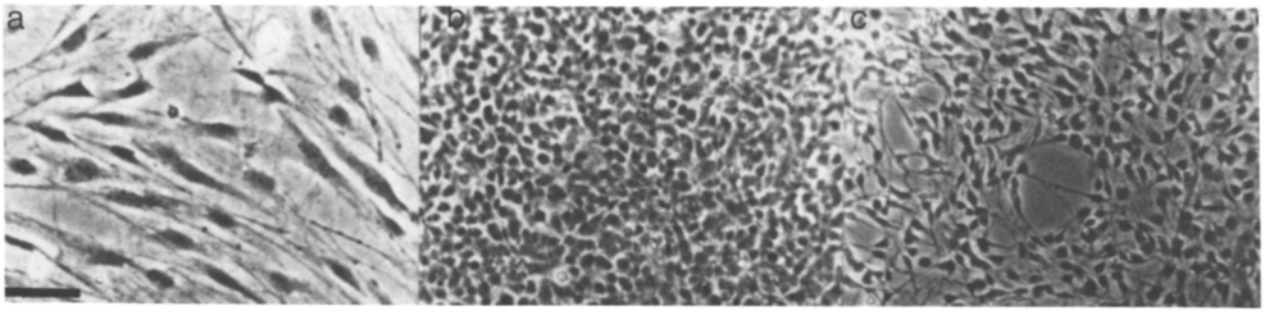

Figure 1. Light micrograph of rat Schwann cells. (*a*) Secondary Schwann cells were plated on plastic dishes and grown in DME containing 10% FBS. (*b*) Transfected secondary Schwann cells with SV-40 large T grown in the presence of 100 μM zinc. These cells have a transformed phenotype. (*c*) Transfected secondary Schwann cells grown in the absence of added zinc. These cells are more spindle-shaped and are contact-inhibited until they reach confluence, after which contact inhibition is lost. Bar, 50 μm.

were free of non-neuronal cells. After an additional 2 wk in plating medium, the dorsal root ganglion neurons had elaborated neuritic halos extending to the edge of the culture dish.

Transfected Schwann cells were suspended in plating medium and seeded onto the neuronal cultures at a density of 500–2,000 cells per dish. The cocultures were maintained for 2 wk in plating medium, after which time they were fixed with 1% glutaraldehyde in 100 mM phosphate buffer containing 0.12 M sucrose and 0.36 mM $CaCl_2$, post-fixed with osmium tetroxide, dehydrated, and embedded in epoxy resin. The polymerized resin was separated from the plastic dish by immersion in boiling water for 1 min. Areas of the culture 10–12 mm from the central mass of neurons were selected for examination by transmission electron microscopy. This effectively eliminated any endogenous (untransfected) Schwann cells that might have survived treatment with the antimitotic agent.

Protein Determination

Protein was measured by the microassay of Bradford in the presence of 0.1 N NaOH, with gamma-globulin as the standard (3).

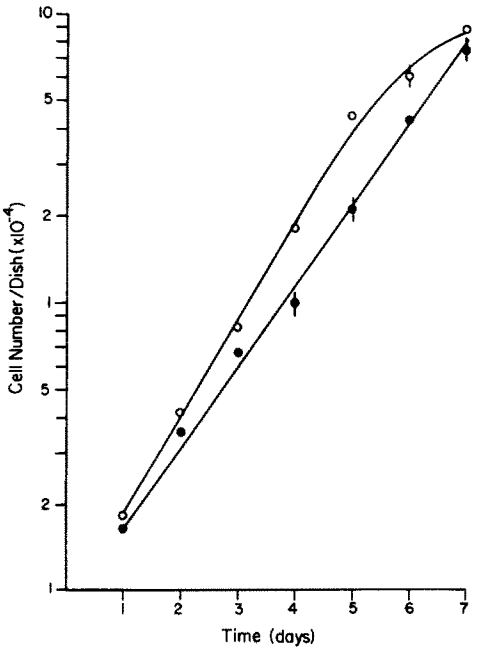

Figure 2. Growth curves for transfected Schwann cells. When transfected Schwann cells were grown in the presence of 100 μM zinc (●) or the absence of added zinc (○), the cell doubling time was ~20 h.

Results

Secondary Rat Schwann Cells

Neonatal rat Schwann cells were isolated by a modification of the method of Brockes et al. (6, 34). With the use of cytosine arabinoside (10^{-5} M) and anti-Thy 1.1 complement killing, pure cultures (99%) of Schwann cells were obtained. Purity was determined by absence of immunofluorescent staining with anti-Thy 1.1 antibody and the presence of staining with anti-Ran-1 (Table I). Previous studies (39) demonstrated that, when first isolated, the Schwann cells were predominantly myelin-forming, as indicated by anti-galactocerebroside and anti-P_0 positive immunofluorescence, but after maintenance in culture for two passages the cells lost these staining properties and began to express markers for non-myelin forming Schwann cells, i.e., Ran-2, A5E3, and GFAP (Table I). The cells also stained with antisera that are known to react with both myelin forming and non-myelin forming Schwann cells (Ran-1, 217c, S-100, and cyclic nucleo-

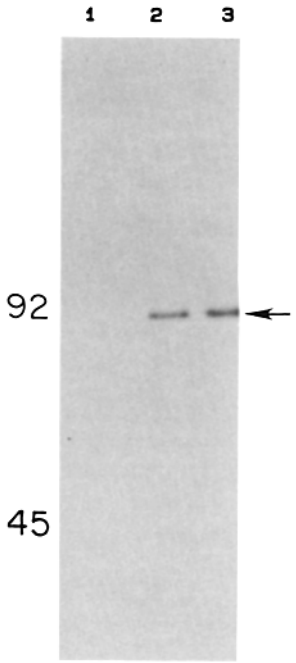

Figure 3. Expression of large T antigen in Schwann cells. Secondary Schwann cells and transfected Schwann cells were labeled with [^{35}S]methionine and the labeled proteins were extracted and precipitated with anti-large T antibody. The precipitate was then analyzed by polyacrylamide gel electrophoresis. Molecular weight markers are given on the left side of the figure. Lane *1*, secondary Schwann cells; lane *2*, transfected Schwann cells grown in the absence of zinc; and lane *3*, transfected Schwann cells grown in the presence of 100 μM zinc. The arrow indicates the location of large T antigen.

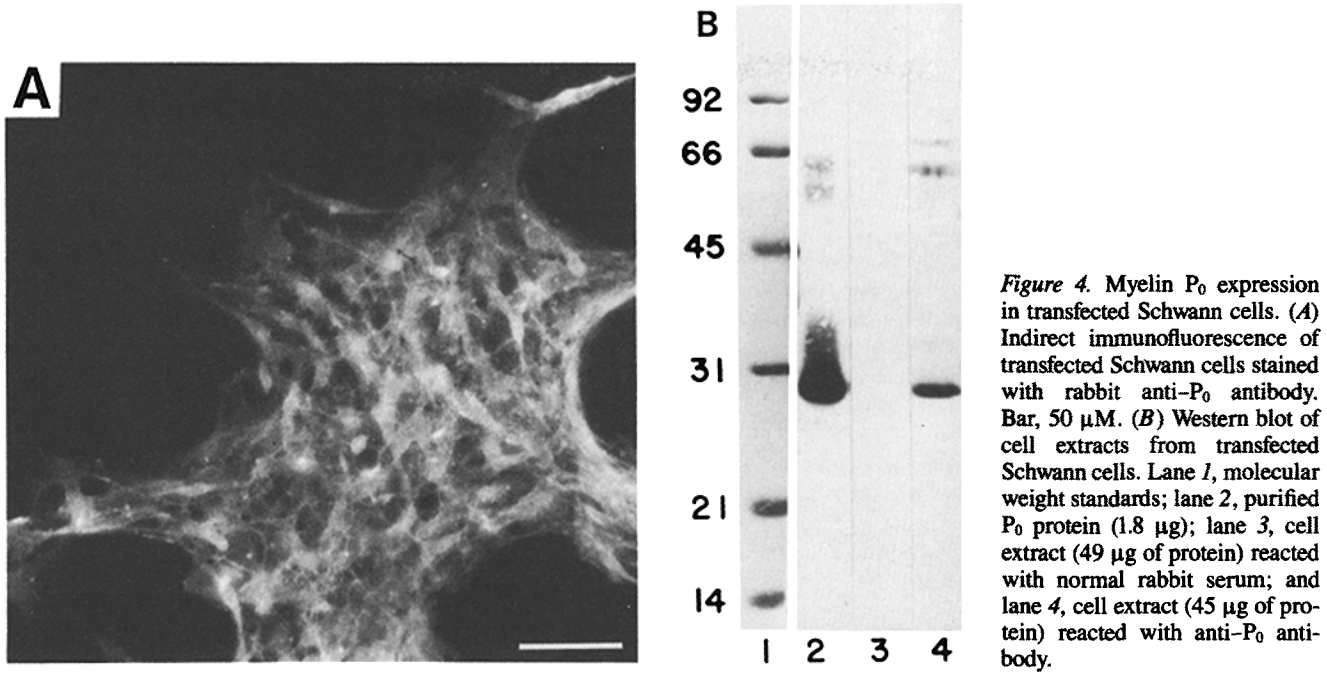

Figure 4. Myelin P_0 expression in transfected Schwann cells. (*A*) Indirect immunofluorescence of transfected Schwann cells stained with rabbit anti-P_0 antibody. Bar, 50 μM. (*B*) Western blot of cell extracts from transfected Schwann cells. Lane *1*, molecular weight standards; lane *2*, purified P_0 protein (1.8 μg); lane *3*, cell extract (49 μg of protein) reacted with normal rabbit serum; and lane *4*, cell extract (45 μg of protein) reacted with anti-P_0 antibody.

tide phosphohydrolase). Biochemically, secondary Schwann cells synthesized myelin-specific neutral glycolipids (galactocerebroside and sulfatide) and myelin proteins, in particular P_0 (data not shown).

Since it was possible that the presence of zinc in the medium could alter the properties of secondary Schwann cells, these cells were examined in medium containing 10% FBS and increasing quantities of zinc or cadmium. Although cadmium was lethal at a level of 0.5–20 μM, neither the growth properties of the Schwann cells nor their biochemical and immunological characteristics were affected by zinc concentrations from 0 to 100 μM (see Properties of Transfected Schwann Cells). If these same cells were maintained in serum-free medium, however, they were sensitive to zinc at concentrations above 20 μM.

Properties of Transfected Schwann Cells

A number of properties of the transfected Schwann cells were investigated. For these studies cells cloned twice by limited dilution were used.

Morphological Properties. Primary Schwann cells were bipolar in appearance, but the cells assumed a flattened morphology when passaged in media containing cholera toxin. However, the secondary Schwann cells (Fig. 1 *a*) were contact-inhibited and on reaching confluence the cells arranged themselves such that the nuclei were aligned in rows resembling palisades, which is characteristic of Schwann cells. None of these morphological features was altered by 100 μM zinc chloride. Transfected Schwann cells grown with zinc in the medium assumed a characteristic transformed phenotype (Fig. 1 *b*): the cells grew in small colonies, were not contact-inhibited, and had a pleomorphic appearance. The cells also appeared smaller and were more tightly packed together. Upon removal of zinc from the medium the cells regained their bipolar appearance after three passages (Fig. 1 *c*), but did not align themselves in palisades. Furthermore, when these cells reached confluence, they tended to grow over each other.

Growth Properties. Studies by Brockes et al. (7, 43, 44) had shown that neonatal rat Schwann cells have a doubling time of 7 d. When these secondary Schwann cells are grown with known Schwann cell mitogens, such as cholera toxin (1 μg/ml), the doubling time is reduced to 48 h (43, 44). Transfected Schwann cells in the absence of cholera toxin, but with medium containing zinc, had a doubling time of 22 h (Fig. 2). Removal of zinc from the medium had no apparent effect on the rate of cell doubling (doubling time 20 h). To eliminate the possible contribution to the zinc concentration by both serum and insulin, which are known to contain low concentrations of this ion, the growth properties of the transfected Schwann cells were determined in a serum-free medium with zinc-free insulin. The growth properties remained unchanged. There are several possible reasons for the decreased generation time of the transfected Schwann cells. The transfected Schwann cells may secrete factors that are mitogenic for Schwann cells. This seems unlikely since conditioned medium from transfected Schwann cells had no effect on untransfected secondary Schwann cells. Alternatively, continued expression of T antigen could be responsible for the shortened cell cycle. A third possibility is that this effect could have arisen through cellular genetic changes that occurred as a result of the type of selection used.

Secondary Schwann cells can be maintained in culture for about 60 doublings before they show senescence. Transfected Schwann cells grown in the presence of zinc have now been maintained for 180 doublings. Morphologically and biochemically, such cells appear similar to transfected Schwann cells at early passage.

Expression of Large T Antigen. Transfected rat Schwann cells grown in medium containing zinc expressed large T antigen predominantly in the nucleus as ascertained by indirect immunofluorescence (see Fig. 6 *B*). Preimmune sera showed no staining of cells. When zinc was removed from

the medium, no nuclear staining was detectable. Nevertheless, if the cells were pulse-labeled with [^{35}S]methionine in medium with and without zinc and then immunoprecipitated with anti-large T antibody, a 90-kD protein was detected (Fig. 3) under both conditions; untransfected Schwann cells treated in an identical manner showed no bands. The cells grown without zinc contained 54% of the amount of large T antigen found in cells grown with zinc. In parallel studies using Northern blots containing total RNA from cells hybridized with SV-40 DNA, we found that the total RNA was reduced to ~34% of the value found in cells grown in the presence of zinc (data not shown). From these results it is clear that even when zinc was eliminated from the medium there was continued, albeit decreased, expression of large T antigen.

In an attempt to estimate the number of copies of the plasmid DNA integrated into the cell line, DNA was isolated from transfected Schwann cells and digested with HindIII, EcoRI, BamHI, BglII, and XbaI restriction endonucleases. The digests were analyzed by Southern hybridization (data not shown) with a ^{32}P-labeled MT-I promoter fragment as probe. The signal was about fivefold higher in the transfected cells than that in untransfected Schwann cells, suggesting that at least five copies of the plasmid DNA had been integrated into the genome.

Expression of Schwann Cell Properties in the Transfected Cells. Indirect immunofluorescence showed that both transfected and untransfected Schwann cells (Table I) stained positively for Ran-1, 217c, S-100, and CNPase, markers specific for all Schwann cells (17–19, 26, 35). The transfected Schwann cells also stained positively for Ran-2, A5E3, and GFAP, which are markers for non-myelin forming Schwann cells (18, 26, 37). This staining profile is different from that of secondary Schwann cells in culture, which are Ran-2-negative but are positive for A5E3 and GFAP. The GFAP-like immunoreactivity could well result from cross-reaction with another intermediate filament. By two-dimensional gel analysis, we have detected the presence of vimentin in secondary Schwann cells, and a preliminary study indicated staining of transfected Schwann cells with anti-vimentin antibody. Markers used to identify myelin-forming Schwann cells, such as anti-P_0 antibody, demonstrated positive staining in all cells (Fig. 4, *A* and *B*). When the immunofluorescence staining patterns of transfected Schwann cells grown without zinc in the medium were compared with those of similar cultures grown in the presence of zinc, the only differences were a disappearance of nuclear staining with anti-large T antigen and the lack of Ran-2 staining in the cells grown in medium without zinc, consistent with a shift to a more differentiated state.

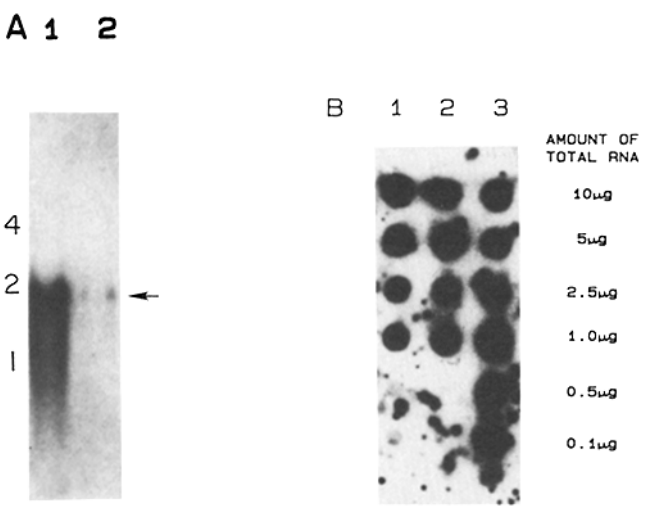

Figure 5. Expression of P_0 mRNA in Schwann cells. (*A*) The hybridization pattern of ^{32}P-labeled EcoRI fragment of pSN63c transferred to pGEM-2 incubated against 10 μg of total RNA per lane. Lane *1*, secondary Schwann cells; lane *2*, transfected Schwann cells. The size markers are given on the left and the arrow indicates the location of P_0 mRNA. (*B*) Dot blot analysis using the same probe. The initial concentration of total RNA was 10 μg. Subsequent wells contained serial dilutions of the starting material and the amount of total RNA per well is indicated. All the dilutions contained 10 μg of yeast tRNA in addition. Lane *1*, transfected Schwann cells grown in the presence of 100 μM zinc; lane *2*, transfected Schwann cells without added zinc; and lane *3*, RNA isolated from sciatic nerves of 7-d-old rat pups.

Biochemical studies confirmed that transfected Schwann cells changed some of their properties when zinc was removed from the medium. There was an increase in CNPase activity (Table II) with values similar to those noted for secondary (untransfected) Schwann cells. Furthermore, in the presence of zinc, transfected Schwann cells incorporated [^{35}S]sulfate into sulfatide, although at ~10% of the level noted for secondary Schwann cells. This level of incorporation increased by fivefold when zinc was removed from the medium (Table II). For secondary Schwann cells 95% of the ^{35}S-labeled counts in the organic phase of chloroform/methanol extraction migrated as sulfatide on TLC plates, whereas

Table II. Markers of Schwann Cell Function

Schwann cells	Ganglioside GM3	CNPase	[^{35}S]Sulfate incorporation into sulfogalactosylceramide
	nmol sialic acid/mg protein	*mol/mg protein/h*	*dpm/mg protein*
Secondary	3.7	10–13	2,990 ± 500
Transfected (plus Zn^{2+})	4.8	3.13 ± 1.15	280 ± 50
Transfected (minus Zn^{2+})	4.1	7.58 ± 1.77	1,200 ± 100

Schwann cells were cultured and transfected as described under Materials and Methods. The amounts of ganglioside and sulfatide and the activity of CNPase were measured as described under Materials and Methods.

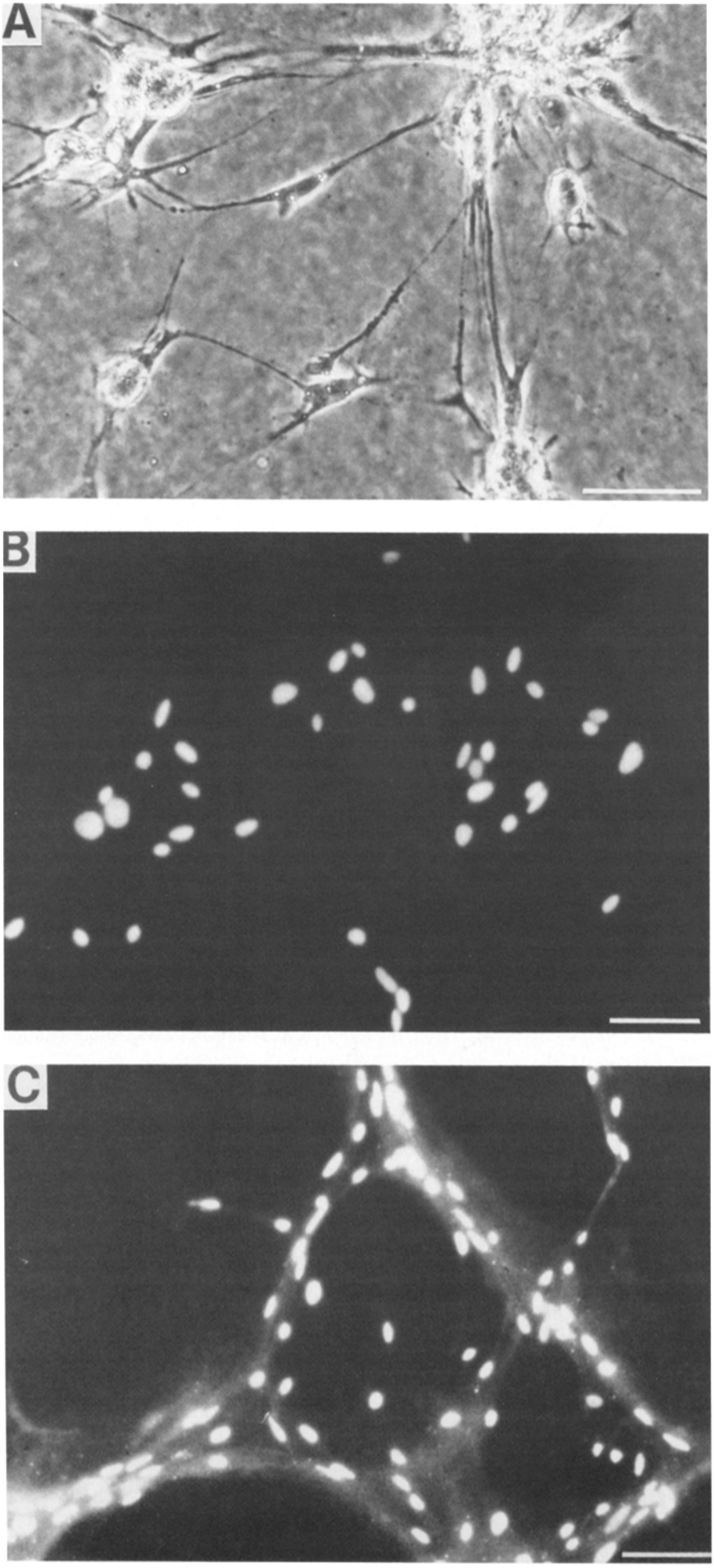

Figure 6. Light micrographs of transfected Schwann cells. (*A*) Transfected Schwann cells plated on basal lamina; untransfected cells had a similar appearance; (*B*) indirect immunofluorescence with anti-large T antibody of transfected Schwann cells plated on plastic showing the nuclear stain; and (*C*) indirect immunofluorescence with anti-large T antibody on transfected Schwann cells plated onto dishes coated with basal lamina. Bar, 50 μm.

for transfected cells only 60% of the organic phase counts migrated as sulfatide, with 30% migrating just above the sulfatide spot. We were unable to identify the latter compound; the band did not correspond to cholesterol sulfate or psychosine sulfate based on the Rf values. Finally, the amount of the major ganglioside synthesized by cultured secondary Schwann cells, N-acetyl GM3, decreased when zinc was removed from the medium of transfected Schwann cells (Table II).

One possibility for the alterations of enzyme activity, resulting in changes in amount of product, was that zinc had a direct effect on the enzyme proteins. To exclude this possibility, the effect of zinc (10 μM to 1 mM) on CNPase and cerebroside sulfotransferase activity was determined in whole brain homogenates, as the activity is much higher in brain than it is in Schwann cells. At 100 μM zinc, CNPase activity was increased by 30% above control and cerebroside sulfotransferase activity was unchanged (data not shown).

These results suggest that the biochemical changes noted are unlikely to be due to a direct action of zinc, but rather appear to be an indication that transfected cells assume properties more closely resembling secondary Schwann cells. In contrast to the changes in glycolipid metabolism, the amount of P_0 mRNA did not change when zinc was omitted from the medium. Both secondary and transfected Schwann cells contained P_0 mRNA of identical size (Fig. 5 *A*), although the amount was 10-fold lower in transfected Schwann cells. Dot blot analysis (Fig. 5 *B*) indicated no change in amount of P_0 mRNA when zinc was removed from the medium, nor was there a change in P_0 protein content as estimated by indirect immunofluorescence and by immunoblots.

Interaction of Schwann Cells with Basal Lamina and Neurites. Transfected Schwann cells grew with a doubling time of 20 h, irrespective of the amount of zinc in the medium (Fig. 2), possibly because of "basal" expression of SV-40 large T antigen. Since interaction with basal lamina is critical in the course of normal development of the Schwann cell (11, 12), we examined the effect of basal lamina synthesized by Englebreth-Holm swarm sarcoma cells on Schwann cell behavior. When plated on basal lamina, both untransfected secondary Schwann cells and transfected cells underwent rapid alterations in their cell morphology. Both types of cells became bipolar (Fig. 6 *A*) and assumed the appearance of the primary Schwann cell in culture. Moreover, the amount of [^{3}H]thymidine incorporated into DNA was dramatically reduced in transfected Schwann cells (Fig. 7). Over 24–48 h, however, the transfected Schwann cells degraded the basal lamina and then began to grow at their original rate. Although it was possible that the basal lamina was turning off T antigen expression and thus stopping transfected Schwann cells from replicating, indirect immunofluorescence staining revealed that transfected Schwann cells grown on basal lamina in the presence of zinc chloride continued to express T antigen (Fig. 6 *C*) in nuclei. Neither fibronectin nor laminin alone, nor extracellular matrix made by bovine aortic endothelial cells had the same effects as basal lamina.

The interaction of transformed Schwann cells with neurites was studied with dorsal root ganglion neurons. Dissociated neurons rapidly coalesce into a central mass from which neurites extended radially up to 15 mm (Fig. 8 *A*). When transfected Schwann cells were added, they distributed themselves throughout the culture but differed in morphology depending on their association with neurites. In neurite-free zones the cells grew as confluent epithelioid sheets. When in contact with neurites, the cells became more fusiform and sent out processes that ensheathed groups of neurites both close to the cell body and at a distance from it (Fig. 8, *B* and *C*). In addition, the slender processes subdivided and segregated some of the bundles of neurites (Fig. 8 *D*). The degree of ensheathment varied in different groups of neurites. Although there was Schwann cell–neurite association we were unable to visualize basal lamina formation. Moreover, the cells neither established a one-to-one relationship with neurites nor formed myelin.

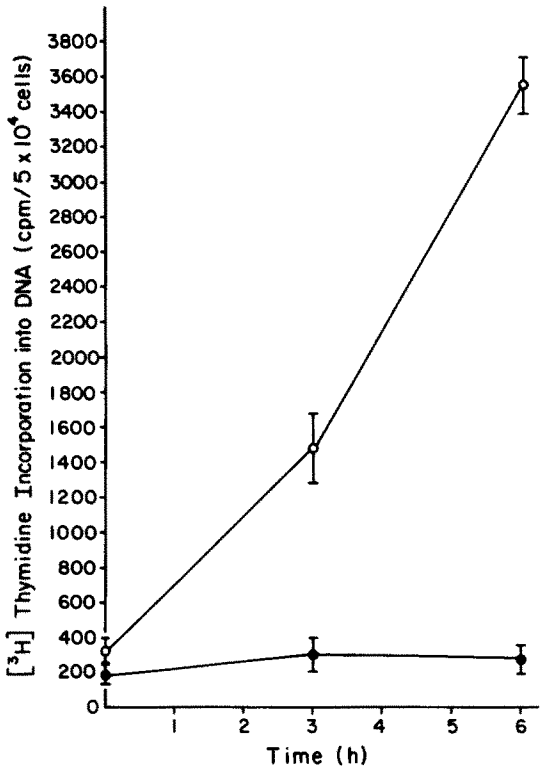

Figure 7. [^{3}H]Thymidine incorporation into DNA. Transfected Schwann cells were grown in medium containing 100 μM zinc but the cells were either plated on plastic dishes (○) or on dishes coated with basal lamina (●).

Discussion

Because of the finite lifetime of secondary Schwann cells in culture (about 60 doublings) and because they divide very slowly (doubling time is 7 d without mitogens), it would be advantageous to have immortalized Schwann cells for studies of Schwann cell–axon interaction in vitro. Transfection of neonatal rat Schwann cells with the SV-40 large T antigen gene by the standard calcium phosphate method resulted in a transfection efficiency of 0.01%. We have obtained cell lines with accelerated growth that have been carried for 180 doublings without degenerative changes taking place. However, the growth properties were not controlled by altering the concentration of zinc in the medium, despite the fact that T antigen is expressed under the control of the metallothionein promoter. Neither decreasing the serum concentration to

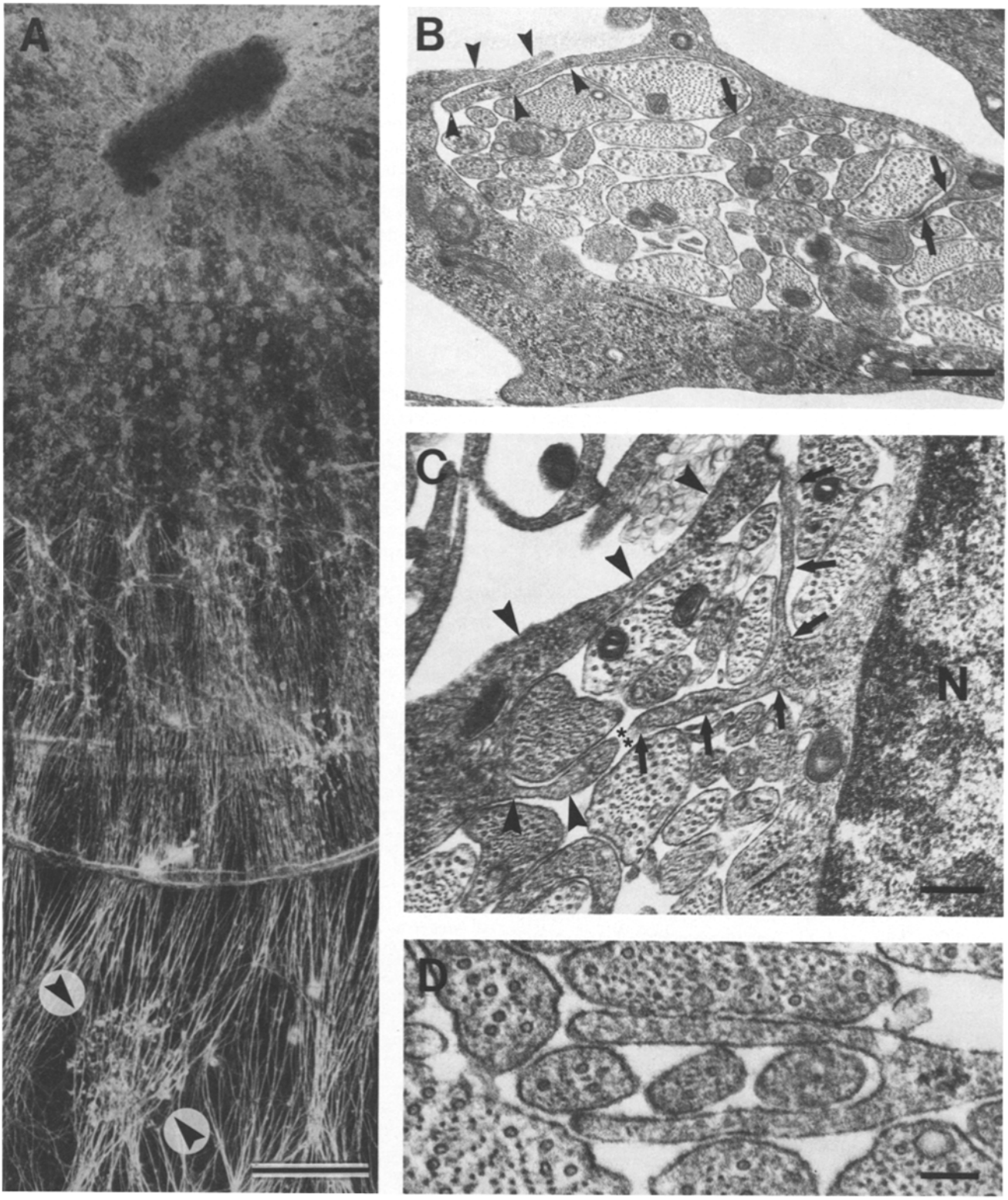

Figure 8. (*A*) Montage of dorsal root ganglion culture seeded with transfected Schwann cells. The dark structure at the top of the figure is the reaggregated mass of neuronal cell bodies. Small clusters of neurons are present at the periphery of the aggregate. Groups of transfected Schwann cells (*arrows*) visible among the distal-most regions of the neuritic outgrowth were chosen for examination by electron microscopy. Photographed with dark field microscopy. Bar, 1 mm. (*B*) A neurite fascicle surrounded by transfected Schwann cell processes. The encircling is completed by the overlapping of two processes (*arrowheads*). Two extensions (*arrows*) are present that penetrate the fascicle and subdivide it. Bar, 0.5 μm. (*C*) Two bifurcating transfected Schwann cell processes (*arrows* and *arrowheads*) segregating a group of axons. One process (*arrows*) emerges close to the perinuclear region of the cell and quickly divides. The second process (*arrowheads*) is more extensive and may be continuous with the first process at the asterisks. *N*, nucleus of transfected Schwann cell. Bar, 0.25 μm. (*D*) Bifurcating transfected Schwann cell process enclosing three small neurites. Bar, 0.1 μm.

0.5% nor growing the cells in serum-free medium with low levels of zinc had any effect on the doubling time of the transfected cells.

Indirect immunofluorescence staining of transfected Schwann cells grown in the absence of zinc showed no large T antigen nuclear staining. However, similar cultures labeled with [^{35}S]methionine under identical conditions showed that large T antigen was being synthesized, albeit at a reduced level (54% of the amount measured in cultures grown in 100 μM zinc). In parallel with this decreased synthesis, the mRNA coding for SV-40 large T antigen was reduced to 34%, indicating that the expression of SV-40 large T antigen gene is regulated by the wild-type MT-I promoter. Recent work on the mouse MT-I promoter has shown that there are five metal regulatory elements in addition to a region responsible for basal activity (15, 51, 52). The continued cell proliferation found in the absence of zinc could be due to continued expression of SV-40 large T antigen, resulting from basal activity of the MT-I promoter. The discrepancy between the absence of indirect immunofluorescence staining and the presence of SV-40 large T antigen by isotope labeling followed by immunoprecipitation probably reflects the lower sensitivity of the indirect immunofluorescence technique. This may also be true for some of the discrepancies in anti-galactocerebroside staining compared with radiolabeling studies.

Transfected Schwann cells in the presence or absence of zinc displayed properties previously noted for secondary Schwann cells. These cells grown with added zinc chloride were Ran-2-positive, a pattern of staining seen with nonmyelin forming cells. Secondary Schwann cells as well as transfected Schwann cells grown in the absence of zinc chloride were Ran-2-negative. Whether the loss of Ran-2 staining represents a more differentiated state is unclear, except that myelin-forming Schwann cells are Ran-2-negative. Moreover, untransfected and transfected Schwann cells were positive for 217c, Ran-1, and S-100 whether or not zinc chloride was present in the medium. The changes in expression of these immunofluorescence markers (Table I) were paralleled by alterations in glycolipid metabolism and in the activity of a Schwann cell–specific enzyme. When zinc was eliminated from the culture medium, both the amount of a myelin-specific lipid (sulfatide) and the activity of the myelin-specific enzyme (CNPase) were increased, whereas the amount of GM3 was decreased. These data are in agreement with the studies described above, suggesting that removal of zinc from the medium leads to increased differentiation of the transfected Schwann cells. However, neither the amount of sulfatide nor the activity of CNPase reached levels found for untransfected secondary Schwann cells (Table II). Moreover, transfected cells contained about one-tenth the amount of P_0 mRNA found in untransfected Schwann cells and the amount did not change in the presence and absence of zinc.

Having established that transfected Schwann cells still maintain properties similar to the untransfected cell as well as some degree of regulatability of T antigen expression, we assessed the usefulness of this cell line in studies of axon–Schwann cell interaction. Two interactions were examined; with basal lamina and with neurites. In the presence of basal lamina, transfected Schwann cells stopped DNA synthesis as measured by [^{3}H]thymidine incorporation, and no cell division was apparent. Neither laminin nor fibronectin alone inhibited DNA synthesis or cell doubling time. It is possible that basal lamina affects cell division in transfected cells by suppression of large T antigen synthesis. This seems unlikely since large T antigen was detected by indirect immunofluorescence and immunoprecipitation of [^{35}S]methionine-labeled cell extracts from transfected cells grown on basal lamina. Such an effect of basal lamina on Schwann cell replication is supported by observations in dystrophic mice where basal lamina synthesis is faulty, and Schwann cells continue to divide in regions where basal lamina is absent (4, 31). In addition to the effect of basal lamina on DNA synthesis, the transfected Schwann cells became bipolar, more closely resembling primary Schwann cells in culture.

In separate co-culture experiments to assess the interaction of transfected Schwann cells with neurites, transfected Schwann cells first adhered to and then segregated neurites into fascicles. They did not, however, establish a 1:1 relationship with these neurites and no myelination was detected. The lack of myelination was not unexpected, since untransfected Schwann cells in culture require additional factors for myelin formation, which were not added in our cultures (38). We are currently using myelinating cultures with transfected Schwann cells to examine the process of formation and maintenance of myelin. It is possible that large T antigen alters the expression of myelin genes and hence prevents myelinogenesis, since SV-40 T antigen and T antigen from another papovavirus, JC virus (59) both affect myelination in transgenic mice.

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