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### Journal

Methods, 3(3)

### ISSN

1058-6687

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### Publication Date

1993-12-01

### DOI

10.1006/ncmn.1993.1057

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Peer reviewed

# Cell Lines Derived from Retrovirus-Mediated Oncogene Transduction into Olfactory Epithelium Cultures

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We describe the isolation and characterization of six immortal cell lines derived from primary cultures of olfactory epithelium (OE) purified from E15 mouse embryos. Cultured cells were immortalized using a replication-defective murine retrovirus containing the cDNA for human *c-myc*, in addition to a dominant selectable antibiotic resistance gene (*neo*). Cells that survived antibiotic selection and displayed process-bearing morphologies were expanded and analyzed for expression of molecular markers characteristic of olfactory receptor neurons, sustentacular cells, and olfactory ensheathing cells. Interestingly, all six cell lines expressed morphological and immunological properties of the ensheathing, or Schwann, cells of the olfactory nerve, but did not express markers characteristic of olfactory receptor neurons. Our results suggest that, while it is possible to generate immortalized OE cell lines using retrovirus-mediated oncogene transfer, there may be limitations to the types of cells that can be immortalized. In addition, we demonstrate the potential usefulness of immortalized OE cell lines for promoter-trap experiments to identify developmentally regulated genes. © 1993 Academic Press, Inc.

To study the molecular events that regulate neurogenesis, our laboratory has developed an *in vitro* system based on primary cultures of olfactory epithelium (OE) purified from mouse embryos (1–3). The OE is a neuroepithelium not unlike the embryonic neural tube in structure. As a tissue for *in vitro* studies of neurogenesis, however, OE has significant advantages over the neural tube. First, it is much simpler, containing only one type of neuron, the olfactory sensory neuron (also called the olfactory receptor neuron), which transduces the sensation of smell from the nose to the main olfactory bulb of the brain. Second, the OE retains both its epithelial morphology and the unique ability to regenerate its neuronal popu-

lation throughout adult life (4). Third, studies of regenerating olfactory receptor neurons in adult animals have already provided insights into cellular interactions that regulate neurogenesis in OE (5–8). Fourth, lineage relationships among cells in the OE neurogenic pathway appear to be relatively straightforward: The last step of neuronal differentiation appears to involve a committed neuronal precursor cell, the INP (for “immediate neuronal precursor”), which divides and gives rise to daughter cells; these daughter cells undergo terminal differentiation to become olfactory receptor neurons (1, 2, 9). A number of laboratories, including ours, have developed molecular markers that can be used to identify cells at different stages in the olfactory neurogenic pathway (1, 10–13). The relative simplicity of the OE neurogenic pathway, plus the availability of markers that enable the cell types in the pathway to be distinguished from one another, makes the OE culture system a particularly attractive one with which to identify molecules involved in the regulation of neurogenesis. One of the goals of the research in our laboratory is to identify genes characteristic of the neuronal precursor phenotype, using, among other strategies, promoter-trap vectors for identifying developmentally regulated genes in cultured cells. Genes that are specifically expressed by the INPs in OE cultures should in principle fulfill this criterion. However, it is not possible to obtain INPs in large numbers, and these cells normally undergo only one or two rounds of division in primary culture (1, 14). Thus, it is necessary to generate a cell line in order to use this approach.

Other investigators have made efforts to obtain cell lines from olfactory epithelium, using several different approaches [see also the article by O. Bernard (23) this issue]. For example, Schubert and co-workers (15), Coon and co-workers (16), and Goldstein and co-workers (17) have derived cell lines by continuous culture of olfactory epithelium cells, selecting for cells that survived multiple passages and gave some evidence of morphological and/or biochemical differentiation. Largent *et al.* (18) approached this problem by attempting to directly immor-

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talize olfactory receptor neurons in transgenic mice. They targeted expression of an oncogene (SV40 T antigen) to the mature olfactory receptor neurons with 5' flanking sequences from a cell-type-specific gene (olfactory marker protein, or OMP, which is expressed only in mature olfactory receptor neurons). Those experiments yielded immortalized cells, but the resulting cell lines do not express neuronal markers characteristic of fully differentiated olfactory receptor neurons, despite the fact that SV40 Tag expression appears to be confined to olfactory receptor neurons *in vivo* in the transgenic founder animals (18). Our approach to this problem has been to try to directly immortalize cells in primary olfactory epithelium cultures, using retroviruses to transduce oncogenes into dividing cells. In this article, we describe experiments in which we infected cultures of olfactory epithelium, purified from embryonic mice, with a recombinant retrovirus containing the *c-myc* proto-oncogene expressed under the control of a constitutively active promoter (19). We derived six stable cell lines from this experiment. Attempts to differentiate these cell lines *in vitro* yielded the surprising finding that all six cell lines expressed both morphological and immunological properties characteristic of the ensheathing, or Schwann, cells of the olfactory nerve, but not properties of olfactory receptor neurons. Our findings corroborate previous experiments suggesting that olfactory Schwann cells are in fact the progeny of a precursor cell type that is present within the olfactory epithelium itself. In addition, our results suggest that there may be limitations to the types of OE cells that can be immortalized under the specific growth conditions and using the particular protocol employed in these experiments.

## METHODS

### *Choice of Retrovirus Vector and Optimization of Virus-Producing Cell Lines*

The vector used for these immortalization experiments was a replication-defective murine retrovirus (MV-7) carrying the human *c-myc* cDNA and designated pMV7myc by Eilers *et al.* (19). We chose this vector anticipating that *myc* could be used successfully to immortalize neural cells of the olfactory epithelium, based on work by others indicating that overexpression of *myc* can immortalize both glial and neuronal precursor cells (e.g., 20–23). Because we work only with mouse cells in our laboratory, we can use murine retroviral vectors, packaged by ecotropic virus-packaging lines such as  $\psi$ -2 and  $\psi$ -cre (24, 25), for our immortalization experiments. Of these two ecotropic packaging lines,  $\psi$ -2 has consistently yielded virus-producing cell lines with the highest titers when transduced with a given vector, so we commonly use it. Care must be taken that no helper activity is present in

the packaging lines, however, or any immortalized cell lines that result from retrovirus-mediated oncogene transduction may themselves produce oncogene-containing retroviruses. To ensure that this does not happen, producer lines should be assayed periodically for the presence of helper virus. Descriptions of such assays are covered in considerable detail in the excellent practical review by Cepko (26).

We commonly use a two-step approach for generating our virus-producer lines. This approach, which utilizes transiently expressed viral particles to infect tunicamycin-treated  $\psi$ -2 cells (27), has been shown by others to produce packaging lines with higher viral titers (28, 29). The general scheme for the two-step approach is as follows: On Day 1, purified plasmid DNA is transfected into subconfluent (approximately  $2 \times 10^6$  cells per 100-mm tissue culture dish)  $\psi$ -2 cells using a standard calcium-phosphate transfection method (30). On Day 2, transfected  $\psi$ -2 cells are refed with fresh medium (DMEM with 10% calf serum). Late that same day (Day 2), new plates of  $\psi$ -2 cells, from which the actual producer lines will be cloned, are plated at very low density ( $2 \times 10^5$  cells per 100-mm tissue culture plate). On the morning of Day 3, the low-density plates of untransfected  $\psi$ -2 cells are treated with tunicamycin (0.2  $\mu$ g/ml of medium, diluted fresh from frozen 1000 $\times$  stock made up in 0.1 N NaOH) to inhibit protein glycosylation and thus overcome the block to retroviral superinfection (27). Late that day, virus-containing culture medium from the transfected  $\psi$ -2 cells is collected and filtered, and polybrene (hexadimethrine bromide, kept as a sterile frozen stock made up in water) is added to a final concentration of 2  $\mu$ g/ml. This medium is then used to infect the recipient, low-density plates of tunicamycin-treated  $\psi$ -2 cells. The afternoon of the next day (Day 4), the infected  $\psi$ -2 cells are placed into selection medium (1 mg/ml G418 in culture medium). These cells are maintained in selection medium for 10–14 days, until clear colonies form and can be picked using cloning rings. We typically test about 10 clones from such an infection and generally obtain producer lines with titers of  $10^5$ – $10^6$  cfu/ml when titrated on recipient NIH3T3 cells.

### *Infection, Selection, and Maintenance of Cell Lines*

Because proviral integration requires host cells to undergo DNA synthesis (cf. 26, 31), we wanted to infect OE cultures at a time when we knew that olfactory neuron precursors (INPs) would be dividing. Our studies on primary cultures had shown that DNA synthesis and cell division by INPs were greatest during the first 18 h after pieces of OE purified from E14–16 mouse embryos were explanted into culture and that, in the absence of specific mitogenic growth factors, the majority of INPs undergo only one round of DNA synthesis and division *in vitro* before their progeny undergo terminal differentiation into NCAM-expressing olfactory receptor neurons (1, 14). A primary objective of our immortalization strategy was

therefore to infect olfactory epithelium cultures as early as possible in the culture period, in the hope of infecting INPs prior to their terminal S-phase.

The procedure used for infecting and culturing cell lines was as follows: Primary suspension cultures of olfactory epithelium, purified from E14.5 CD-1 mouse embryos (Charles River), were prepared and grown in serum-free low-calcium medium as described previously (3). The medium in these cultures was altered such that the F12 component of the medium was replaced with F12-VCM [F12-virus containing medium, i.e., Ham's F12 supplemented with serum-free culture additives, 6 g/liter glucose, and 5 mg/ml crystalline BSA as described (3) and then conditioned for 18 h over a confluent monolayer of  $\psi$ -2 cells packaging pMV7myc]; in addition to F12-VCM, polybrene was added to the culture medium to a final concentration of 2  $\mu$ g/ml. After 14 h of suspension culture in serum-free, virus-containing medium, cells were dissociated by enzymatic digestion as described (3). Dissociated cells were then replated at high density [ $>10^4$  cells/well of 96-well tissue culture trays that had been treated with poly-D-lysine (1 mg/ml overnight in the cold) followed by laminin (50  $\mu$ g/ml for 3 h at 37°C)] in medium consisting of 50% F12-VCM, 50% DMEM containing 20% fetal bovine serum, 2 mM L-glutamine, and 100 units/ml penicillin-streptomycin. This second round of exposure to the virus was intended to maximize the chance of infecting any dividing cells that might remain in OE cultures after the first 14 h of the culture period. After this second feeding, cells were left to grow with the culture medium unchanged for 5 days, after which they were placed into selection in DMEM-F12 containing 10% defined, supplemented bovine calf serum (Hyclone), 10% fetal bovine serum (Hyclone), 2 mM L-glutamine, 100 units/ml penicillin-streptomycin, plus 200  $\mu$ g/ml G418. Selection medium was changed every 3–5 days, and the cells were maintained for 2 months before being expanded and characterized.

Because there is as yet no molecular marker specific for INPs, our strategy was to first identify cell lines that were both resistant to the dominant selectable antibiotic G418 and displayed an appropriate morphology under phase-contrast optics. Colonies of cells that were G418-resistant, and in which the majority of cells extended processes and exhibited bi- or multipolar morphologies even at high densities, were chosen as it was expected that such cells had the best chance of representing immortalized neuronal precursors (e.g., 16–18). Cells that grew in contiguous epithelial sheets were eliminated as being likely to represent immortalized basal epithelial cells.

Six cell lines fulfilled these criteria and survived subsequent subculturing. These cell lines have been called the OEmyc790 series of cell lines. For continuous growth, these cell lines are cultured in untreated 100-mm tissue culture dishes at 37°C in a 5% CO<sub>2</sub>/95% air atmosphere and replated at one-third the original density when approximately 50–60% confluent (typically 3–4 days). These

lines have been maintained in this manner for more than 2 years and have successfully survived cryopreservation and subcloning by limiting dilution.

#### *Characterization of OEmyc70 Cell Lines Using Molecular Markers for Olfactory Epithelium Cell Types*

OEmyc790 cell lines were characterized for their expression of specific molecular markers by a variety of techniques. For immunocytochemical analysis, cells were plated onto acid-cleaned glass coverslips (13 mm round, thickness No. 1, Propper Mfg., Long Island City, NY) that had been treated sequentially with poly-D-lysine (Sigma, 1 mg/ml) followed by merosin (Telios, 10  $\mu$ g/ml). Different fixation procedures and secondary antibodies were found to be optimal for different primary antibodies; details for each antibody used are given in Table 1 and Fig. 1. Primary antibodies used include rabbit anti-S100 (Dako Z311); rabbit anti-glial fibrillary acidic protein (GFAP; Dako Z334); mouse anti-vimentin, clone V9 (ICN Biomedicals); mouse anti-neural cell adhesion molecule (NCAM), clone AG1D5 (1); mouse anti-mucin, clone 3C2 (32); and rat anti-NCAM, clone H28 (33). For immunoblotting, standard procedures were used for polyacrylamide gel electrophoresis, electrophoretic transfer, and antibody binding (34). Detection of primary antibodies was accomplished by binding of affinity-purified phosphatase-labeled secondary antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD), followed by development in BCIP/NBT according to manufacturer's instructions (Kirkegaard and Perry).

Reverse transcriptase-polymerase chain reaction analysis of odorant receptor gene expression was performed in collaboration with Drs. Y. Kubota and H. Sakano (University of California, Berkeley). Total RNA was prepared from OEmyc790 cell lines using the single-step purification method of Chomczynski and Sacchi (35). cDNA was made using the InVitrogen cDNA Synthesis Kit according to manufacturer's instructions and used as template for amplification of odorant receptor transcripts using degenerate primers corresponding to the second, third, sixth, and seventh transmembrane regions of the odorant receptor genes (10). Sequences for the primers used are given in the footnotes to Table 1. Four combinations of primers (TM2–TM6, TM3–TM7, TM2–TM7, and TM3–TM6) were used in an attempt to determine whether OEmyc790 cell lines express odorant receptor gene transcripts.

#### *Infection of OEmyc790 Cell Lines with the U3LacZ Promoter-Trap Vector*

$\psi$ -2 cells that package the U3LacZ promoter-trap retrovirus (36) were the generous gift of S. Reddy, H. von Melchner, and E. Rulley (Massachusetts Institute of Technology). OEmycD10 cells were infected with the U3LacZ retrovirus under conditions permissive for cell proliferation (growth at low density in high serum). The

mechanics of infection were such that a large number of individual cultures (18) from one clonal cell line were each infected with virus at a low multiplicity of infection (MOI). The promoter-trap strategy requires that tagged genes eventually be cloned from the progeny of only one cell, showing regulated *lacZ* expression, which has been obtained from each of these individual cultures. This ensures that independent events are studied, i.e., that each clone of cells analyzed has a provirus integrated in a different gene. Infection of cultures at low MOI should ensure that any recipient cell has only one integrated U3LacZ provirus, a necessary prerequisite for reliable amplification of flanking sequences by inverse PCR (37).

Individual cultures were expanded to the minimum number of cells sufficient for analysis of *lacZ* expression and cloning with the fluorescence-activated cell sorter (between  $10^6$  and  $10^7$  total cells per individual infected culture).  $\beta$ -Galactosidase activity was assessed in the 18 independent cultures using the substrate fluorescein di- $\beta$ -D-galactoside (FDG), which is hydrolyzed and retained intracellularly in *lacZ*-expressing cells (38). For FACS analysis and sorting, individual cultures were then trypsinized, loaded with FDG by hypotonic shock according to manufacturer's instructions (Molecular Probes FluoReporter LacZ Flow Cytometry kit), chilled, and analyzed in a Becton-Dickinson FACS Star cell sorter. Expressing cells from each individual culture were then directly cloned into 96-well plates by the FACS, expanded, and reanalyzed for  $\beta$ -galactosidase expression using the chromogenic substrate 4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal) (39).

## RESULTS AND DISCUSSION

From these experiments, six cell lines (C7s.D, D6s.AB8, C6s.BG9, D10, C4, and D4) were isolated and further characterized. Under normal growth conditions, immediately after plating, the cells in these lines tend to have either spindle-shaped or fibroblastic morphologies. With further time and growth in culture (to 30–60% confluence), the cells display two distinct morphologies—flat epithelioid cells and bi- or multipolar process-bearing cells with phase-bright cell bodies. Most cell lines contained a mixture of these two morphologies, although particular cell lines such as C7 consisted mainly of the bipolar cell type (see Fig. 1). Culturing the cell lines in the same medium for a week or more resulted in a greater proportion of cells adopting a process-bearing morphology as well as a marked inhibition of cell growth, and cells in some of the lines displayed a very distinctive alignment into parallel arrays (e.g., Fig. 1C). Interestingly, similar results have been observed with a *myc*-immortalized cell line obtained by retroviral oncogene transduction into neural crest cell cultures (22).

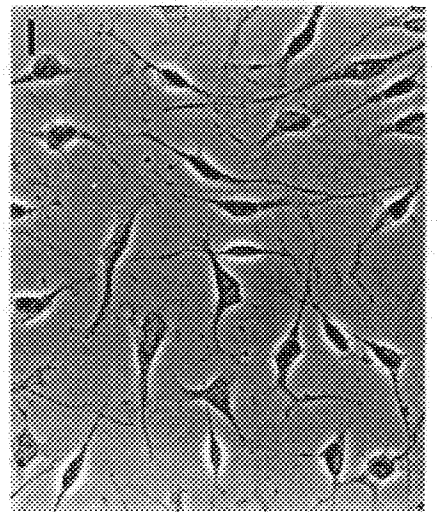
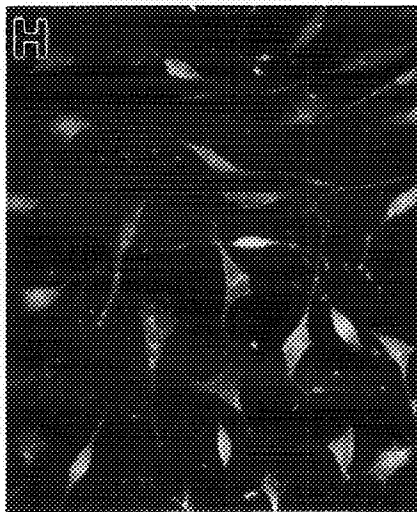
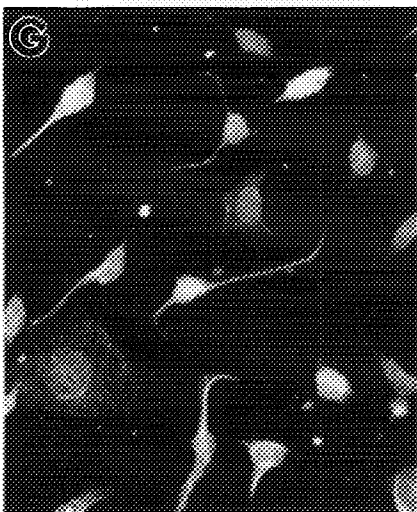
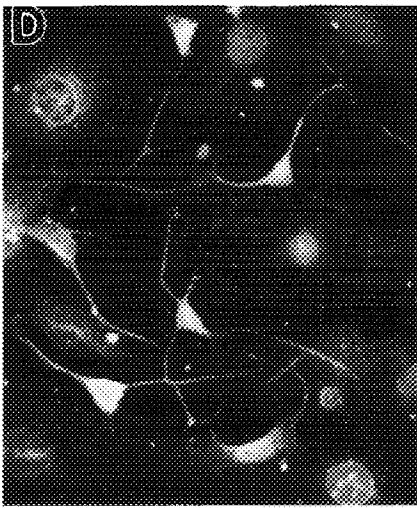
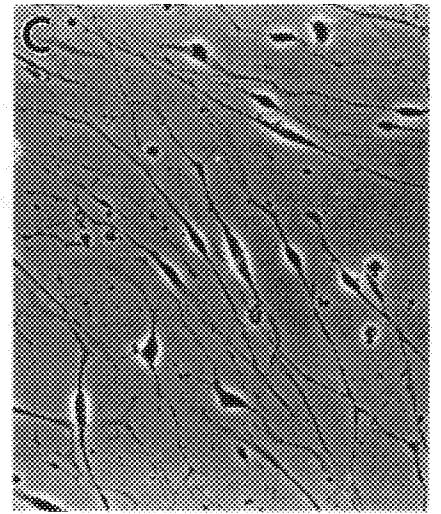
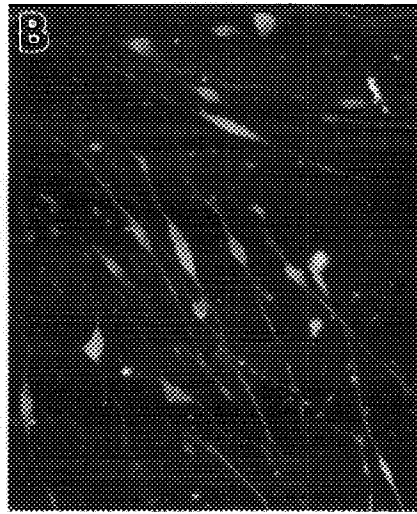
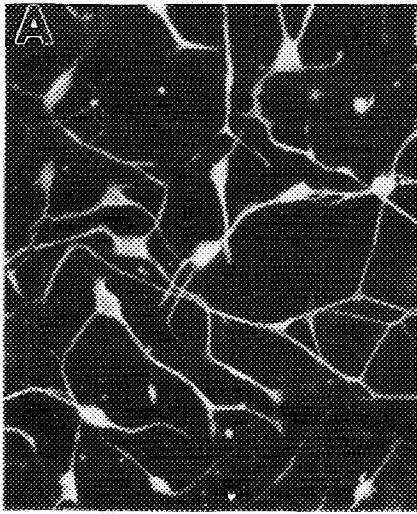
### *Characterization of OEmyc790 Cell Lines with Molecular Markers Indicates That They Do Not Represent Immortalized Olfactory Receptor Neurons*

Although the morphologies of cells of the OEmyc790 cell lines were not strikingly similar to those of olfactory receptor neurons in our primary cultures of embryonic mouse OE (cf. Ref. 1), they were similar to those observed in OE cell lines generated by others (16–18) and also appeared similar to those of cultured cells reported to represent purified neonatal rat olfactory receptor neurons (40). We therefore screened the OEmyc790 cell lines for molecular markers characteristic of olfactory receptor neurons.

The AG1D5 monoclonal antibody to the NCAM recognizes the 140- and 180-kDa forms of the NCAM polypeptide (41). The AG1D5 antigen is specifically expressed by olfactory receptor neurons in cultures of purified mouse OE and can be detected by immunocytochemistry within hours of these cells' terminal differentiation (1). However, neither immunoblotting nor immunocytochemical analysis of OEmyc790 cell lines could demonstrate expression of the NCAM by process-bearing cells of the OEmyc790 cell lines. Even when cell lines were shifted to low (1%) serum for several days to inhibit cell division and/or were grown under conditions that appeared to select for greater numbers of process-bearing cells in the cultures [e.g., C6 glioma conditioned medium and 40 mM potassium (cf. legend to Fig. 1) or 10–20  $\mu$ M forskolin (not shown)], no NCAM expression was detected with the AG1D5 monoclonal antibody (Table 1). A different monoclonal antibody to NCAM, the H28 antibody, which recognizes all three forms of the NCAM polypeptide (33), was also used to analyze NCAM expression by these cell lines. For two cell lines, D10 and C7, occasional expression was seen in the areas of contact between flat, epithelioid cells present in these cultures. No process-bearing cells in these cultures were observed to express H28-detectable NCAM.

We also performed immunocytochemical analysis to determine whether any OEmyc790 cell lines expressed vimentin detectable by the V9 monoclonal antibody. *In vivo*, in the adult rat olfactory epithelium, olfactory receptor neurons have been reported to express vimentin detectable with this antibody (42). Our immunocytochemical analysis of the OEmyc790 cell lines showed little detectable vimentin expression, and that only in a subpopulation of the flat epithelioid cells present in some of the cell lines (Table 1).

The results of immunochemical experiments using anti-NCAM and anti-vimentin antibodies suggested that OEmyc790 cell lines probably did not express the biochemical characteristics of olfactory receptor neurons, even when these cells were cultured under conditions in which cell division was inhibited and morphological "differentiation" (the appearance of extended cell processes) was induced. However, it seemed possible that expression



of cell-surface adhesion proteins such as NCAM might be abnormal in cells such as these, which had been immortalized by constitutive overexpression of an oncogene. Accordingly, we tested the cell lines for yet another biochemical marker of olfactory receptor neurons. To determine if odorant receptor genes were expressed by these cell lines, reverse transcriptase-polymerase chain reaction analysis of cDNAs generated from their cellular RNA was performed (Y. Kubota and H. Sakano, unpublished results). Using degenerate primers taken from conserved regions of the transmembrane domains of members of the odorant receptor gene family (cf. Ref. 10), no PCR products that corresponded to odorant receptor transcripts could be amplified from cDNAs obtained from the five cell lines tested (Table 1).

This negative result, taken together with the negative results obtained with the antibodies to NCAM and vimentin, led us to conclude that the OEmyc790 cell lines probably did not represent immortalized olfactory receptor neurons. We also performed an immunocytochemical analysis of the cell lines using the 3C2 monoclonal anti-mucin antibody. The 3C2 antibody recognizes a carbohydrate differentiation antigen expressed primarily on the supporting, or sustentacular cells, of the olfactory epithelium (32). In light of our negative results with markers characteristic of olfactory receptor neurons, it was important to consider the possibility that we might have immortalized the sustentacular cells of the epithelium. However, no immunoreactivity of the cell lines to this antibody was observed. Thus, it appeared unlikely that these cell lines represent immortalized sustentacular cells.

#### *Analysis with Glial Markers Indicates That OEmyc790 Cell Lines Represent Immortalized Olfactory Ensheathing Cells*

From the analysis described above, it remained possible that the OEmyc790 cell lines could in fact represent immortalized immediate neuronal precursors, which do not express the differentiation markers characteristic of olfactory receptor neurons (cf. Refs. 1, 2). However, we noted a striking similarity between the morphologies of the OEmyc790 cell lines and those that had been observed both for primary cultures of olfactory ensheathing, or Schwann, cells and for immortalized Schwann cell pro-

genitors of the neural crest (cf. Refs. 22, 43, 44). This morphological similarity, coupled with the availability of specific molecular markers that distinguish olfactory ensheathing cells from olfactory receptor neurons in primary cultures (43–46), led us to explore the possibility that our cell lines might represent immortalized olfactory ensheathing cells.

Olfactory ensheathing or Schwann cells support, but do not myelinate, the axons of olfactory receptor neurons (reviewed in 47). These cells differ from myelinating Schwann cells of the peripheral nervous system (PNS) in a number of other ways. For example, olfactory ensheathing cells express the intermediate filament protein GFAP, which is not expressed by myelinating Schwann cells of the PNS, but is expressed by central nervous system astrocytes (43, 45, 46, 48). They also express the cytoplasmic protein S100, which, like GFAP, appears to be a general marker for nonmyelinating Schwann cells *in vivo* and *in vitro* (43, 49).

We therefore undertook an immunocytochemical analysis of these cell lines for molecular markers of olfactory ensheathing cells. All six OEmyc790 cell lines expressed easily detectable levels of both GFAP and S100 (Table 1). The typical pattern of staining observed is illustrated for three of the cell lines—C7s.D, D6s.AB8, and C6s.BG9—in Fig. 1. Staining of the process-bearing cells was more intense than staining of the flat epithelioid cells with both S100 and GFAP antisera, but both morphological types clearly expressed both proteins in all cell lines. This was true whether these cells were grown in reduced serum medium containing cyclic AMP, a condition that is known to promote Schwann cell differentiation (50), or whether they were grown in reduced serum medium containing C6 glioma conditioned medium (Table 1, Fig. 1).

These positive results using markers for olfactory ensheathing cells, taken together with the negative results obtained using markers for olfactory receptor neurons and sustentacular cells, led us to conclude that the OEmyc790 cell lines probably represent immortalized olfactory ensheathing cells. We have never observed ensheathing cells in short-term (1–3 days), serum-free primary cultures of OE purified from E14–15 mice, so we were initially puzzled as to the cell type that could have been immortalized to

**FIG. 1.** Immunoreactivity of OEmyc790 cell lines with glial cell markers. OEmyc790 cell lines were plated onto glass coverslips that had been coated with poly-D-lysine (1 mg/ml), followed by merosin (10 mg/ml). Cells were grown for 2–4 days in either “C6 glioma conditioned medium” (50% DME-F12 + 1% calf serum/50% C6 glioma conditioned medium/40 mM KCl) or “low serum + cAMP medium” (DME-F12 + 1% calf serum + 1 mM dibutyryl cAMP). Cells were fixed with either acetone (5 min at room temperature) or PPG (4% paraformaldehyde/0.2% picric acid/0.05% glutaraldehyde/0.1 M sodium phosphate, pH 7.0) for 15 min, also at room temperature. The antibodies and staining conditions used were as follows: For anti-S100 staining, cells were grown for 2–4 days in C6 glioma conditioned medium and then fixed in PPG. Cells were stained with rabbit anti-S100 IgG (Dako Z311), 1:200 dilution. For anti-GFAP staining, cells were grown in low serum + cAMP and then fixed in acetone. These cells were stained with rabbit anti-GFAP IgG (Dako Z334), 1:100 dilution. Both primary antibodies were visualized with FITC-conjugated goat anti-rabbit IgG (Capell), used at a 1:100 dilution. (A–C) OEmyc790 C7s.D stained for GFAP, stained for S100, and viewed in phase contrast, respectively. (D–F) OEmyc790 D6s.AB8 stained for GFAP, stained for S100, and viewed in phase contrast. (G–I) OEmyc790 C6s.BG9 stained for GFAP, stained for S100, and viewed in phase contrast.

give rise to these cell lines. However, GFAP-positive cells have been observed to appear in cultures of rat olfactory epithelium grown in high (10%) concentrations of serum, and such observations have led to the proposal that the olfactory ensheathing cells are derived from progenitors that reside within the olfactory epithelium itself (51). We also have observed that a small fraction of the cells growing in contiguous epithelial sheets in OE explant cultures acquire GFAP immunoreactivity when cultured for extended periods (not shown). It is our current hypothesis that the olfactory ensheathing cell progenitors originate in the *in vitro* cell population that consists of keratin-expressing basal epithelial cells, a cell population that we know is capable of proliferating for long periods in culture (1). It is presumably this cell type that was immortalized by the pMV7myc retrovirus in these experiments, in which cultures were placed into serum-containing medium shortly (<24 h) after dissection and plating.

It is unknown if the olfactory ensheathing cell progenitor, which we postulate has been immortalized in these experiments, can also generate INPs and thence olfactory receptor neurons. It is possible that we are simply ignorant of the conditions necessary for stimulating neuronal differentiation in these cell lines. However, INPs in primary OE cultures undergo DNA synthesis, division, and neuronal differentiation in the absence of serum, synaptic target tissue, or extrinsic factors (1). This suggests that INPs are committed neuronal progenitors, programmed

for neuronal differentiation, and that no special conditions are normally required in order for this differentiation process to take place. Thus, our current hypothesis concerning the OEmyc790 cell lines is that the olfactory ensheathing cell lineage is separate from the neurogenic lineage of olfactory receptor neurons and that either the circumstances of cell immortalization (constitutive expression of an oncogene) or the culture conditions employed (high serum, absence of mitogenic stimulation specific for INPs at the time of infection) have selected for immortalization of progenitors in the ensheathing cell lineage.

In this light, it is worth mentioning that no group—including ours—has yet succeeded in immortalizing cells from olfactory epithelium that, even when switched to conditions that slow cell division and promote apparent morphological differentiation of cells, appear to express the molecular markers characteristic of differentiated olfactory receptor neurons (15–18). This is the case even for cells immortalized by directing oncogene expression with regulatory elements from the OMP gene, a gene whose expression is characteristic of mature olfactory receptor neurons (18). It may be that olfactory neuron precursors are particularly refractory to immortalization by retrovirus-mediated oncogene transfer. It is certainly the case that these cells display only a limited capacity for division *in vitro* and that specific mitogens are necessary in order to allow them to divide more than once (1, 14).

TABLE 1  
Immunological Characterization of OEmyc790 Immortalized Cell Lines

| Primary antibody                 | C4  | D4  | D10                    | D6s.AB8                 | C6s.BG9                | C7s.D                  |
|----------------------------------|-----|-----|------------------------|-------------------------|------------------------|------------------------|
| $\alpha$ S100 <sup>a</sup>       | +++ | ++  | ++                     | ++                      | ++                     | ++                     |
| $\alpha$ GFAP <sup>a</sup>       | +++ | +++ | ++                     | ++                      | ++                     | ++                     |
| $\alpha$ Vimentin <sup>b</sup>   | nd  | –   | ( $\pm$ ) <sup>c</sup> | –( $\pm$ ) <sup>c</sup> | ( $\pm$ ) <sup>c</sup> | ( $\pm$ ) <sup>c</sup> |
| $\alpha$ NCAM AG1D5 <sup>d</sup> | –   | –   | –                      | –                       | –                      | –                      |
| $\alpha$ Mucin <sup>e</sup>      | –   | –   | –                      | –                       | –                      | –                      |
| $\alpha$ NCAM H28 <sup>f</sup>   | –   | –   | $\pm$ <sup>g</sup>     | –                       | –                      | $\pm$ <sup>g</sup>     |
| Odorant receptor <sup>h</sup>    | –   | –   | nd                     | –                       | –                      | –                      |

<sup>a</sup> See legend to Fig. 1.

<sup>b</sup> Mouse anti-vimentin IgG (ICN, clone V9, ascites fluid diluted 1:500). Fixation: acetone. Growth condition: DME-F12 +1% calf serum +1 mM cAMP. Visualized with rhodamine goat anti-mouse IgG (Tago), 1:100 dilution.

<sup>c</sup> Immunoreactivity of the flat, epithelioid cells.

<sup>d</sup> AG1D5 mouse anti-NCAM ascites fluid diluted 1:500. Fixation: acetone. Growth condition: 50% DME-F12 +1% calf serum/50% C6 glioma conditioned medium/40 mM KCl. Visualized with rhodamine goat anti-mouse IgG (Tago), 1:100 dilution.

<sup>e</sup> 3C2 mouse anti-mucin IgM, applied as full-strength hybridoma supernatant. Fixation: PPG. Growth condition: 50% DME-F12 +1% calf serum/50% C6 glioma conditioned medium/40 mM KCl. Visualized with Texas Red goat anti-mouse IgM (Jackson), 1:100 dilution.

<sup>f</sup> Rat H28 hybridoma supernatant to NCAM used at full strength. Fixation: acetone. Growth condition: 50% DME-F12 +1% calf serum/50% C6 glioma conditioned medium/40 mM KCl. Visualized with Texas Red Goat anti-rat IgG (Jackson), 1:100 dilution.

<sup>g</sup> Staining was present only in areas of contact between epithelioid cells.

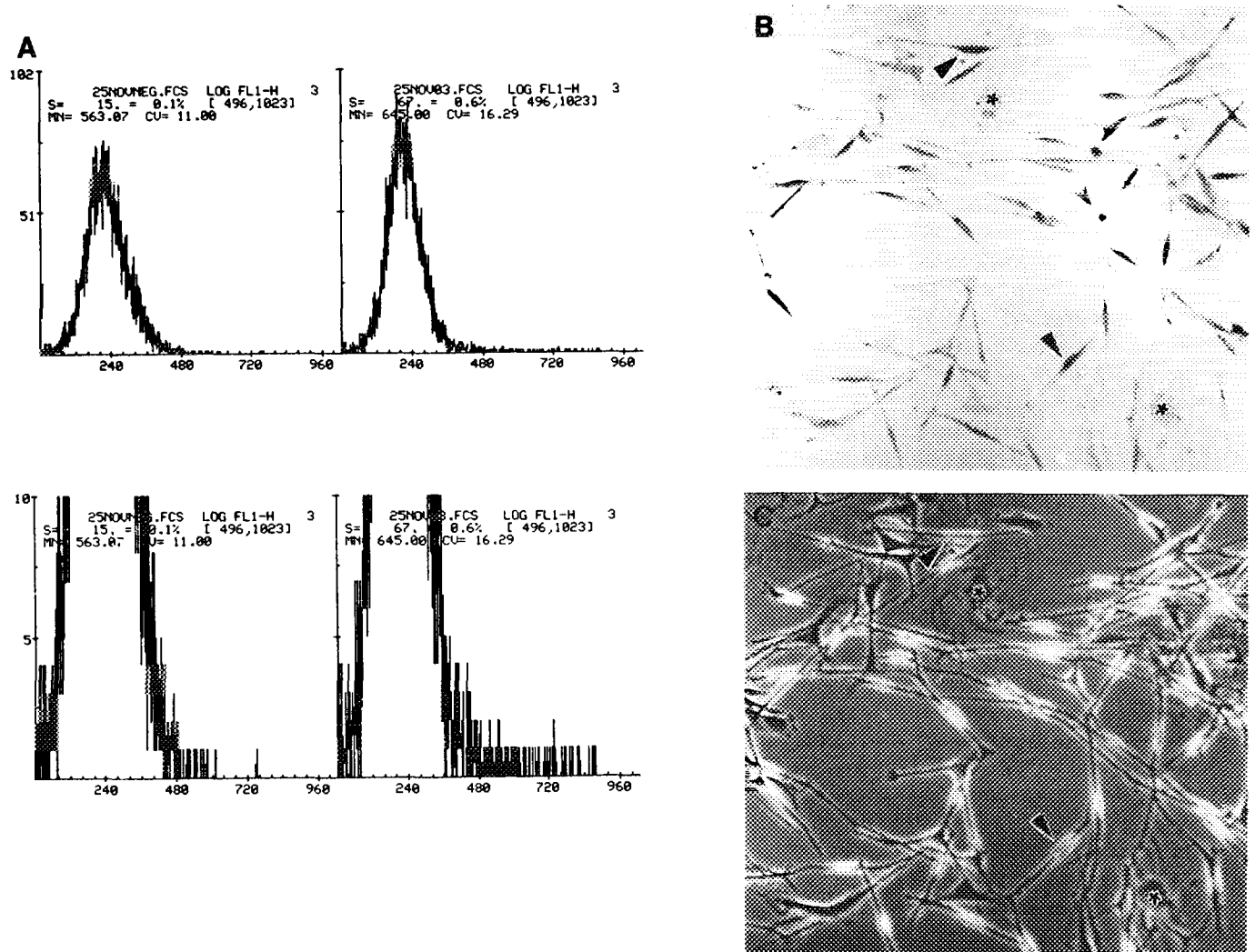
<sup>h</sup> Odorant receptor results are from RT-PCR experiments performed by Y. Kubota and H. Sakano (unpublished), with degenerate primers to the transmembrane regions most conserved in the odorant receptor gene family. The sequences of these primers correspond to amino acids 59–64, 118–127, 240–245, and 282–290 (10). TM2 primer: 5'-CGG AAT TCC C(G/A/T/C)A TGT A(C/T)(C/T) T(G/A/T/C)T T(C/T)C T-3', PMY(L/F)FL; TM3 primer: 5'-ATG GC(G/C/T) TA(C/T) GA(C/T) (A/C)G(AA/G/C) TA(C/T) GT(G/C) GC(G/C/T) AT(A/T/C) TG-3', MAYDRYVAIC; TM6 primer: 5'-ATA AGC TTA G(G/A)T G(G/A/T/C)(G/C) (T/A)(G/A/T/C)(G/C) C(G/A)C A(G/A/T/C)G T-3', TC(A/G)SHL; TM7 primer: 5'-TA(T/G/A)AT(G/A)AA(C/G)GG(G/A)TT(T/G/C)A(G/A)CAT(T/C/G/A)GG-3', PMLNPFYIY.



It may also be the case that it is necessary to employ a conditional allele of an oncogene for immortalizing these neuronal progenitor cells and that approaches such as those used by Noble and colleagues (52) will prove more fruitful in this respect. Experiments to immortalize INPs using these and other conditional immortalization strategies are currently being conducted in our laboratory.

#### Promoter-Trap Analysis of OEmyc790 Cell Lines

For preliminary experiments to determine whether promoter-trap vectors could be used to identify developmentally regulated genes in immortalized OE cell lines, we have used the retrovirus promoter-trap vector U3LacZ (36). In this vector, a reporter gene (*lacZ*) is inserted into the U3 region of an enhancerless Moloney murine leu-



**FIG. 2.**  $\beta$ -Galactosidase expression by OEmycD10 cells infected with the U3LacZ promoter-trap retrovirus. Cultures of OEmycD10 cells were infected with the U3LacZ promoter-trap retrovirus and expanded as described in the text. (A) Analysis and sorting with the fluorescence-activated cell sorter (FACS). Cells were loaded with fluorescein di- $\beta$ -D-galactopyranoside (FDG) by hypotonic shock according to manufacturer's instructions (Molecular Probes FluReporter Lac Z Flow Cytometry kit), chilled on ice, and analyzed for fluorescence using a Becton-Dickinson FACS Star. Fluorescence profiles shown are frequency histograms, giving the number of cells of a given fluorescence intensity. X axis: log fluorescence intensity. Y axis: number of fluorescent events (cells). In the top panel, the left-hand profile shows uninfected cells; the right-hand profile shows a culture that had been infected with the U3LacZ virus at a low multiplicity of infection. In this culture, 0.6% of total cells had fluorescence intensity greater than background (496–1023). The bottom panel shows the same two profiles, magnified to display the highly fluorescent events more clearly. The most intensely fluorescent 0.2% of cells from this culture were cloned directly into 96-well tissue culture trays and expanded for subsequent analysis. (B, C) Retesting of sorted subclones using X-gal staining. Subclone 2A, expanded from FACS cloning of U3LacZ-infected OEmycD10, was fixed in 0.5% glutaraldehyde in phosphate-buffered saline + 1 mM  $MgCl_2$  and assayed for  $\beta$ -galactosidase expression using 4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal) according to published procedures (39). (B) Phase-contrast picture, showing both flat cells and bipolar process-bearing cells. (C) X-gal staining of this same culture. LacZ expression appears to be high in those cells that are spindle-shaped and have extended process (arrowheads), but is negligible in the flat cells (asterisks). This suggests that the U3LacZ promoter trap may have integrated into a gene whose expression is upregulated when these cells take on a process-bearing morphology.

kemia virus long terminal repeat. When the U3LacZ provirus integrates near an active transcriptional promoter in the cellular DNA of host cells, fusion transcripts are produced and the infected host cells express *Escherichia coli*  $\beta$ -galactosidase in a regulated manner corresponding to that of the endogenous gene whose promoter has been "trapped" (36, 53, 54). For these experiments, the OEmyc790 cell line D10 was chosen because it exhibits strong contact inhibition of its growth, thus making it a good candidate for a cell line in which some genes would show regulated expression with differentiation.

Multiple cultures of the OEmyc790 cell line D10 were infected with the U3LacZ vector at a low multiplicity of infection as described under Methods, and then lacZ-expressing cells were cloned by using the fluorescence-activated cell sorter (Fig. 2A). Cloned cells were then expanded and individual clones retested for expression using X-gal as a substrate for  $\beta$ -galactosidase. An example of one subclone of U3-infected OEmycD10 (2A), retested using the X-gal method, is shown in Fig. 2. Figure 2B is a phase-contrast picture of the subclone; Fig. 2C shows X-gal staining of this same culture.  $\beta$ -Galactosidase expression appears to be highest in those cells that have extended processes (arrowheads), suggesting that the U3LacZ promoter trap may have integrated into a gene whose expression is upregulated with this morphological differentiation. This gene can now be identified and cloned, using either of two strategies: inverse PCR, to identify cellular DNA sequences flanking the integrated promoter-trap provirus (37); or 5' rapid amplification of cDNA ends, to amplify fusion transcripts from cellular RNA (55).

These results indicate that immortalized OE cell lines can indeed provide useful material for promoter-trap analyses of differential gene expression in this system. The challenge in the future will be to develop cell lines that represent immortalized olfactory neuron precursors, which could then be used to identify genes of importance in regulating neurogenesis and neuronal differentiation in this uniquely regenerative nervous system tissue.

## ACKNOWLEDGMENTS

We thank Martin Eilers and J. Michael Bishop for the PMV7myc retrovirus vector; Richard Mulligan for  $\psi$ -2 cells; Sita Reddy, Harald vonMelchner, and Earl Ruly for the U3LacZ promoter-trap vector; Pat Levitt and Steve Prouty for 3C2 anti-mucin; Christo Goridis for H28 anti-NCAM; Yoshi Kubota and Hitoshi Sakano for RT-PCR analysis of odorant gene expression; Miyuki Yamamoto for AG1D5 anti-NCAM; and Ron Doucette for helpful advice on immunocytochemical markers for olfactory ensheathing cells. We are grateful to Arthur Lander for helpful discussions and critical reading of the manuscript. This work was supported by a grant to A.L.C. from the National Science Foundation (IBN-9253103).

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