ABSTRACT Immortalized cell lines have been generated from embryonic mouse neuroepithelium by infection with a retrovirus containing the c-myc oncogene. The morphology and the antigenic phenotype of the cloned cell lines are characteristic of normal neuroepithelium. Although the cell lines are stable and do not spontaneously differentiate, morphological changes can be induced with both acidic and basic fibroblast growth factor. Fibroblast growth factor at 5 ng/ml stimulates differentiation of the neuroepithelial cells, and it has been shown that the cloned cell line 2.3D can differentiate into astrocytes, containing glial fibrillary acidic protein, and neurons, expressing the A2B5 marker and neurofilaments. This indicates that some cells in the neuroepithelium at embryonic day 10 are multipotent and are not restricted to either the glial or neuronal cell lineage. The cell lines also can be induced with interferon γ to express class I and class II histocompatibility antigens. The response of the c-myc-immortalized cell lines to these two factors is similar to that observed with freshly isolated neuroepithelium and suggests that such immortalized precursor populations are representative of the cells found in the developing neuroepithelium.

The two major cell types in the mammalian central nervous system, neurons and glia, are developmentally derived from the neuroepithelium that forms the neural tube. Neuroepithelial cells have been shown to give rise to both types of neural cells in vitro (1, 2), and precursor cells committed to a particular cell lineage have been identified within mouse neuroepithelial cells as early as embryonic day 10 (E10) (3). The factors and genes that regulate precursor cell differentiation in the mammalian nervous system are largely unknown, and the establishment of cell lines representative of these neural precursor populations would greatly assist in their identification. A possible method for establishing such neural cell lines is the introduction of the nuclear protooncogenes of the myc family into neural cells. These genes have been classified as having immortalizing activity because they can cooperate with an activated ras gene in the transformation of primary cells (4) and can immortalize primary cells without inducing tumorigenicity (4, 5). Furthermore, the introduction of the c-myc oncogene has been shown to block differentiation in erythroleukemic cell lines (6, 7) and to favor proliferation rather than differentiation of pre-B cells in Eμ-myc transgenic mice (8). In contrast, avian retroviruses containing v-myc transform chicken embryo neuroretinal cells in vitro, apparently without interfering with their continued morphodifferentiation (9).

We report here the generation of cell lines derived from mouse E10 neuroepithelial cells, by the introduction of the c-myc oncogene with a murine retrovirus delivery system. The morphology and antigenic phenotype of these cell lines are characteristic of normal E10 neuroepithelium. Although the cloned cell lines do not spontaneously differentiate, they can be induced with acidic or basic fibroblast growth factor (FGF) to form identifiable glial and neuronal cells. It is proposed that the integration of proviral c-myc into defined neural precursor populations results in the establishment of phenotypically stable, but inducible, cell lines suitable for the study of neural differentiation at a cellular and molecular level.

MATERIALS AND METHODS

Construction of c-myc Retrovirus. The c-myc retrovirus used to infect neuroepithelial cells was constructed by using the shuttle vector pDel as described (10). This vector carries the bacterial neomycin resistance (NeoR) gene that confers resistance to the antibiotic G418 (11). The XhoI fragment of the murine c-myc cDNA was inserted into the BamHI site of the pDel vector. This fragment contains the entire c-myc coding region (12). A schematic diagram of the recombinant retroviral plasmid is shown in Fig. 1A. To avoid possible effects induced by a helper virus in subsequent experiments, virus stocks were produced by transfection of ψ-2 fibroblasts (14) with the retroviral vector. Transfected ψ-2 cells were selected for resistance to G418, and a cell line producing the highest titer of the c-myc virus (10⁴ viruses per ml) was used for all the experiments.

Infection of Neuroepithelial Cells. Virus-producing ψ-2 cells were cultured in 24-well Linbro plates in 1 ml of Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum at 5 × 10⁵ cells per well and were irradiated with 2800 rads (1 rad = 0.01 Gy). After 24 hr, single-cell suspensions of neuroepithelial cells isolated from mesencephalon of 10-day embryo CBA/CaH mice were cultured on the irradiated cells at various concentrations ranging from 10⁵ to 5 × 10⁵ cells per well. After culturing the cells for 2–5 days, 0.4 mg of Genetecin (G418) (GIBCO) was added to each well, and fresh medium containing G418 was subsequently added every 3–4 days. Cell lines were cloned by limiting dilution by using 3T3 fibroblasts as an underlayer.

Immunofluorescent Staining. Cell lines were cultured on glass coverslips contained in 24-well Linbro plates at a density of 2 × 10⁵ cells per ml. After incubation for 48 hr coverslips were removed and transferred to a humidified Terasaki plate. To stain for surface antigenic determinants, 50 μl of the primary antibody was added per coverslip and incubated for 30 min at room temperature. Coverslips were washed by gentle immersion into beakers of isotonic phosphate-buffered saline, and 50 μl of fluorescein-labeled second antibody was applied to each coverslip and incubated for a further 30 min. Coverslips were washed as before and

Abbreviations: FGF, fibroblast growth factor; GFAP, glial fibrillary acidic protein; IFN-γ, interferon γ; E10, embryonic day 10; NeoR, neomycin resistance.

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with the virus-producing ψ-2 cell lines. A ψ-2 line producing equivalent titers of the pDol virus carrying the Neo<sup>6</sup> gene was used as a control. The majority (>98%) of the neuroepithelial cells used for the infection expressed the cytokertin intermediate filament marker as shown by immunofluorescent staining with the antibody K2F2, indicating that this population was not significantly contaminated with mesenchymal elements. The G418-resistant cells were evidence 7–10 days after the addition of the drug, whereas, in control cultures containing only neuroepithelium, all the cells were dead within 48 hr of G418 addition. After 10–14 days of culture, no ψ-2 cells survived, leaving only the infected neuroepithelial cells to proliferate. pDol-Neo virus-infected cells grew slowly and only survived for 2–3 months. In marked contrast, cells infected with pDol-c-myc virus grew rapidly after an initial lag period of 1–2 weeks and have continued to proliferate for >12 months. The frequency of precursors capable of giving rise to Neo<sup>6</sup> cell lines was assessed by limiting dilution and found to be 2 × 10<sup>-4</sup>.

Clonality of the Cell Lines. To establish that the cell lines did indeed harbor c-myc virus and to investigate their clonal composition, DNA from selected lines was digested with EcoRI and with Xba I restriction endonucleases and subjected to Southern blot analysis (27) with a radioactive c-myc probe. Fig. 1B shows the results for the lines 2.3Q and 2.3D as well as for 2.3D.12, which is a clonal derivative of 2.3D.

**RESULTS**

**Infection of Neuroepithelial Cells.** To maximize the efficiency of infection, neuroepithelial cells were co-cultivated fixed by the addition of acid alcohol [95% (vol/vol) absolute ethanol and 5% (vol/vol) acetic acid] for 20 min at -20°C. The coverslips were inverted and mounted in glycerol containing 2.6% (wt/vol) 1,4-diazobicyclo[2.2.2]octane (Dabco, Merck, Melbourne, Australia). To visualize the glial fibrillar acidic protein (GFAP) antigen and the neurofilament proteins, cells were fixed in acid alcohol prior to the addition of the primary antibody.

**Biological Reagents.** A2B5, an anti-ganglioside antibody (15), was used at a dilution of 1:100 of ascites fluid. Anti-GFAP antibody was a polyclonal antiserum from Dakopatts (Sydney, Australia) and was used at a dilution of 1:30. Anti-neurofilament antibody (16) was obtained from Immuno Nuclear (Stillwater, MN) and was used at a dilution of 1:150. K2F2, an anti-cytokeratin antibody (17), was obtained from D. Hewish (CSIRO, Melbourne, Australia) and was used at a dilution of 1:100. WEHY-NEP-6, an anti-glia cell precursor monoclonal antibody, was prepared in our laboratory (3) and used at a dilution of 1:10. Anti-H-2K<sup>6</sup> monoclonal antibody was from hybridoma clone 11-4.1 and anti-IA<sup>6</sup> monoclonal antibody was from hybridoma clone 10-2.16 (18); both hybridomas were obtained from the American Type Culture Collection.

Acidic FGF from bovine brain was obtained from R & D Systems (Minneapolis, MN). Recombinant bovine basic FGF was obtained from Collaborative Research (Waltham, MA). Recombinant interferon γ (IFN-γ) was obtained from Genentech (South San Francisco, CA).

**Fig. 1.** (A) Schematic representation of recombinant retroviral plasmid used to produce the c-myc retrovirus. The expression of the c-myc gene is controlled by the promoter and enhancer in the Moloney long terminal repeats (LTRs). The Neo<sup>6</sup> gene is expressed through the simian virus 40 early promoter and enhancer (SV). pBRori is the pBR322 origin of replication. R, EcoRI; B, BamHI; X, Xba I. (B) Southern blot analysis of DNA from neuroepithelium (lanes NE) and neuroepithelial cell lines (lanes 2.3Q, 2.3D.12, and 2.3D). Cellular DNA (2 μg) was digested with EcoRI (RI) or Xba I (X), size-fractionated on 0.7% agarose, and transferred onto a Zeta-Probe membrane (Bio-Rad) in 0.4 M NaOH. The filter was hybridized to <sup>32</sup>P-labeled c-myc cDNA clone (11). Hybridization and washing conditions were as described (13). Solid arrowheads mark the position of the endogenous c-myc-bearing fragments, and the open arrowheads indicate the proviral c-myc DNA bands. Sizes of fragments are in kilobase pairs. The intensities of the bands in various lanes vary because of unequal amounts of DNA loaded onto the gel.

**Fig. 2.** (A) Expression of c-myc in neuroepithelial cell lines. Poly(A)<sup>+</sup> RNA from several cell lines (indicated above the lanes) was fractionated on a formaldehyde/agarose gel and transferred to nitrocellulose filters for hybridization with <sup>32</sup>P-labeled probe (13). The c-myc probe was as above and the Neo probe was a 1.4-kilobase HindIII–Sal I fragment from pSV2 Neo (11). Lines 2.3D.17 and 2.3S.19 are clones isolated from 2.3D and 2.3S cell lines, respectively. Astroglia were from cultured populations of 19-day embryo cerebellar cells. (B) The same filters were rehybridized to a 32P-labeled Neo probe. The full-size viral RNA corresponds to the 6-kilobase transcript hybridizing to the c-myc probe; the smaller 3-kilobase RNA corresponds to the subgenomic simian virus 40–Neo mRNA. The amount of poly(A)<sup>+</sup> RNA loaded onto the gel for the 2.3S.19 cells and the astroglia was at least 3 times lower than in other lanes.
Table 1. Phenotype of neuroepithelial cell lines

<table>
<thead>
<tr>
<th>Cell(s)</th>
<th>Antibody markers</th>
<th>A2B5</th>
<th>GFAP</th>
<th>NEP</th>
<th>IAI*</th>
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<tr>
<td>E10 NE</td>
<td></td>
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<tr>
<td>2.3D, -S, + -Q</td>
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<td>95% of lines screened</td>
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<td>2.3A</td>
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<td>2.3R</td>
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Cells cultured on glass coverslips were examined for expression of various markers by immunofluorescence with the following antibodies: A2B5, anti-ganglioside; anti-GFAP; K2F2, anticytokeratin; WEHY-NEP-6, anti-gliial cell precursor; anti-H-2K* monoclonal antibody clone 11-4.1; and anti-I-A* monoclonal antibody clone 10-2-16. All cells listed were K2F2-positive, H-2-negative, H-2-positive in the presence of IFN-γ, and I-A-negative. The antigenic phenotype of the E10 neuroepithelium (NE) was similar to that of 95% of the cell lines screened. -, Absence of marker; +, presence of marker.

*Cells were incubated with IFN-γ (1 unit/ml) for 24 hr prior to staining; +, indicates the ability to express these antigens in the presence of IFN-γ.

Xba I cuts within each proviral long terminal repeat, releasing a 5.5-kilobase fragment. Since, for 2.3Q cells, the intensity of this band was about half that of the 8.5-kilobase fragment from the endogenous c-myc alleles, it is clear that this line carries only a single provirus and is, therefore, clonal. The line 2.3D harbors multiple inserts, as judged by the relative intensity of the Xba I fragments. Individual proviral inserts can be distinguished in EcoRI digests: there is only one EcoRI site within the provirus, and the size of the released fragment thus depends on the location of the nearest EcoRI site in the flanking DNA. By this criterion, 2.3D cells carry three inserts, two within EcoRI fragments of similar size. Since the number and size of the proviral fragments was identical in the subclone 2.3D.12 (Fig. 1B), the 2.3D line is also clonal.

Expression of c-myc mRNA. Analysis of mRNA from several neuroepithelial cell lines for c-myc expression showed that both the proviral and endogenous c-myc genes are expressed in all cell lines (Fig. 2A). As expected from the number of integrated proviruses, the level of the 6-kilobase proviral c-myc transcript is higher in the 2.3D line than in the 2.3Q line. Significantly, the 2.3-kilobase endogenous c-myc transcript is expressed in all the lines examined. Thus, the introduction of the proviral c-myc gene has not silenced the endogenous c-myc gene. This result contrasts with those obtained for B lymphocytes in transgenic mice carrying a c-myc oncogene driven by the immunoglobulin heavy chain enhancer, where no expression of the endogenous c-myc gene could be detected (19, 20). However, it is similar to that reported for some fibroblast cell lines where expression of the endogenous c-myc gene is not suppressed by the introduced c-myc gene (21).

Tumorigenicity of the Neuroepithelial Cell Lines. The c-myc virus-infected cells were not tumorigenic. Syngeneic animals injected with 5 × 10⁶ cells from several lines were monitored for 3 months, but no tumors were detected. Thus, by itself, deregulated c-myc expression appears not to be sufficient to transform neuroepithelial cells. However, after continual passage in vitro for over a year the 2.3S and -Q cell lines were capable of generating tumors when injected subcutaneously into mice. The transformation of these cell lines was not associated with any discernible alteration in morphology or surface phenotype. Furthermore, RNA gel blot analysis has shown no significant changes in the expression of either the endogenous or the proviral c-myc genes (data not shown). We presume that transformation resulted from accumulation of additional genetic change(s) during long term culture.

Phenotype of Neuroepithelial Cell Lines. The majority of the cell lines generated from the neuroepithelium appear to represent cells at a very early stage of neural differentiation. They have the morphological appearance of normal neuroepithelium cultured in vitro and express similar antigenic markers (Table 1). All the cell lines express the cytokeratin intermediate filament; this marker is also found in E10 neuroepithelium but is lost during neural differentiation and is not present in mature glial or neuronal cells (3). A further indication that these lines are of an immature phenotype is that the majority of the cell lines (95%) do not express neurofilaments or the neuronal surface marker A2B5 (15) [also expressed on some glial cell precursors (22)] or the astrocyte-associated intermediate filament GFAP. In addition, the lines do not express the surface marker NEP normally present on the glial precursor cells prior to expression of GFAP (3). In an attempt to induce these lines to differentiate or to shift antigenic phenotype, we have used several agents known to induce cellular differentiation, such as phorbol esters, retinoic acid, and 8-azacytidine, but have observed no surface phenotypic changes in any of the lines examined. However, IFN-γ and acidic and basic FGF have been effective in initiating phenotype changes.

Induction of Surface Antigens by IFN-γ. The neuroepithelial cell lines are similar to E10 neuroepithelium in that they normally do not express surface class I and class II

![Fig. 3](image-url) Neuroepithelial cell line 2.3D incubated with IFN-γ (1 unit/ml) for 48 hr in vitro and then stained for the expression of surface H-2K* with monoclonal antibody 11-4.1 and sheep anti-mouse immunoglobulin antibody coupled with fluorescein. Phase-contrast (A) and fluorescent (B) micrographs are shown. (× 270.)
histocompatibility antigens. This property is also shared by mature neurons and glia; however, it has been shown that some mature neural cells can be induced to express both classes of molecules after incubation with IFN-γ (23). We have found in all the 140 neuroepithelial cell lines tested that classes I and II histocompatibility molecules can be induced within 48 hr after IFN-γ treatment (Fig. 3 and Table 1). The expression of class I antigens is similarly induced on freshly isolated E10 neuroepithelium (3). However, the expression of class II antigens is not seen on normal neuroepithelium and is only found on GFAP-positive astrocytes. The significance of this finding is unclear, although it may indicate that these lines are biased toward the glial differentiation pathway. As shown in in Fig. 2A the pretreatment of the neuroepithelial cell lines with IFN-γ had no effect upon endogenous or proviral c-myc gene expression.

**FGF Stimulates Differentiation.** Acidic and basic FGF are found in relatively high concentrations in the brain (24) and have been shown to enhance neuronal survival *in vitro* and *in vivo* (25). Results from our laboratory indicate that FGF is a potent proliferative stimulus to freshly isolated E10 neuroepithelium and, at concentrations >5 ng/ml, induced morpho-differentiation (M. Murphy, J. Drago, and P.F.B., unpublished observations). Cells from the 2.3D cell line were

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Fig. 4. Neuroepithelial cell line 2.3D cultured for 72 hr in basic FGF (50 ng/ml). Although this line does not normally express neural markers the FGF-stimulated line contained GFAP-positive (A and B) as well as A2B5 (C and D)- and neurofilament (E and F)-positive cell types. Phase-contrast (A, C, and E) and fluorescent (B, D, and F) photomicrographs are shown. (×270.)
incubated with FGF (5 ng/ml) and showed obvious morphological changes within 24 hr of incubation, and some of the cells begin to round up and form aggregates that sometimes detached from the plate. Staining of coverslips of these lines after treatment with FGF for the presence of neuronal and glial markers has revealed cells containing GFAP (Fig. 4 A and B) as well as a process bearing population that is both A2B5-positive (Fig. 4 C and D) and also contains neurofilament protein (Fig. 4 E and F). A large percentage of the cells (40–50%) express neurofilaments by 3 days, whereas GFAP is not detected until 7 days after the addition of FGF.

DISCUSSION

We have used a recombinant retrovirus bearing the murine c-myc protooncogene to immortalize neuroepithelial cells isolated from the neural tube of E10 mouse embryos. It is clear from these studies that c-myc is efficient in immortalizing these neuroepithelial cells without transformation. Although this property of immortalization has been reported (5) in rat embryo fibroblasts, this is the first report to our knowledge demonstrating that immortalization of freshly isolated embryonic cells results in the maintenance of a stable phenotype in cells that are normally capable of differentiation. Our results differ from those obtained with chicken embryo neuroretinal cells infected with the v-myc MC29 retrovirus (9), in which continuous neuronal differentiation was observed. Several differences exist between the two systems that may explain the discrepancy. First, the neuroretinal cells are efficiently transformed in vitro by the avian v-myc retroviruses. Second, the infected neuroretinal cells were not selected after the infection with MC29, as are the Neoφ cells in this report; therefore, it is difficult to assess the stage at which viral integration really occurred. The reasons why elevated c-myc expression can be associated with blocking differentiation in some cell lines (6, 7) and yet with allowing differentiation to proceed in others (9, 19) are unclear. It has been suggested (19) that the level of c-myc expression sets the balance between proliferation and cellular differentiation. High levels of c-myc expression promote proliferation and, therefore, inhibit differentiation, as observed in erythroleukemia cell lines (6, 7), whereas reduction in c-myc expression is associated with differentiation, as seen in the promyelocytic leukemia cell line HL-60 (26). It is also possible that deregulation of the endogenous c-myc is important in determining whether the cells are capable of differentiation or not. In most of the cases where exogenous c-myc was introduced into cells expression of the endogenous c-myc was turned off (20). However, in all the c-myc-immortalized cell lines so far examined endogenous c-myc was expressed (Fig. 2). Although the ratio between the endogenous and the exogenous c-myc mRNA varies from line to line, the endogenous c-myc was expressed in all the lines examined at levels comparable to that found in normal E10 neuroepithelium (data now shown).

The advantage of generating neuroepithelial cell lines frozen at a defined stage of differentiation is that it provides an inexhaustible supply of material for analysis at the cellular and molecular levels. The production of other neural cell lines by c-myc integration representative of different stages of differentiation should be possible. Furthermore, the infection of pure or enriched populations of neural cells with proviral c-myc should allow the selection of cell lines of predetermined phenotype.

It is clear from these experiments that, although the cell lines have a stable phenotype, they retain the ability to respond to discrete epigenetic stimuli and to undergo differentiation into mature neural cells. This observation confirms the nature of the cell type immortalized and indicates that we are indeed producing lines of neural precursor cells. The observation that both neuronal and glial cells can be generated from a cloned cell line indicates that many of the neuroepithelial cells are multipotent and are not restricted to a particular pathway at this stage of development. It has been shown that a proportion of E10 neuroepithelial cells are committed to a particular neural pathway (3), and further screening of cell lines or cell lines established from older embryos may reveal such lineage restrictions.

The ability of immortalized cell lines to respond to factors, such as FGF, that also stimulate normal neuroepithelium differentiation is further evidence that the immortalized cells retain many of the properties of normal precursors and, therefore, should be useful in the study of factor and gene regulation of neural differentiation.

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