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A Multipotent EGF-Responsive Striatal Embryonic Progenitor Cell Produces Neurons and Astrocytes

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The mitogenic actions of epidermal growth factor (EGF) were examined in low-density, dissociated cultures of embryonic day 14 mouse striatal primordia, under serum-free defined conditions. EGF induced the proliferation of single progenitor cells that began to divide between 5 and 7 d *in vitro*, and after 13 d *in vitro* had formed a cluster of undifferentiated cells that expressed nestin, an intermediate filament present in neuroepithelial stem cells. In the continued presence of EGF, cells migrated from the proliferating core and differentiated into neurons and astrocytes. The actions of EGF were mimicked by the homolog transforming growth factor α (TGFα), but not by NGF, basic fibroblast growth factor, platelet-derived growth factor, or TGFβ. In EGF-generated cultures, cells with neuronal morphology contained immunoreactivity for GABA, substance P, and methionine-enkephalin, three neurotransmitters of the adult striatum. Amplification of embryonic day 14 striatal mRNA by using reverse transcription/PCR revealed mRNAs for EGF, TGFα, and the EGF receptor. These findings suggest that EGF and/or TGFα may act on a multipotent progenitor cell in the striatum to generate both neurons and astrocytes.

The adult mammalian CNS comprises a large number of cell types that arise over a short period of time from a small number of cells in the neural tube. Although the sequence of events, proliferation, migration, differentiation, occurring during this process has been well described (for review, see McConnell, 1988; McKay, 1989), little is known about the epigenetic signals responsible for its initiation. Two polypeptide growth factors, basic fibroblast growth factor (bFGF) and NGF, have been demonstrated to regulate the *in vitro* proliferation of neuronal precursors of the mammalian CNS. Gensburger et al. (1987) reported that bFGF induced a twofold enhancement of the proliferation of embryonic day 13 (E13) rat cortical neuronal precursors, while Murphy et al. (1990) demonstrated that bFGF enhanced, up to 100-fold, the division and survival of E10 mouse neuroepithelial cells. Cattaneo and McKay (1990) reported that NGF enhanced (2.5-fold) the proliferation of E13.5–E14.5 rat striatal neuronal precursors, whose initial production and/or survival was induced by bFGF.

Epidermal growth factor (EGF) has been demonstrated to have numerous actions on CNS nerve cells *in vitro*. In E15 rat aggregating brain cell cultures, EGF enhanced glial cell maturation (Honegger and Guentert-Lauber, 1983; Almazan et al., 1985) and altered the synthesis and secretion of soluble proteins (Guentert-Lauber and Honegger, 1983; Tenot et al., 1989). EGF has been reported to induce neuritogenesis in E8 chick telencephalic neuronal precursors (Rosenberg and Noble, 1989), stimulate the division of postnatal rat protoplasmic (type I) astrocytes (Simpson et al., 1982; Raff et al., 1983), enhance the survival of postnatal day 0–1 (P0–P1) rat neurons from numerous brain regions (Morrison et al., 1987, 1988), inhibit myelin basic protein expression in P4 mouse oligodendrocytes (Sheng et al., 1989), and exert neuronotrophic effects on dopaminergic neurons of the embryonic ventral mesencephalon (Casper et al., 1991; Ferrari et al., 1991). Recently, Anchan et al. (1991) found that EGF and transforming growth factor α (TGFα) enhanced the proliferation of E18 or P2 rat retinal neuroepithelial cells. These *in vitro* studies suggest that EGF or an EGF-like molecule may play a role in the normal development of the mammalian CNS.

The present study asks whether EGF is a mitogen for precursor cells of the embryonic mouse striatum. We find that EGF and TGFα induce the proliferation of a multipotent progenitor cell that gives rise to both neurons and astrocytes. The phenotype of the cells produced and the presence of EGF, TGFα, and EGF receptor mRNAs in the E14 striatum are consistent with a role for these factors in striatal cell production.

Materials and Methods

Primary cultures. Striata were removed from 14-d-old CD_1 albino mouse embryos (Charles River) and mechanically dissociated with a fire-polished Pasteur pipette in serum-free medium composed of 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and F-12 nutrient (GIBCO). Cells (2500/cm^2) were plated on poly-L-ornithine–coated (15 μg/ml; Sigma) glass coverslips in 24 well Nunclon (0.5 ml/well) culture dishes. The culture medium was a serum-free medium composed of DMEM/F-12 (1:1) including glucose (0.6%), glutamine (2 mM), sodium bicarbonate (3 mM), and HEPES buffer (5 mM) [all from Sigma except glutamine (GIBCO)]. A defined hormone mix and salt mixture (Sigma) that included insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (60 μM), and selenium chloride (30 nM) was used in place of serum. Cultures contained the above medium together with 20 ng/ml EGF (purified from mouse submaxillary; Collaborative Research) or TGFα [human recombinant; GIBCO/Bethesda Research Laborato-

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ries (BRL)]. After 10–14 d *in vitro* (DIV), media (DMEM/F-12 plus hormone mixture) and EGF were replaced. This medium change was repeated every 2–4 d. The number of surviving cells at 5 DIV was determined by incubating the coverslips in 0.4% trypan blue (GIBCO) for 2 min, washing with phosphate-buffered saline (PBS; pH 7.3), and counting the number of cells that excluded dye with a Nikon Diaphot inverted microscope.

For examination of the clonal nature of EGF-induced proliferation, cells were diluted to yield approximately 1 cell/well of 96-well (100 μl/well) Nunclon plates. The presence of a single cell in a well was confirmed by using phase-contrast microscopy.

Antibodies. Rabbit antiserum to neuron-specific enolase (NSE; used at 1:500) was purchased from Dakopatts, mouse monoclonal antibody to glial fibrillary acidic protein (GFAP; 1:100) was obtained from Boehringer Mannheim, and a mouse monoclonal antibody to bromodeoxyuridine (BrdU; undiluted) was purchased from Amersham. Rabbit antiserum to nestin (Rat 401; 1:1500) was a gift from Drs. M. Marvin and R. McKay (MIT, Cambridge, MA), and a mouse monoclonal antibody to neurofilament (168 kDa; clone RMO 270, 1:50) was generously supplied by Dr. V. Lee (University of Pennsylvania, Philadelphia, PA). The rabbit antisera to GABA (1:3000), methionine-enkephalin (1:1000), and substance P (1:500) were purchased from Incstar. Fluorescein-conjugated affinipure goat antibody to mouse IgG and rhodamine-conjugated affinipure goat antibody to rabbit IgG were obtained from Jackson. BrdU was purchased from Sigma, and Fluorsave, from Calbiochem.

Immunocytochemistry. Indirect immunocytochemistry was carried out with cells that had been cultured for 6–30 DIV on glass coverslips. All coverslips were fixed with 4% paraformaldehyde for 30 min followed by three (10 min each) washes in PBS. For dual labeling experiments, primary antibody and antiserum were added together. Following the PBS rinse, coverslips were incubated in the primary antibody and/or antiserum in PBS/10% normal goat serum/0.3% Triton X-100 for 2 hr at 37°C. Coverslips were washed three times (10 min each) in PBS and incubated with secondary antibodies (1:100) for 30 min at 37°C. Coverslips were then washed three times (10 min each) in PBS, rinsed with water, placed on glass slides, and coverslipped using Fluorsave as the mounting medium. Fluorescence was detected and photographed with a Nikon Optiphot photomicroscope.

The neuronal specificity of NSE antiserum used in this study was confirmed by using a second source of rabbit antiserum to NSE (1:1000; gift of Dr. R. Hawkes, University of Calgary), which showed the identical pattern of staining demonstrated in Figures 4–6 (data not shown). Furthermore, the morphological characteristics and patterns of staining (in Figs. 4–6) were not reproduced when rabbit antiserum to glial-specific enolase (1:5000; gift of Dr. R. Hawkes) or rabbit antiserum to galactocerebroside (1:200; Advanced Immunochemical Services) were used as primary antisera (data not shown). The staining observed by using antiserum to nestin was not reproduced when the primary antiserum was omitted or substituted by preimmune serum.

BrdU labeling and detection. BrdU (1 μM) was added to cells with the normal medium change at 14 DIV. BrdU-free medium was reintroduced with the normal medium change 7 d later. Cells were examined for BrdU incorporation by indirect immunocytochemistry as detailed above: fixation was with 4% paraformaldehyde, antibody to BrdU (undiluted) and antiserum to NSE (1:500) were combined, and secondary antibodies were fluorescein-conjugated affinipure goat anti-mouse IgG and rhodamine-conjugated affinipure goat anti-rabbit IgG (1:100) combined.

Reverse transcription and polymerase chain reaction (PCR). Total RNA was extracted from mouse embryonic striata (E14) using the acid guanidinium-thiocyanate-phenol extraction procedure of Chomczynski and Sacchi (1987). This RNA was transcribed into first-strand cDNA using oligo-dT priming. In brief, 0.5 μg of RNA was transcribed for 1 hr at 42°C in 10 μl of a reaction mixture containing 50 mM Tris-HCl (pH 8.3), 60 mM KCl, 2 mM $MgCl_2$, 7.5 mM dithiothreitol, 2.5 μM oligo-dT (16–18mer, Pharmacia), 2 mM of each deoxynucleotides, 0.1 mg/ml nuclease-free bovine serum albumin (BSA), 5 U of RNasin (Promega), and 200 U of mouse muloney leukemia virus reverse transcriptase (Superscript, BRL). Each reaction yielded enough cDNA for 10 amplifications by PCR: 50 μl of the PCR reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg/ml nuclease-free BSA, 0.6mM of each dNTP, 1 U of Taq polymerase (Pharmacia), and 1 μg of the 3′ and 5′ primers. The specific primers used for amplification of EGF mRNA were taken from the mouse EGF sequence given in Gray et al. (1983); specifically, the 5′ primer corresponded to bases 3261–3281 and the 3′ primer was complementary to bases 4200–4179. For amplification of TGFα mRNA, the 5′ primer was a 21mer corresponding to the sequence coding for amino acids 20–26 in Lee et al. (1985) and the 3′ primer was a 21mer complementary to amino acids 111–117. For the EGF receptor, the 5′ primer corresponded to bases 1358–1382 and the 3′ primer was complementary to 1644–1623 of the rat sequence given in Petch et al. (1990). PCR amplification was performed for 40 cycles (1 min at 94°C, 2 min at 45°C, and 2 min at 72°C) in a Coy (Diamed) thermocycler. The PCR products were separated on 2% agarose gels containing ethidium bromide, photographed to verify the expected size, and subsequently blotted onto nylon membranes (Zetaprobe, Bio-Rad). The EGF receptor amplification products were hybridized with an oligonucleotide complementary to the EGF receptor (bases 1528–1504) sequence. This probe was end-labeled using terminal deoxynucleotidyl transferase and radiolabeled ^{35}S-dATP (New England Nuclear, NEM-034 H). The EGF amplification products were probed with a 960 base pair (bp) insert of the pmEGF-26F12 cDNA (American Tissue Type Collection, 37486) labeled according to the oligonucleotide labeling procedure of Feinberg and Vogelstein (1983), as well as by an oligonucleotide probe complementary to the EGF (bases 3953–3972) sequence end-labeled using terminal deoxynucleotidyl transferase and radiolabeled ^{35}S-dATP. The TGFα amplification products were probed with an oligonucleotide complementary to amino acids 93–99, also labeled using terminal deoxynucleotidyl transferase and radiolabeled ^{35}S-dATP. Thus, all amplification products were probed with oligonucleotides complementary to sequences that were not overlapping with the primers. Hybridizations were done overnight according to standard protocols (Sambrook et al., 1989), and posthybridization washes were performed at 55°C using 2×, 0.5×, and 0.1× SSC (sodium citrate/sodium chloride; see Sambrook et al., 1989). The DNA blots were exposed to x-ray film for 4–48 hr. Positive controls from mouse submaxillary gland (EGF) and mouse liver (EGF receptor) were run in parallel. Negative controls without reverse transcription were blank.

Results

Proliferation of an EGF-responsive progenitor cell

Although 2500 viable E14 striatal cells/cm^2 were plated (on poly-L-ornithine–coated coverslips, in the absence of serum), trypan blue staining after 5 DIV demonstrated that fewer than 1% of the plated cells were viable. In the presence of EGF (20 ng/ml), only 13 ± 3 viable cells/cm^2 ($n = 14$ independent culture preparations) were identified after 5 DIV. Thereafter, one to three cells per well would begin to divide (Fig. 1) and form proliferating clusters. The clonal nature of these clusters of cells was established by plating single cells (by limiting dilution of the cell suspension) in single wells of 96-well plates. Under these conditions, we observed the proliferation of a single cell into a cluster (Fig. 2*A–D*) that was indistinguishable from that observed with our standard plating procedure. The majority of cells in the proliferating cluster expressed nestin immunoreactivity (IR) (Fig. 2*E,F*), suggesting that they contained this intermediate filament protein found in CNS neuroepithelial stem cells (Frederiksen and McKay, 1988; Lendahl et al., 1990). At this stage (8–12 DIV), these same clusters were negative for neuronal or glial cell markers (see below). Figures 1 and 2 are representative of identical observations made in 48–96 replicates per experiment, carried out in 14 independent culture preparations. This pattern of proliferation was also observed when dissociated cells derived from E14 cerebellum, cortex, thalamus, septum, spinal cord, or ventral mesencephalon were plated under these experimental conditions (data not shown). In all cases, removal of EGF at any stage resulted in a cessation of proliferation.

Up until 7 DIV, addition of EGF resulted in proliferation, suggesting that addition of EGF at the time of plating is not required for the survival of the progenitor cells, although the cells did not begin to divide for 5–7 d. The actions of EGF were mimicked by equivalent concentrations of TGFα, a homolog of

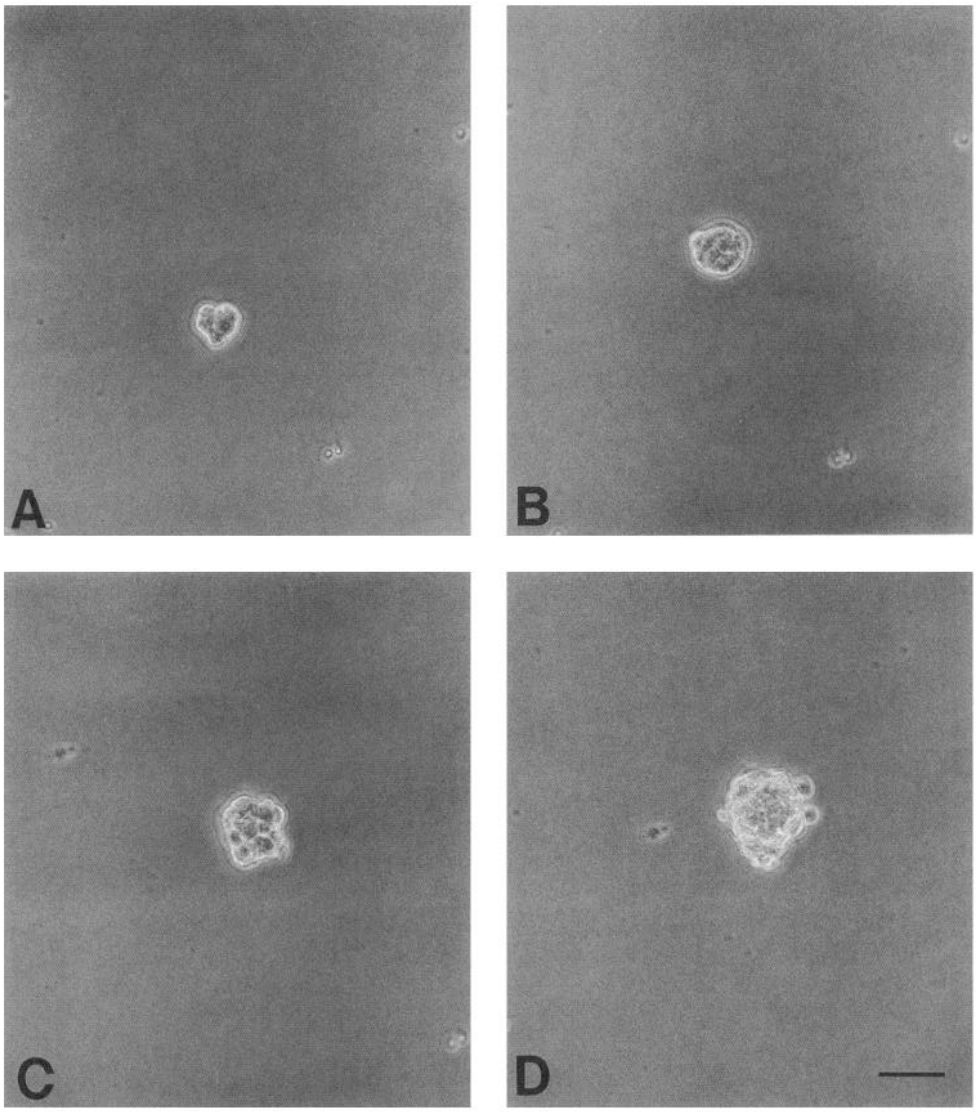

Figure 1. Initial cell division of an EGF-responsive striatal progenitor cell: phase-contrast micrographs of the initial cell divisions of an EGF-responsive progenitor at 5 DIV (*A*), 6 DIV (*B*), 6.5 DIV (*C*), and 7 DIV (*D*). Scale bar, 50 μm for *A–D*.

EGF (data not shown). However, if cells were exposed to equivalent concentrations of bFGF, NGF, platelet-derived growth factor, or TGFβ, at the time of plating under the experimental conditions described above, no proliferating clusters were observed.

Immunocytochemical identification of neurons and astrocytes produced in response to EGF

The maturation of a colony of proliferating cells originating from a single progenitor is shown in Figure 3. The progenitor began dividing at 5–7 DIV, and by 13 DIV a proliferating cluster of cells had developed (Fig. 3*A*). By 18 DIV, the proliferating core had grown and cells were beginning to migrate from the center (Fig. 3*B*). At 21 DIV (Fig. 3*C*), all cells appeared to originate from the centrally located cluster. However, by 25 DIV (Fig. 3*D*), new proliferating clusters were observed (arrows).

A polyclonal antiserum directed against NSE and a monoclonal antibody against GFAP were used to determine if neurons and astrocytes were produced by the EGF-responsive progenitor. Prior to 14 DIV, no positive staining was observed. If BrdU was present in the medium during the 14–21 DIV period, it was incorporated into proliferating cells that were subsequently (25 DIV) identified as NSE-IR (Fig. 4), suggesting that neuronal proliferation was active during this period. At 25 DIV, NSE-IR cells were present in either EGF- or TGFα-generated cultures (Fig. 5). Dual-labeling, indirect immunocytochemistry at 25 DIV revealed numerous NSE- (Fig. 6*A*) and GFAP-IR (Fig. 6*B*) cells present in the EGF-generated culture. These two distinct populations of NSE- and GFAP-IR cells were present in all 16 independent EGF-generated culture preparations.

Many of the cells in the EGF-generated cultures, which had the morphology of the NSE-IR cells, were also immunoreactive for the 168 kDa form of neurofilament protein (Fig. 6*E*). The presence of CNS neurotransmitters in the EGF-generated cells was examined by indirect immunocytochemistry with anti-neurotransmitter antibodies. Cells with a neuronal morphology and immunoreactivity for GABA (Fig. 6*F*), substance P (Fig. 6*G*), and methionine-enkephalin (Fig. 6*H*) were present in 25 DIV EGF-generated cultures ($n = 3$ independent culture preparations). These cultures did not contain cells with immunoreactivity for neuropeptide Y, somatostatin, 5-HT, tyrosine hydroxylase, or glutamate (data not shown).

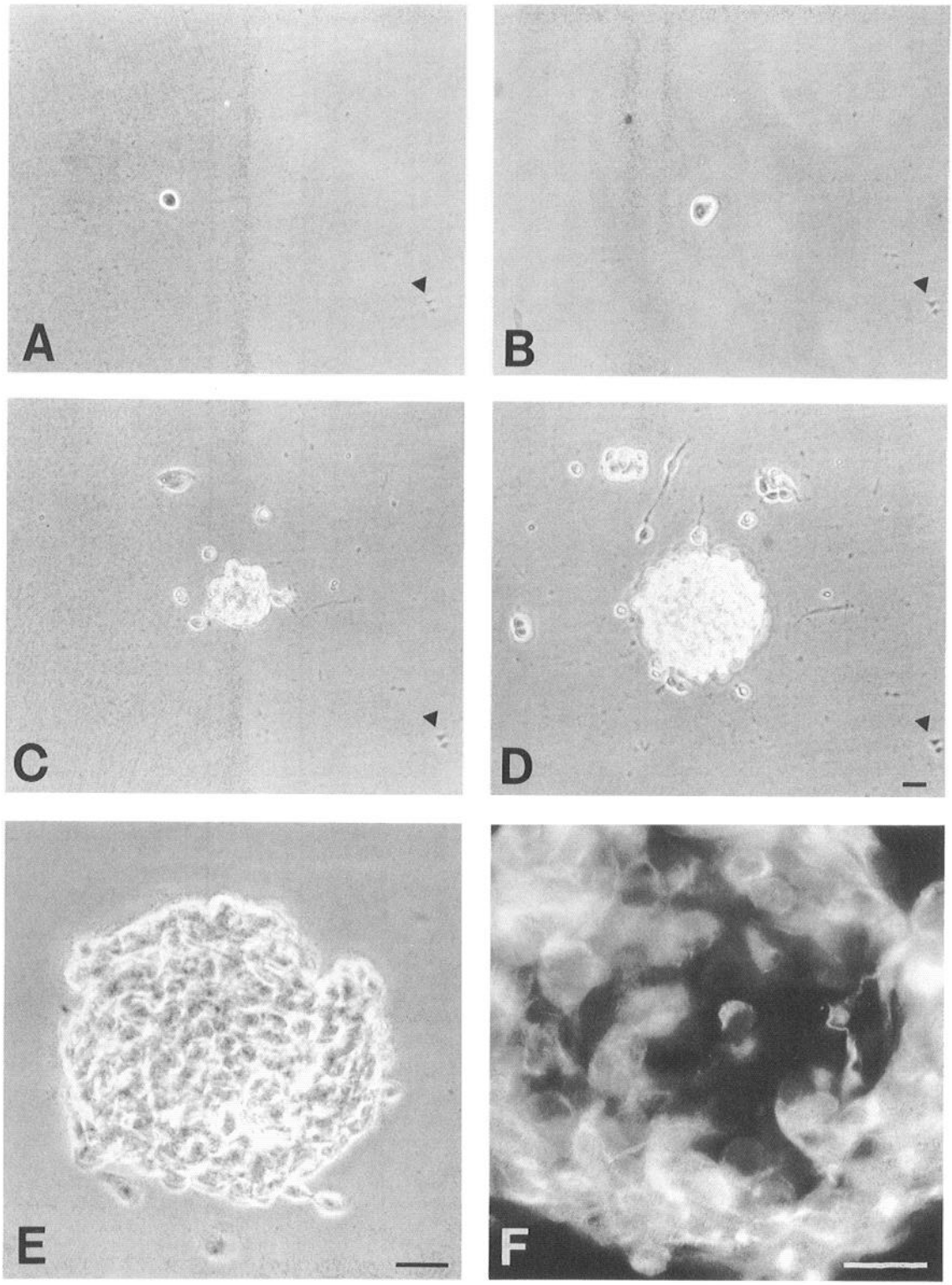

Figure 2. EGF-induced development of a cluster of undifferentiated cells from a single cell. With limiting dilution of the cell suspension, single cells were plated and identified at the base of 96-well plates, as described in Materials and Methods. *A–D,* Phase-contrast micrographs of a single cell after 3 DIV (*A*). The same cell began to divide after 5 DIV (*B*) and generated a cluster of cells observed after 7 DIV (*C*) and 10 DIV (*D*). Scratches in the plastic (*arrowheads*) serve to identify the field. *E* and *F,* Phase-contrast micrograph (*E*) of 10 DIV cluster of cells, generated in response to EGF; the majority of cells in this cluster contained nestin-IR (*F*). Scale bars, 10 μm.

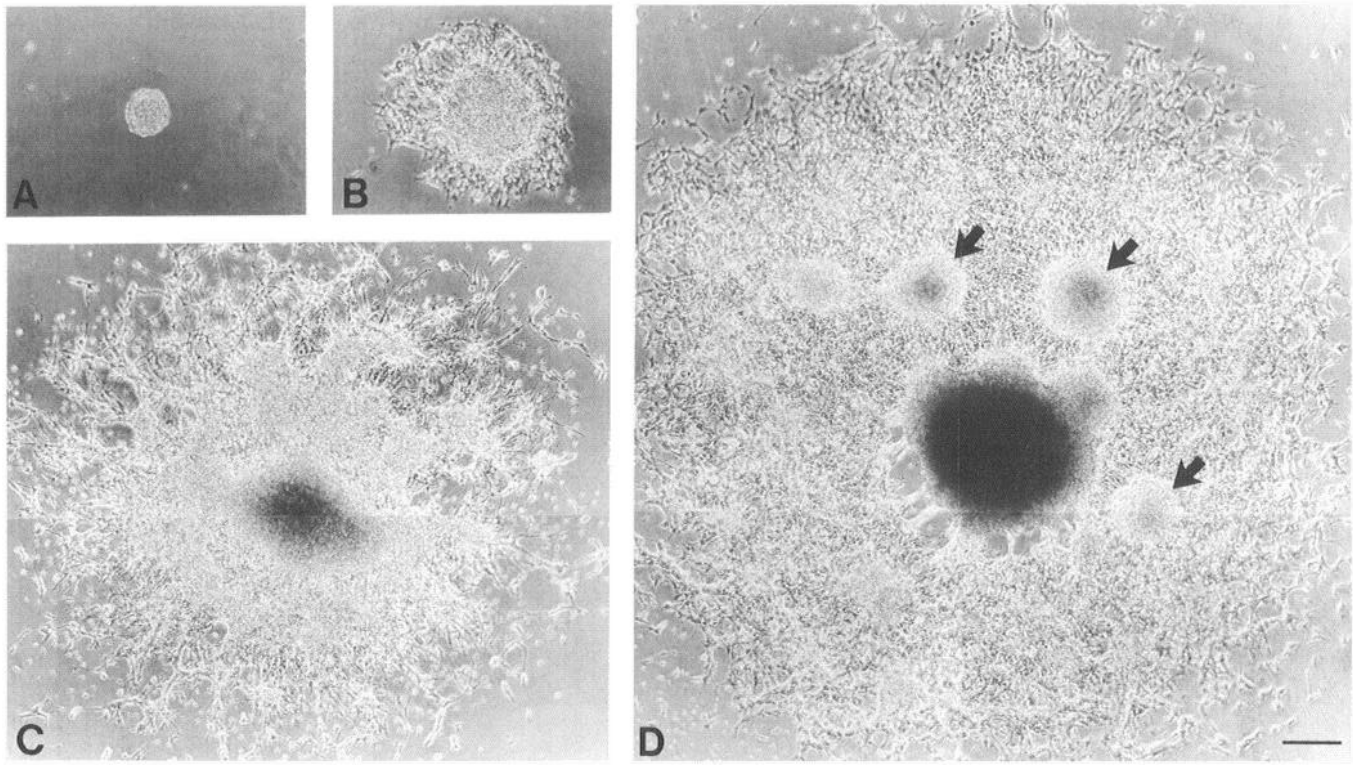

Figure 3. Time-lapse examination of the later stages of an EGF-induced proliferating cluster of striatal cells. Micrographs illustrate the same proliferating cluster at various developmental periods. *A,* At 13 DIV, the EGF-responsive progenitor had generated a small cluster composed of hundreds of cells. *B,* Two days later (15 DIV), cells had migrated away from the central proliferating core (immunocytochemical staining for NSE and GFAP were negative at this time). *C,* By 21 DIV, the central core continued to produce cells that migrated away from the center. *D,* At 25 DIV, the proliferating core was still growing and many more cells had migrated from the proliferating core. In addition, new or secondary proliferating clusters were apparent (*arrows*). Scale bar, 200 μm for *A–D.*

EGF, TGFα, and EGF receptor mRNA in the E14 striatum

The EGF- and TGFα-induced proliferation of multipotent progenitor cells of the E14 striatum suggests that these factors may play a role in striatal development. Although EGF and TGFα mRNA has been found in the adult mammalian CNS (Rall et al., 1985; Wilcox and Derynck, 1988) and the results of binding studies suggest that large numbers of EGF receptors are present during development (Adamson and Meek, 1984; Quirion et al., 1988), it is not known if the mRNAs for EGF, TGFα, and EGF receptor are present in the embryonic striatum. To address this question, we reverse transcribed mRNA isolated from the E14 striatum and used PCR followed by Southern blotting and hybridization with probes specific to EGF, TGFα, and EGF receptor. These experiments revealed that EGF, TGFα, and EGF receptor mRNAs were present in the E14 striatum (Fig. 7).

Discussion

We have studied the actions of EGF on E14 striatal cells in low-density, serum-free cultures. Our results demonstrate that a single progenitor cell will proliferate in response to EGF and give rise to a cluster of undifferentiated cells with the properties of neuroepithelial stem cells. In the continued presence of EGF,

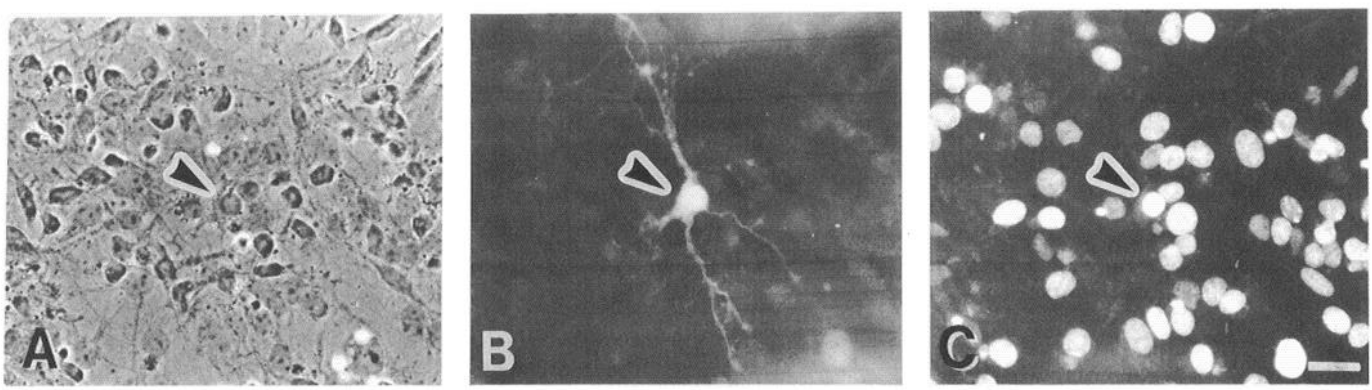

Figure 4. BrdU incorporation into NSE-IR cells. Between 14 and 21 DIV, 1 μM BrdU was included in the incubation media. After 25 DIV, cells were fixed and double stained with antibodies directed against NSE and BrdU. Micrographs demonstrate a cell (*arrowhead, A*) that is NSE-IR (*B*) and had incorporated BrdU (*C*). Scale bar, 20 μm.

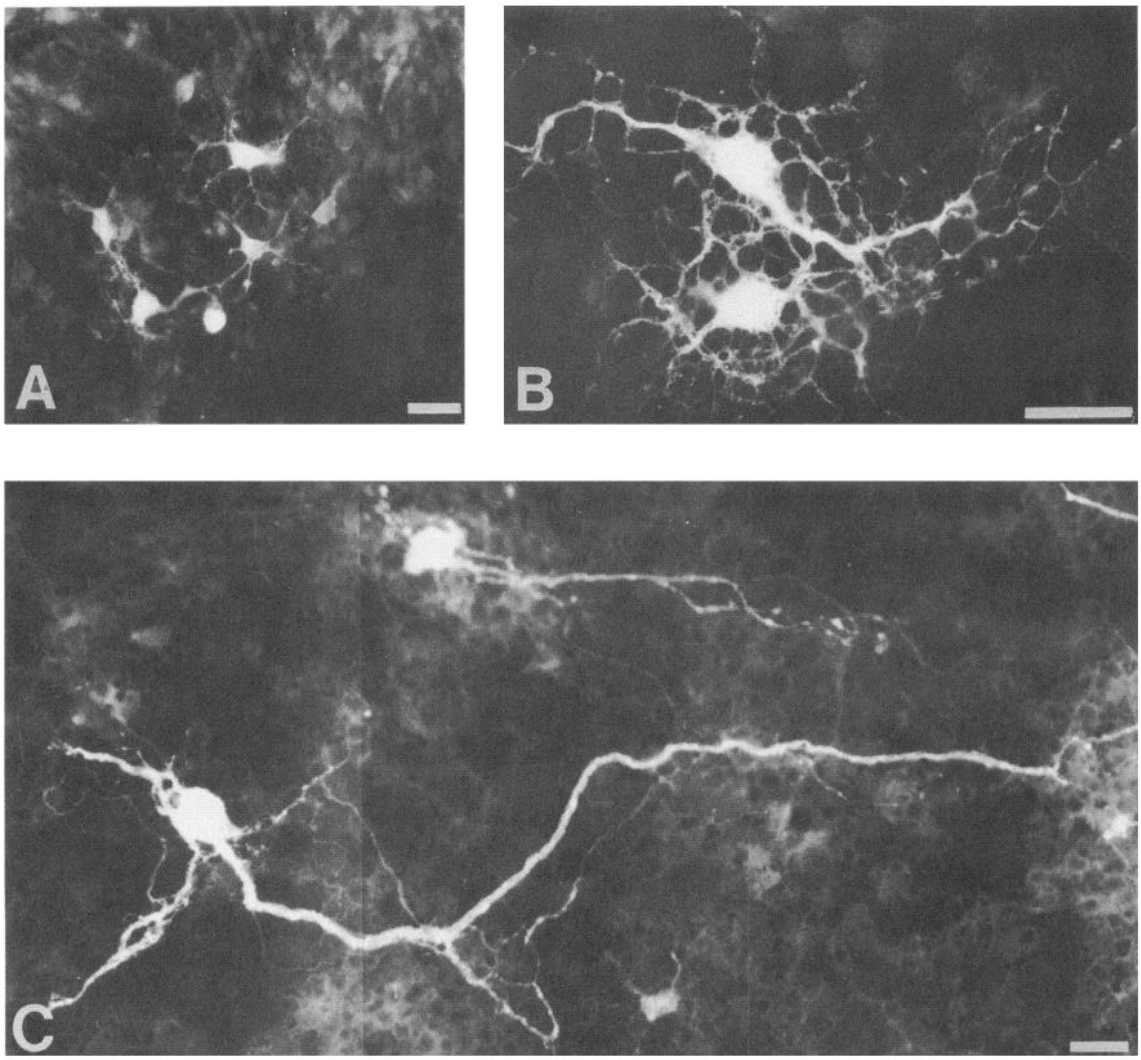

Figure 5. EGF- and TGFα-generated cultures contained NSE-IR cells. After 25 DIV, NSE-IR cells were present in both EGF-generated (*A, B*) and TGFα-generated (*C*) cultures. Some of the NSE-IR cells found had elaborate processes that were quite short and rarely more than two or three times the diameter of the cell body, while others had longer processes. Scale bars, 20 μm.

these cells continue to proliferate and differentiate into neurons and astrocytes. Cells with neuronal morphology express immunoreactivity for GABA, substance P, and met-enkephalin, three principal neurotransmitters of the adult striatum. EGF actions were mimicked by TGFα, but not any of the other factors examined. The mRNAs for EGF, TGFα, and the EGF receptor are present in the E14 striatum. Taken together, these findings suggest that EGF and/or TGFα may play a role in cell production in the developing striatum.

In establishing that a single cell gives rise to a cluster of undifferentiated, nestin-IR cells, we used a limiting dilution technique (Fig. 2) that was distinct from that used for demonstrating that single clusters give rise to neurons and astrocytes (Figs. 3–6). The tissue source and culture media in each series were identical. The former approach (utilizing 96-well culture dishes) was used, as the latter (utilizing glass coverslips for immunocytochemistry) was not amenable to single-cell analysis. Thus, our evidence for a single cell giving rise to neurons and astrocytes is indirect. Nevertheless, in *every* experiment when EGF-induced proliferation of a single cell was followed (as in Fig. 2; $n = 10$ cells in three independent culture preparations), a cluster of undifferentiated cells was produced. In addition, in *every* experiment when an EGF-generated cluster was followed for 25 DIV (as in Figs. 3–6; $n = 47$ clusters in 11 independent culture preparations), neurons and glia were produced. These findings strongly support our conclusion that single EGF-responsive progenitor cells give rise to neurons and glia.

Neurons and glia are primarily generated during different developmental periods (neurons preceding glia) (reviewed in McKay, 1989), findings that support the presence of separate precursors for neurons and glia. Lineage analysis studies in a variety of species, however, have provided convincing evidence for neuronal and glial cells being derived from multipotent precursor cells (Turner and Cepko, 1987; Frederiksen et al., 1988; Wetts and Fraser, 1988; Galileo et al., 1990). In fact, Turner et al. (1990) have reported that nearly all of the proliferating cells in the E13 and E14 mouse retina are multipotent and that these progenitors exist in the postnatal retina (Turner and Cepko, 1987). Raff and colleagues have characterized a CNS multipotent progenitor cell (O-2A) that will differentiate, *in vitro*, into oligodendrocytes and type 2 (stellate) astrocytes depending on intrinsic and environmental signals (reviewed in Raff, 1989;

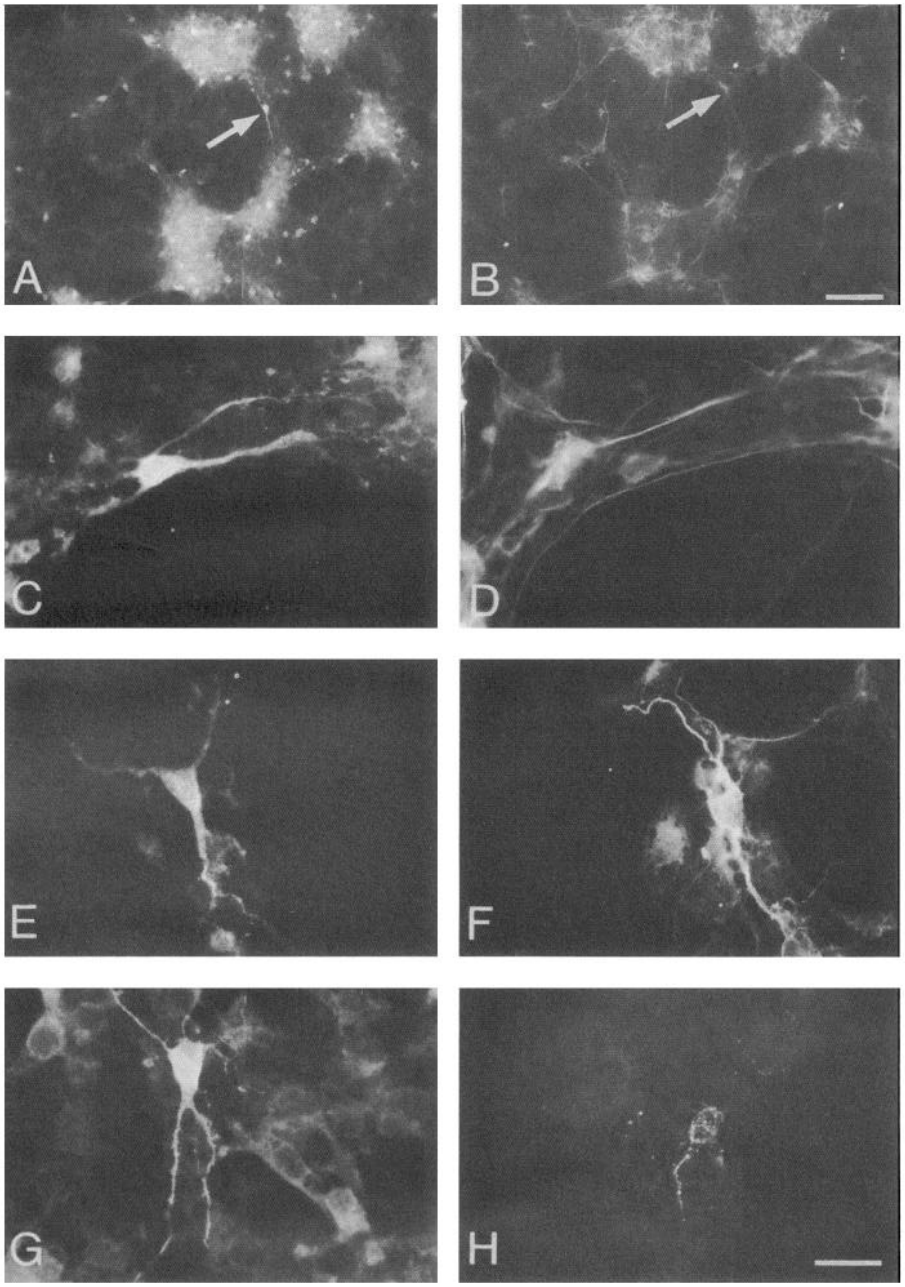

Figure 6. Indirect immunocytochemistry for antigens characteristic of neurons and astrocytes in EGF-generated cultures. Dual labeling with antisera to NSE (*A*) and GFAP (*B*) revealed numerous positive stained cells in 25 DIV, EGF-generated cultures. *C* and *D* are high-power photomicrographs of NSE-IR (*C*) and GFAP-IR (*D*) cells. The *arrows* in *A* and *B* point to cells seen in *C* and *D*, respectively. Many of the cells with neuronal morphology were neurofilament (168 kDa)-IR (*E*). In addition, cells with a neuronal morphology and immunoreactivity for GABA (*F*), substance P (*G*), and met-enkephalin (*H*) were present in EGF-generated cultures. Scale bars: *B* (for *A*, *B*), 200 μm; *H* (for *C–H*), 20 μm.

Lillien and Raff, 1990). Using a recombinant retrovirus carrying the LacZ gene to study the development of rat cerebral cortex precursor cells, Williams et al. (1991) found that up to 18% of the labeled clones gave rise to neurons and oligodendrocytes. In our study, we observed single EGF-responsive progenitor cells, induced into a unique pattern of proliferation. The clonal nature of this proliferation was established by plating single cells (in the presence of EGF) in single wells of 96-well plates and observing the identical pattern of proliferation. These individual, responsive cells generated a cluster of undifferentiated nestin-IR cells, many of which subsequently migrated from the proliferating core and initiated new proliferating cores or differentiated into neurons and astrocytes. In microculture studies of E13.5–E14.5 septal blast cells, Temple (1989) identified several classes of multipotent stem cells that produced neuronal and non-neuronal cells, whose production was dependent upon live conditioning cells. One particular class of multipotent cell (<1% of the initial septal cell suspension) was found to divide continuously, produce neurons and astrocytes, and produce additional blast cells with the same characteristics of the initial cell. These properties are remarkably similar to the EGF-responsive progenitor cell we describe in this report. It is reasonable to suggest that this low-abundant, highly proliferative multipotent progenitor cell may be present in various regions of the CNS. In support of this hypothesis is our finding that EGF-responsive multipotent progenitor cells are present in low-density cultures derived from cerebellum, cortex, septum, thalamus, spinal cord, and ventral mesencephalon of the embryonic mouse brain.

Until recently, only bFGF had been demonstrated to enhance the proliferation of embryonic CNS neuroblasts (Gensburger et al., 1987; Murphy et al., 1990). In the latter study, glial cell

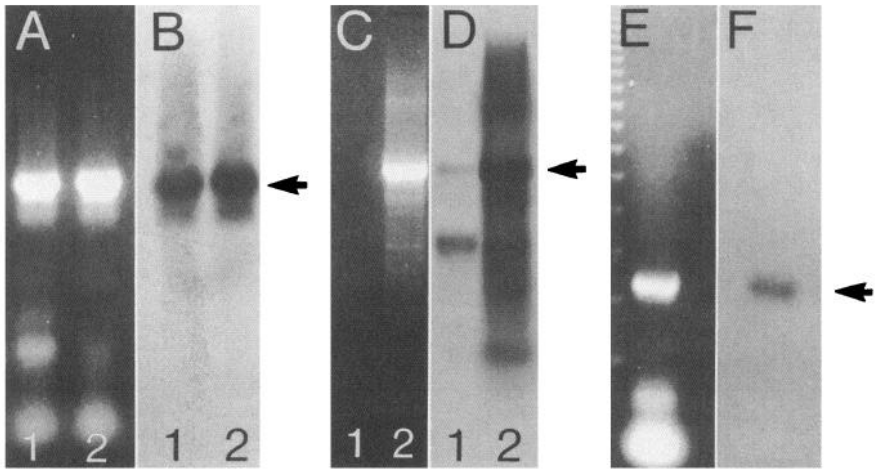

Figure 7. EGF, TGFα, and EGF receptor mRNA are present in the E14 mouse striatum: ethidium bromide–stained gels (*A, C, E*) and Southern blots (*B, D, F,*) of PCR product for EGF receptor (*A, B*), EGF (*C, D*), and TGFα (*E, F*). The EGF receptor (*A, B*) PCR product from E14 striatum (*1*) and liver (*2*) produced a strong single band of the expected size (286 bp; *arrow*). The bands gave a strong hybridization signal in Southern blots using an oligonucleotide probe synthesized from the rat EGF receptor sequence (*B*; see Materials and Methods). Amplification with the EGF primers produced a detectable band of the expected size (939 bp; *C, arrow*) on ethidium bromide stained gels for tissue from the submaxillary gland (*2*) but not for E14 striatum (*1*). However, a cDNA probe for EGF (*D*) revealed two bands on a Southern blot, one corresponding in size to the band found in the tissue obtained from the submaxillary gland (*arrow*). Identical results were obtained with an oligonucleotide probe (see Materials and Methods). The weak band in (*1*) is not due to overflow form (*2*) as it is also seen when we loaded the PCR product on nonadjacent lanes. Amplification with the TGFα primers produced a detectable band of the expected size (294 bp; *E, arrow*), which gave a strong hybridization signal in Southern blots using an oligonucleotide probe synthesized from the rat TGFα sequence (*F*) (see Materials and Methods).

proliferation was also observed in response to bFGF; however, the issue of separate or common precursors was not addressed. Although ineffectual alone, NGF was found to induce the proliferation of embryonic striatal neuroblasts that had been primed by bFGF (Cattaneo and McKay, 1990). Recently, Anchan et al. (1991) reported that EGF and TGFα stimulated retinal neuroepithelial cell proliferation. These authors demonstrated increased ^{3}H-thymidine labeling in low-serum-containing cultures exposed to EGF or TGFα. In our serum-free cultures, without the addition of EGF, no proliferation is observed. In addition, the removal of EGF, after proliferation has occurred for 2 or 3 weeks, results in a cessation of proliferation. This raises the question of whether, in the retinal cultures, EGF was (1) stimulating proliferation of progenitor cells or (2) enhancing proliferation by increasing the survival of progenitors that then went on to divide under intrinsic influence or under the influence of undefined factors (i.e., serum factors or factors released by other cells in the cultures). Furthermore, while Anchan et al. (1991) concluded that EGF and TGFα stimulated the proliferation of a multipotent progenitor cell, no clonality experiments were reported. We have been able to show (Fig. 2*A–D*) a single cell that divides *only* in response to EGF and gives rise to a cluster of undifferentiated cells, which continue to divide and differentiate into neurons and astrocytes.

Neurogenesis in the murine striatum occurs in the ventricular and subventricular zone beginning at E12 and continuing until the early postnatal period (Fentress et al., 1981). Our dissection procedure involved removal of the ventricular/subventricular region, dissociation into single cells, and plating at low density in a completely defined medium. Under these conditions, the majority of cells die, except the EGF-responsive progenitors that begin to proliferate after 5 DIV. In the continued presence of EGF, proliferation continues and differentiated cells are observed after 18–21 DIV. Previous investigations of the mitogenic actions of EGF (Simpson et al., 1982; Raff et al., 1983; Anchan et al., 1991) on embryonic or early postnatal cells did not reveal this pattern of proliferation. The explanation for our unique observation and prolonged time course may be the complete absence of serum and low plating density, respectively. When we examined EGF actions on low-density cultures, plated on a substrate that was precoated with serum, the proliferation observed was not of the pattern described above (e.g., clusters of round, undifferentiated cells, etc.); rather, the dividing cells adopted a flat morphology. In addition, proliferation of protoplasmic astrocytes in the absence of added growth factors was observed (B. A. Reynolds, W. Tetzlaff, and S. Weiss, unpublished observations). The utility of serum in promoting cell proliferation, differentiation, and survival of cultured cells notwithstanding, our findings suggest that the *in vitro* actions of defined growth factors may be influenced in an undefined manner by previous or concomitant exposure of cells to serum. Plating at a higher density (25,000 cells/cm^2) significantly accelerated the time course of proliferation and differentiation, with BrdU-labeled neurons and astrocytes present as early as 7–10 DIV (Reynolds, Tetzlaff, and Weiss, unpublished observations). While such a time course may be more compatible with cell production/differentiation *in vivo*, the presence of other surviving, nonproliferating cells precludes examination of a direct action of EGF on the progenitor cell.

Little is known regarding the signaling molecules responsible for production of specific phenotypes of CNS neurons. Recently, bFGF was demonstrated to enhance (threefold) the production of GABAergic neurons from E14 rat cerebral hemispheres (Deloulne et al., 1991). In our study, indirect immunocytochemistry of EGF-generated cells revealed populations with neuronal morphology that were immunoreactive for GABA, substance P, and met-enkephalin, three of the principal neurotransmitters of projection neurons of the adult striatum (Graybiel, 1990). On the other hand, other striatal phenotypes, for example, the somatostatin/neuropeptide Y interneurons, were not found. Several

questions remain unanswered by this study. These include (1) why only specific phenotypes are produced, (2) whether these neurotransmitters are colocalized, and (3) what is the ontogeny of the phenotypes produced and would others be produced at later stages? A detailed examination of the neuronal phenotypes produced by EGF-generated cultures, and the influence of other defined growth factors, is the subject of current investigations.

The results of ligand binding studies demonstrated that the number of binding sites for ^{125}I-EGF in the rodent brain increased during gestation (Adamson and Meek, 1984) and was higher in the first postnatal week than in the adult (Quirion et al., 1988), consistent with a role for this factor in CNS development. Our *in vitro* results suggest that EGF-like factors may be signaling molecules, which stimulate the proliferation of striatal neuroepithelial stem cells, resulting in an increase in the number of stem cells and the production of neuroblasts and glioblasts. The presence of EGF, TGFα, and EGF receptor mRNA in the E14 striatum suggests that EGF and/or TGFα may play a role during striatal development. Although reverse transcription/PCR is not quantitative, the signal for TGFα mRNA was stronger than that detected for EGF mRNA. Utilizing a quantitative nuclease protection assay, Lazar and Blum (1992) have recently shown that in the E14 whole brain and adult striatum of the mouse, TGFα mRNA is present in significantly greater quantities than EGF mRNA. Thus, in the developing mouse striatum, TGFα mRNA may be present in greater quantities than EGF mRNA. Understanding the expression of EGF-like growth factors and their receptors in the developing striatum would also be of considerable interest given recent reports of stem cells in the adult striatum. Morshead and van der Kooy (1992) have identified a population of cells in the rostrolateral region of the lateral ventricle of the adult mouse that continue to proliferate. Interestingly, the fate of half of the postmitotic progeny is death, suggestive of a stem cell under restrictive influences. In addition, we have recently found that EGF-responsive progenitor cells may be isolated from the adult striatum, consistent with the persistence of these stem cells in the adult brain following the end of neurogenesis (Reynolds and Weiss, 1992). While the cells produced by the adult progenitor cells are similar to those reported in this study, our findings suggest that the adult cells may be under restrictive influences of the substrate or extracellular matrix. Previous studies have demonstrated the presence of EGF- and TGFα-IR (Fallon and Seroogy, 1984; Code et al., 1987; Kudlow et al., 1989), EGF and TGFα mRNA (Rall et al., 1985; Wilcox and Derynck, 1988), and EGF receptor-IR (Gomez-Pinilla et al., 1988; Werner et al., 1988) in the adult rodent and human CNS.

Examination of factors that influence the *in situ* proliferation of these cells in the developing striatum, coupled with the *in situ* identification and localization of EGF-like factors and EGF receptors, will serve to elucidate the role for these factors in regulating the activity of striatal stem cells.

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