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Generation and Characterization of Embryonic Striatal Conditionally Immortalized ST14A Cells

Elena Cattaneo* and Luciano Conti
Institute of Pharmacological Sciences, University of Milano, Milano, Italy

Neural progenitor cells have been isolated from the embryonic central nervous system (CNS) of several mammalian species. These exhibit properties of immature cells, including expression of the intermediate filament protein Nestin, the ability to self renew, and to give rise to terminally differentiated cell types. In this study we describe some of the properties of ST14A cells, which were established via retroviral transduction of the temperature-sensitive mutant of the SV40 Large T Antigen into primary cells derived from the embryonic day 14 (E14) rat Striatum primordia. At 33° C, ST14A cells proliferate and express Nestin, whereas at the nonpermissive temperature, cell growth becomes restricted in coincidence with the disappearance of the immortalizing oncoprotein. We also describe the ability of ST14A cells to differentiate and express MAP2. Furthermore, we analyzed the expression of specific growth factors and growth factor receptors in the ST14A cells, and found that nerve growth factor (NGF) and Trk receptors are most commonly expressed. J. Neurosci. Res. 53:223–234, 1998. © 1998 Wiley-Liss, Inc.

Key words: SV40 Large T Antigen; immortalization; CNS progenitor cells; differentiation

INTRODUCTION

The cell types of the adult mammalian brain originate from the proliferating stem cells located in the ventricular zone of the embryonic brain (McKay, 1989; Jacobson, 1991; Gage et al., 1995). Several groups have shown that these stem cells (and their committed derivates) can be isolated and expanded in vitro in the presence of specific mitogens. Under these conditions, the cells maintain features of immature cells such as Nestin expression (Frederiksen and McKay, 1988; Lendahl et al., 1990), the ability to self renew and to give rise to multiple differentiated cell types (Gage et al., 1995; Svendson et al., 1995). Significant advances in understanding the factors and genes that regulate the differentiation of immature central nervous system (CNS) cells have been made over the last few years (Johe et al., 1996). One strategy employed by various groups has been to attempt to derive homogenous cell populations that are representative of these immature CNS cells (Whittemore et al., 1994; Woodring et al., 1989; Ryder et al., 1990; Bernard et al., 1989; Redies at al., 1991; Jung et al., 1994; Bartlett et al., 1988; MacDonald et al., 1996; Snyder et al., 1992; Mehler et al., 1993). Indeed, although the use of primary cultures provides an important yet limited and heterogenous supply of cells, the development of clonal cell lines represents an interesting tool for studies on cell lineage and cell differentiation, as well as on the expression of exogenous genes (Lendhal and McKay, 1990; Cattaneo and McKay, 1991; Cattaneo et al., 1996). Oncogene-containing retroviral vectors that are capable of infecting dividing cells have been used in the past to obtain immortalized immature CNS cells (Cepko 1988, 1989). In fact, oncogenes such as myc and Large T Antigen have been shown to exhibit immortalizing properties without fully transforming the cells (Land et al., 1986; Fanning, 1992). Several immortalized neuronal and glial cell lines have been developed using this technique (Bernard et al., 1989; Ryder et al., 1990; Snyder et al., 1992). Following the discovery of temperature-sensitive mutants of specific oncogenes (for example, the SV40 Large T Antigene gene; Jat and Sharp, 1989) a new set of conditionally immortalized cell lines has been created which exhibit some hallmarks of the respective primary cell types from which they derive (Frederiksen et al., 1988; Almazan and McKay, 1992; Mehler et al., 1993; Redies et al., 1991;

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*Correspondence to: Elena Cattaneo, Institute of Pharmacological Sciences, University of Milano, Via Balzaretti 9, Milano 20133, Italy. E-mail: elenacat@imiucca.csi.unimi.it

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Renfranz et al., 1991). Such cell lines are also suitable for experiments on genetic manipulation, to express particular genes of interest (Cattaneo et al., 1996; Corti et al., 1996; Martinez-Serrano et al., 1995), or endogenous genes can be functionally altered by homologous recombination, thereby creating recessive or loss of function mutations that are useful in investigation of gene function. Finally, conditionally immortalized neuronal progenitor cell lines are also particularly suitable for cell transplantation experiments to study neurodegenerative processes, as the nonpermissive temperature for the immortalizing oncoprotein corresponds to the body temperature of rodents (Bjorklund, 1993; Gage et al., 1991; McKay, 1992; Martinez-Serrano and Bjorklund, 1997).

In the present paper, we studied some of the properties of the ST14A cell line that was derived from the embryonic day (E14) rat striatum primordia by retroviral transduction of the temperature-sensitive *ts* A58/U19 allele of SV40 Large T Antigen. As expected, the growth rate of ST14A cells could be altered by shifting the cells from the permissive temperature (33°C) to the nonpermissive temperature (39°C). Furthermore, in the absence of serum, a considerable increase in the expression of the neuronal cytoskeletal component microtubule-associated protein (MAP)2 (Dotti et al., 1987) was observed, while Nestin expression was decreased. Finally, we demonstrate that ST14A cells expressed specific neurotrophins and neurotrophin receptors.

MATERIALS AND METHODS

Antibodies and Growth Factors

Rabbit polyclonal antibody against the different Trks were utilized at 1:1,000 (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-glial fibrillary acidic protein (GFAP) polyclonal antibody (Sigma, Italy) was applied at 1:1,000. Rat 401 anti-Nestin antiserum (provided by R.D.G. McKay) was applied at 1:3. Monoclonal antibody against MAP2 (Boehringer Mannheim, Indianapolis, IN) was used at 1:1,000 dilution. Monoclonal antibody against SV40 Large T Antigen (Oncogene Science, DBA, Italy) was applied at 1:1,000. Secondary antibodies (goat anti-rabbit, or goat anti-mouse, Kirkegaard and Perry Laboratories, CELBIO, Italy) were used at 1:10,000 dilution. The mitogenic effect of basic fibroblast growth factor (bFGF; Biosource, CELBIO, Italy) and epidermal growth factor (EGF) (Biosource, CELBIO, Italy) was tested by exposing the in vitro culture to these factors at a final dilution of 10 ng/ml.

Primary Culture and Establishment of Cell Lines

Timed-pregnant Sprague-Dawley rats (Charles River, Italy) were sacrified by decapitation at E14. The fetuses were removed into ice-cold calcium- and magnesium-free Hanks' balanced salt solution (HBSS; GIBCO, Burlington, Ontario) supplemented with 3.9 g/liter HEPES, and the striatum primordia were dissected out. Cells were dissociated in the absence of trypsin and plated in Dulbecco's Modified eagle Medium (DMEM; Life Technologies, Bethesda, MD) supplemented with 0.11 g/liter sodium pyruvate, 3.7 g/liter $NaCO_3$, 0.29 glutamine, 3.9 g/liter HEPES, 100 units/ml penicillin-streptomycin (Life Technologies) plus 10% fetal calf serum (FCS; Imperial, UK). Plating density was $5 \times 10^4 - 2 \times 10^5$ cells/cm^2.

The following day, the primary cells were infected for 2 hours with filtered conditioned medium from ?2 cells packaging the retrovirus transducing the tsA58/U19 Large T Antigen and the neomycin resistance gene, to which 8 μg/ml polybrene had been added. After infection, the virus-containing medium was replaced with fresh DMEM+10% FCS, and the cells placed at 33°C. Two days later, the cultures were passaged and fed with fresh complete medium in the presence of the selective agent, the neomycin analog G418 (Geneticin, GIBCO) at 200 μg/ml. The medium was replaced every 4 days. Within 3 weeks, G418-resistant colonies were observed. Single colonies were picked with cloning rings and expanded into 96-well plates. The clones were subsequently grown at 33°C in DMEM+10% FCS and expanded up through several 100-mm plates, whereupon they were cryopreserved.

Immunofluorescence Analysis

The cells were plated onto 12-mm glass coverslips previously coated with poly-DL-ornithine at 1.5 μg/ml. According to specific experiments, the cells were grown in completed medium at 33°C or 39°C. Cells were rinsed twice with fresh phosphate-buffered saline (PBS; 8 g/liter NaCl, 0.2 g/liter KCl, 1.15 g/liter Na_2HPO_4, 0.2 g/liter KH_2PO_4) and fixed with MeOH/Acetone (for TAg staining) for 10 min or with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min. After permeabilization with 0.4% Triton X-100 for 15 min, the coverslips were rinsed with fresh PBS and incubated for 18 hr with primary monoclonal antibody Pab 419 (Oncogene Science) or monoclonal antibody Rat 401 anti-Nestin (from R. McKay). Cells were rinsed several times with PBS and exposed to fluorescein-conjugated goat anti-mouse antibody at 1:100 dilution (Vector, DBA, Italy) for 1 hr at room temperature. Coverslips were mounted using Permafluor (Italscientifica, Italy).

Western Blot Analyses

The lysis of the cells and the Western blot procedure was as previously described (Cattaneo et al., 1996; Conti et al., 1997). After transfer to nitrocellulose, the blots were blocked in 10% nonfat dry milk in TBS-T (20 mM Tris, pH 7.5, 500 mM NaCl, 0.01% Tween 20) overnight

at 4°C. Blots were then incubated for 1 hr at room temperature in primary antibody. After washing with TBS-T, membranes were exposed to horseradish peroxidase-conjugated secondary antibody for 1 hr at room temperature. Immunoreactivities were detected using the enhanced chemiluminescence method (ECL, Amersham, Italy) according to the manufacturer's instructions.

RNA Preparation

Cells were lysed in 4 M guanidinium isothiocyanate (containing 25 mM sodium citrate, pH 7.5, 0.5% sarcosyl, and 0.1% β-mercaptoethanol) and total RNA was isolated by phenol-chloroform extraction according to Chomczynski and Sacchi (1987).

RNA Probe Synthesis for RNase Protection Assay

A transcription kit (Promega, Madison, WI) was used to generate cRNA probes and [^{32}P]CTP was used as a radiolabeled nucleotide. The following plasmids (containing the appropriate cDNAs) were used: Plasmid RObFGF103 (provided by Dr. Andrew Baird, Whittimore Institute, La Jolla, CA) containing a 1.016-bp portion of the rat bFGF cDNA was linearized with NcoI and used to generate a ^{32}P-labeled 524-base antisense cRNA probe that included 477 bases of bFGF sequence and 47 bases of the Bluescript polylinker region. Plasmid BSrNGF (Dr. Scott Whittemore, University of Miami, Miami, FL), containing a 721-base portion of the rat NGF cDNA, was linearized with Nco I and in vitro transcribed to generate a 447-base antisense cRNA probe containing 401 bases of NGF sequence and 46 bases of the polylinker region. A Bluescript II SK+ plasmid, containing the full coding sequence of rat ciliary neutrophic factor (CNTF) (Dr. Michael Sendtner, University of Wurzburg, Germany), was linearized with Eco RI and used as a template for a T3 RNA polymerase to generate a 670-base antisense cRNA probe yielding a protected fragment of 600 bases. Plasmid containing a 870-bp portion of the rat brain derived neurotrophic factor (BDNF) cDNA (provided by Dr. George Yancopoulos, Regeneron Pharmaceutical, Terrytown, NY) was linearized with SmaI and used to generate a ^{32}P-labeled 696-base antisense cRNA probe that included 656 bases of BDNF sequence and 40 bases of the Bluescript polylinker region. Plasmid containing a 800-base portion of the rat NT3 cDNA (provided by Dr. George Yancopoulos) was linearized with Eco RI and in vitro transcribed to generate a 579-base antisense cRNA probe containing 544 bases of NT3 sequence and 35 bases of the polylinker region.

RNase Protection Assay

The RNase protection assay was performed on a 10–20 μg sample of total RNA as described by Riva et al. (1992). In brief, after ethanol precipitation, total RNA was dissolved in 20 μl of hybridization solution (80% formamide, 40 mM piperazine N,N′,-bis(2-ethanesulfonic acid), pH 6.4, 400 mM sodium acetate, pH 6.4, and 1 mM EDTA) containing 150,000 cpm of each ^{32}P-labeled cRNA probe specific activity (sp. act.) $> 10^8$ cpm/μg). After heating at 85°C for 10 min, the cRNA probes were allowed to hybridize to the endogenous RNAs at 45°C overnight. At the end of the hybridization, the solution was diluted with 200 μl of RNase digestion buffer (300 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, pH 7.4) containing a 1:400 dilution of an RNase cocktail (1 μg/μl RNase A and 20 U/μl RNase T1) and incubated for 30 min at 30°C. Proteinase K (10 μg) and sodium dodecyl sulfate (10 μl of 20% stock solution) were then added to the sample and the mixture was incubated at 37°C for an additional 15 min. At the end of the incubation, the sample was extracted with phenol/chloroform and ethanol precipitated. The pellet, containing the RNA:RNA hybrids, was dried and resuspended in loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, 2 mM EDTA), boiled at 95°C for 5 min, and separated on a 5% polyacryl-amide gel under denaturing conditions (7 M urea). The protected fragments were visualized by autoradiography and their sizes were determined by the use of ^{32}P-end-labeled (T4 polynucleotide kinase) MspI-digested pBR322 fragments.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was prepared from the cells according to the method of Chomczynski and Sacchi (1987). Total RNA (0.5 μg) was treated with 0.35 U/μl DNase I (Promega) at 37°C for 30 min. Single strand cDNA was synthesized with 0.5 μM poly-U primers and 100 U/μl M-MLV reverse transcriptase (BRL, Gaithersburg, MD) at 37°C for 60 min. PCR was performed in a total volume of 30 μl containing 0.2 μg cDNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM each of 5' and 3' primers, and 0.02 U/μl Taq polymerase (Perkin Elmer, Oak Brook, IL). The PCR products were electrophoresed on a 1% agarose gel and visualized by staining with ethidium bromide.The primer sequences used for the amplification amplified a fragment between the coding regions 289–307 and 1368–1385 from the p75^{NGFR} cDNA (Salvatore et al., 1995). The sequences of β-actin primers were as previously described (Kojima et al., 1994).

Proliferation Assay

The cells were seeded at a density of 3×10^4 cells into each well of a six-well dish and incubated in DMEM+10% FCS or in serum-free medium (SFM,

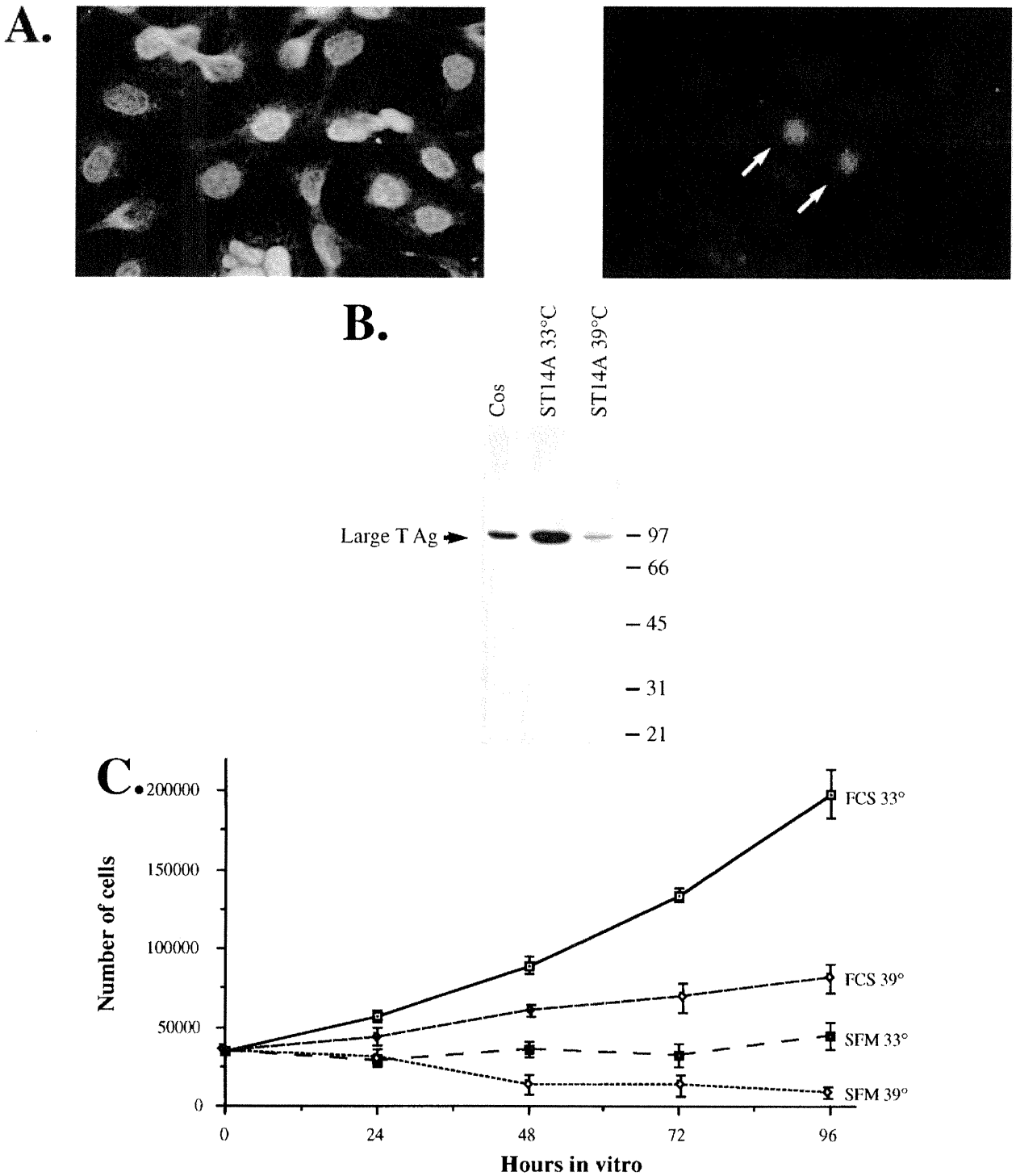

Fig. 1. ST14A cells express the Large T Antigen and proliferate at the permissive temperature. **A:** Immunoreactivity for the SV40 Large T Antigen in ST14A cells grown at the permissive (**left**) and nonpermissive (**right**) temperature. Arrows in the right panel indicate the only two immunoreactive nuclei found in one well (of a 24-well plate) of ST14A cells grown at 39°C. **B:** Western blot analyses showing levels of the Large T Antigen protein in lysates from ST14A cells grown at 33°C and at 39°C. The arrow points at the 94-KDa T Ag band. Lysates from Cos cells were loaded as a positive control. Fifty μg of proteic extracts were loaded into each lane (only 20 μg were loaded for the control). Immunoreactive bands were detected by enhanced chemiluminesence (ECL). **C:** Growth curve of ST14A cells grown at the two temperatures (33°C and 39°C) and in two different culture conditions, i.e. FCS (medium supplemented with 10% fetal calf serum) and SFM (serum free medium). The cells were seeded at a density of 3×10^4 cells into each well of a six-well dish and the amount of cells was monitored at different time points by counting the number of live cells by Coulter Counter.

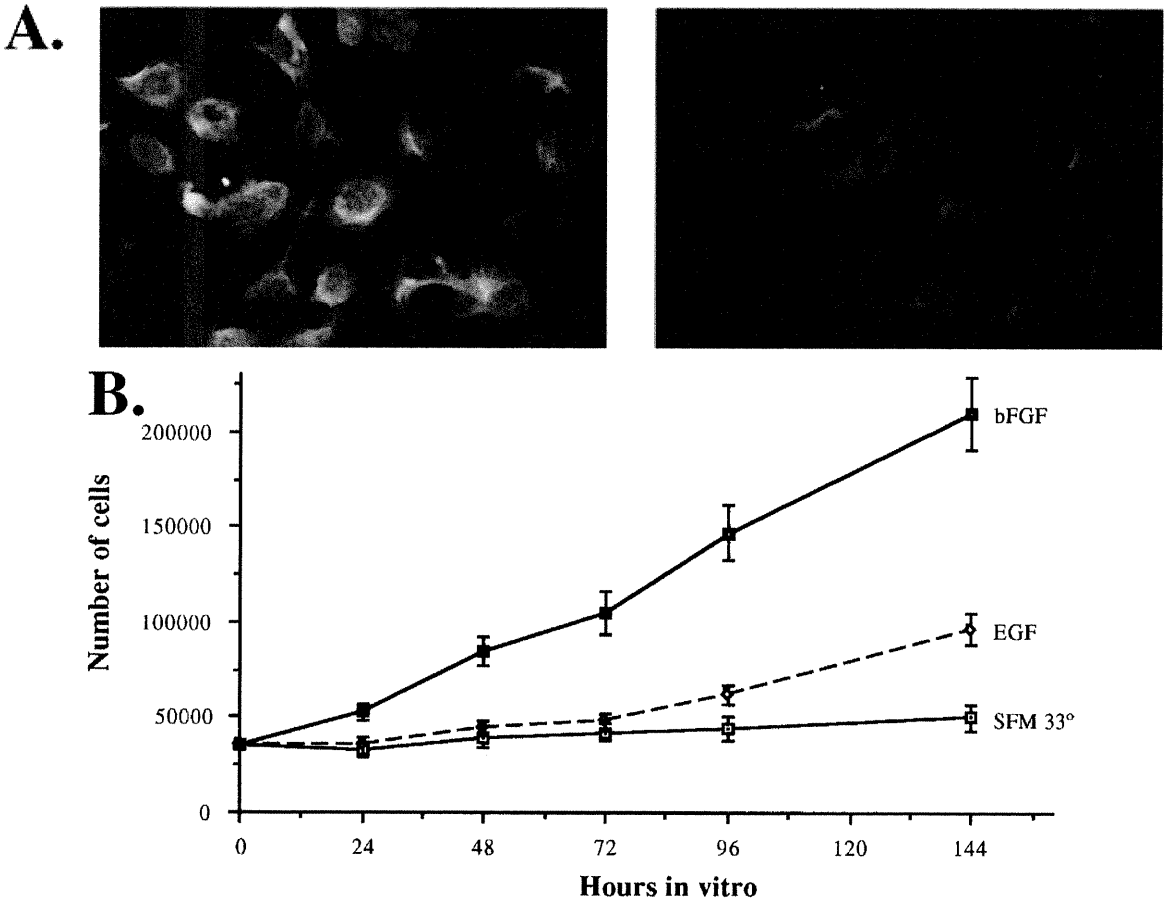

Fig. 2. ST14A cells express Nestin and proliferate in response to EGF and basic fibroblast growth factor (bFGF) stimulation. **A:** Nestin immunoreactivity in ST14A cells grown at the permissive temperature (**left**) and nonpermissive temperature (**right**). **B:** Growth curve of ST14A cells at the permissive temperature (33°C) in the absence of serum (SFM) and in the presence of 10 ng/ml EGF (EGF line) or 10 ng/ml bFGF (FGF line). The cells were seeded at an initial density of 3×10^3 cells/cm^2 and their number at different time points was evaluated as described in Figure 1.

composition: 1:1 F12: DMEM including 5 mg/liter insulin, 100 mg/liter transferrin, 20 nM progesterone, 30 nM selenite salt, 60 mM putrescine, 2 mM glutamine, 0.11 g/liter sodium pyruvate, 3.7 g/liter $NaCO_3$, 3.9 g/liter HEPES) at the appropriate temperature. Cells growth was monitored at different time points by counting the number of live cells by Coulter Counter (model ZM from Coulter Instruments, Milan, Italy).

RESULTS

Immortalization and Large T Antigen Expression

Infected primary cells were selected by their resistance to G418 and by their capacity to grow at low cell density. Isolated colonies were individually picked and expanded. From a total of 10^7 primary cells infected, 15 stable clones were selected. The generated cell clones were given the name "ST" (indicating striatal derivation) followed by a number indicating the colony. These ST lines grew with doubling times of approximately 30–72 hr depending on the line. For each line, cell growth was contacted-inhibited. Immunostaining with monoclonal antibody Pab 419 revealed the characteristic nuclear localization of the immortalizing Large T Antigen oncogene at 33°C (see below). Typically, cells in a given line were heterogeneous with respect to the intensity of nuclear staining. After a short exposure time to the nonpermissive temperature (39°C), the immunoreactivity to Pab 419 was completely abolished in all cell clones (see below). Subsequent tests on one particular established cell line included the following: morphological and growth examination of the cells at 33°C and at 39°C, followed by immunohistochemical detection of Nestin, MAP2, and glial fibrillary acidic protein.

The ST14A cell line was selected for further studies because it exhibited a high proliferative ability at 33°C (see below) and the most abundant expression of Nestin at the permissive temperature.

Expression of the immortalizing oncoprotein in the ST14A line at the permissive (33°C) and nonpermissive temperature (39°C) was examined by immunocytochemistry (Fig. 1A) and immunoblot analysis (Fig. 1B). In both cases, a strong immunoreactivity was observed at 33°C (Fig.1A, left) with the Pab 419 antibody, which reveals a band corresponding to the molecular weight of the immortalizing oncoprotein on Western blot (Fig. 1B). At 39°C, reactivity was almost completely abolished (Fig. 1A, right, and B). In Figure 1A (right) the arrows indicate the only two nuclei (in a 24-well plate) that were found still to be immunoreactive at 24 hr after the shift in temperature.

The proliferative ability of ST14A cells under different culture conditions was evaluated by growth curve analyses. Figure 1C shows the variations in cell number with time, at the two growth temperatures and in two different culture conditions. In serum-containing medium at 33°C, the cells proliferated with a doubling time of approximately 36 hr, exhibiting a polygonal fibroblast-like morphology. At this temperature, the proliferation of the cells was maintained by the presence of both the immortalizing oncoprotein and the serum. Indeed, the removal of serum at 33°C caused the cells to stop dividing. As shown, after plating 3.5×10^4 cells, only 20% more cells (4.4×10^4) were observed at 120 hr (see graph, Fig. 1C). This slight increase in cell number was probably due to those cells that had completed their last cell divisions after serum deprivation. At the nonpermissive temperature of 39°C, in the absence of the Large T Antigen and in a serum-containing medium, the cells showed a flat morphology. As expected, the cells also exhibited a limited growth potential. Indeed, 120 hr after initiation of the study, cell number had only doubled. Finally, in a serum-deprived medium, the number of cells was found decreased with time and they appeared very flat, vacuolated, and in poor condition. Under these culture conditions, we observed cell death within 2–3 days of incubation and, after 96 hr, only one-third of the initially plated cells was still alive as judged by trypan blue exclusion (not shown).

ST14A Cells Express Antigens Typically Found in CNS Progenitor Cells

As shown in Figure 2A, left, ST14A cells grown at 33°C express Nestin, a protein that belongs to a new class of intermediate filament proteins that is only transiently expressed during the development of the CNS and is therefore considered to be a marker of CNS precursor cells (as well as of radial glial and oligodendrocyte precursor cells; Frederiksen and McKay, 1988; Lendahl et al., 1990; Gallo and Armstrong, 1995). As shown, the cells exhibit a peculiar cytoplasmic Nestin immunoreactivity with an enhanced perinuclear staining. Shifting of the cells to 39°C (Fig. 2A, right) as well as growing them at 33°C in SFM (see below, Fig. 3A) results in a reduction in Nestin expression, as expected under conditions that promote cell differentiation.

Cultured CNS progenitor cells derived from the embryonic rat striatum are known to respond to bFGF and EGF stimulation by undergoing cell proliferation (Gensburger et al., 1987; Cattaneo and McKay, 1990; Reynolds et al., 1992; Vescovi et al., 1993; Kilpatrick and Bartlett, 1993; Temple and Quian, 1996; Murphy et al., 1990; Gage et al., 1995). We therefore examined the effects of EGF and bFGF on the proliferative ability of ST14A cells plated in SFM. Figure 2B shows the growth curve of ST14A cells in SFM conditions at the permissive temperature. As can be seen, bFGF exerts a clear and immediate mitogenic effect. Indeed, the number of cells increases rapidly, with a doubling time of approximately 24–30 hr. On the other hand, the effect of EGF was delayed and an increase in cell number was only observed after 96 hr.

Differentiation of the ST14A Cell Line

Studies conducted on CNS progenitor cells isolated from different embryonic brain regions have revealed that removal of growth factors from the culture medium halted the proliferation of the progenitor cells, which then differentiate and exhibit neuronal and glial markers. We therefore tested the effect of serum withdrawal on the differentiation of ST14A cells at the permissive and nonpermissive temperature. However, as we found that the removal of serum in association with the shift in temperature (up to 39°C) induced cell death within 2–3 days of incubation, we analyzed most of the expression of the various antigens at 33°C in SFM. Figure 3A shows a Western blotting analysis of Nestin expression under the growth conditions detailed above. As is visible, Nestin expression decreased with time, becoming only weakly detectable after 168 hr. A parallel blot was reacted with an anti-MAP2 antibody (Fig. 3B). MAP2 is a protein associated with the microtubules and it is expressed in neuronal (and glial) cells (Dotti et al., 1987). As shown in Figure 3, MAP2 was expressed in ST14A cells under normal growth conditions (FCS lane). However, we observed a downregulation of MAP2 content after 72 hr in SFM and a subsequent drastic upregulation, reaching its maximum at 168 hr. This phenomenon could be attributed to an initial accomodation of the cells to the new culture conditions. Indeed, during this time course, the cells exhibited some morphological changes, looking more phase-bright after 48–72 hr in SFM conditions and then flat at 168 hr. On the contrary, GFAP, an antigen that

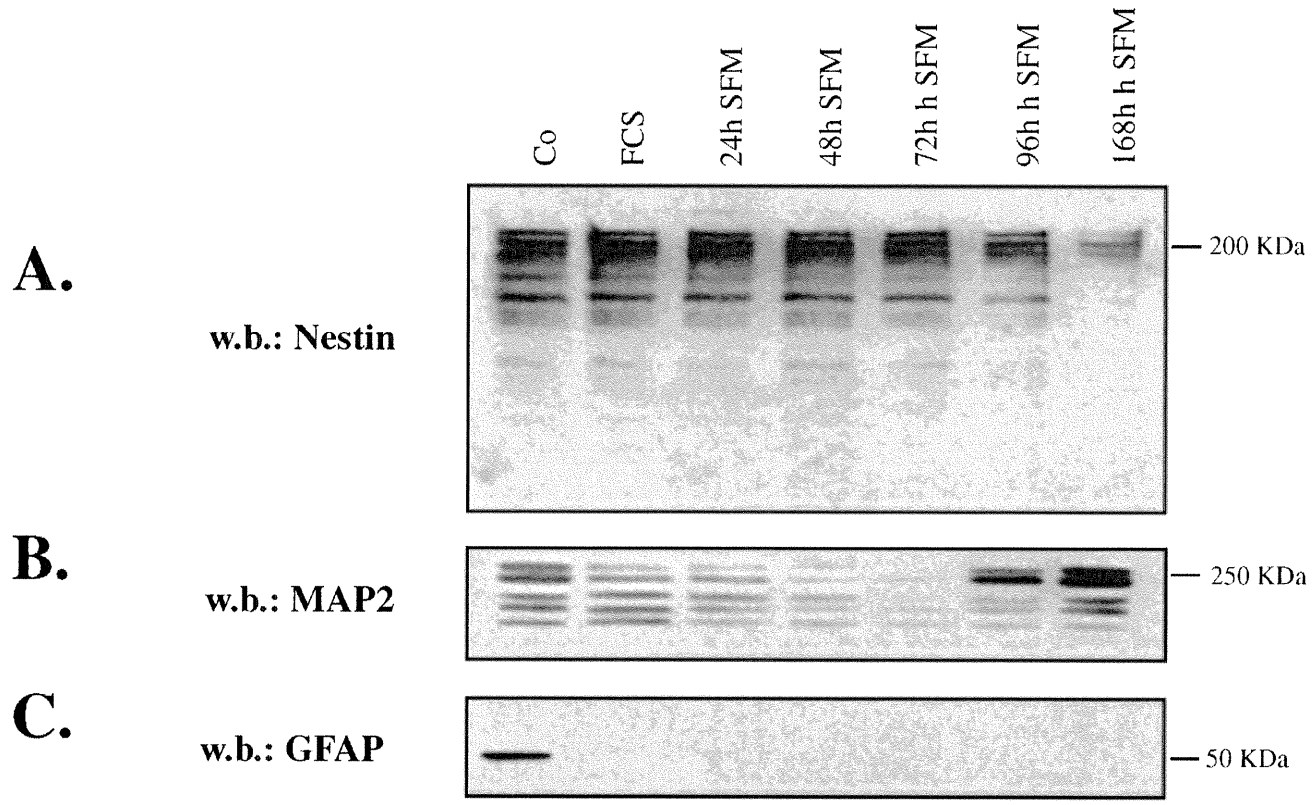

Fig. 3. Differentiation of ST14A cells. Western blot (w.b.) analyses showing Nestin (**A**), microtubule-associated protein (MAP)2 (**B**), and glial fibrillary acidic protein (GFAP; **C**) levels in ST14A cells incubated for various time intervals in the absence of serum and growth factors. As a control, lysates from the embryonic day (E)14 rat striatum or from the adult rat cortex were loaded for Nestin and MAP2 detections, respectively (20 μg were loaded for the controls). For the GFAP Western blot, lysates from cultured astrocytes were loaded (10μg). In the other lanes, 50 μg of proteic extracts were loaded. Immunoreactive bands were detected by ECL.

is specific to glial cells (Debus et al., 1983), was never expressed at the permissive temperature, either in the presence or absence of serum (Fig. 3C).

Production of Neurotrophins and Growth Factors

Neurotrophic factors exert a plethora of effects on different subtypes of neurons (Glass and Yancopoulos, 1993; Curtis and DiStefano, 1994; Snider, 1994). Since these polypeptides can also influence the in vitro behavior of CNS progenitor cell lines, possibly by an autocrine mechanism, we examined ST14A cells for the expression of the various neurotrophin messenger RNAs by RNase protection assay. The assay was performed using total RNA extracted from ST14A cells grown both at the permissive and at the nonpermissive temperatures. As shown in Figure 4, a significant amount of the 401 base pairs protected fragment from the NGF probe was present in ST14A cells grown both at 33°C and at 39°C. NT3 (544 bp), BDNF (656 bp), and bFGF (477 bp) transcripts were present to a lesser extent, remaining constant at 33°C and 39°C. On the other hand, a lower signal was detected with the CNTF anti-sense probe.

Expression of Neurotrophin Receptors

We evaluated the pattern of expression of neurotrophin receptors in ST14A cells. Figure 5A is a Western blotting showing expression of the TrkA receptor and, to a lesser extent, of the TrkB and C receptors in ST14A cells. The expression of the low-affinity receptor $p75^{NGFR}$ was investigated by RT-PCR analysis using specific oligos (Fig. 5B). Equal amounts of mRNA extracted from ST14A cells grown at 33°C and at 39°C were processed and the PCR products were electrophoresed on a 1% agarose gel and visualized by staining with ethidium bromide. RT-PCR analyses indicated the presence of a band of the expected length of 1,097 bp, corresponding to $p75^{NGFR}$ mRNA.

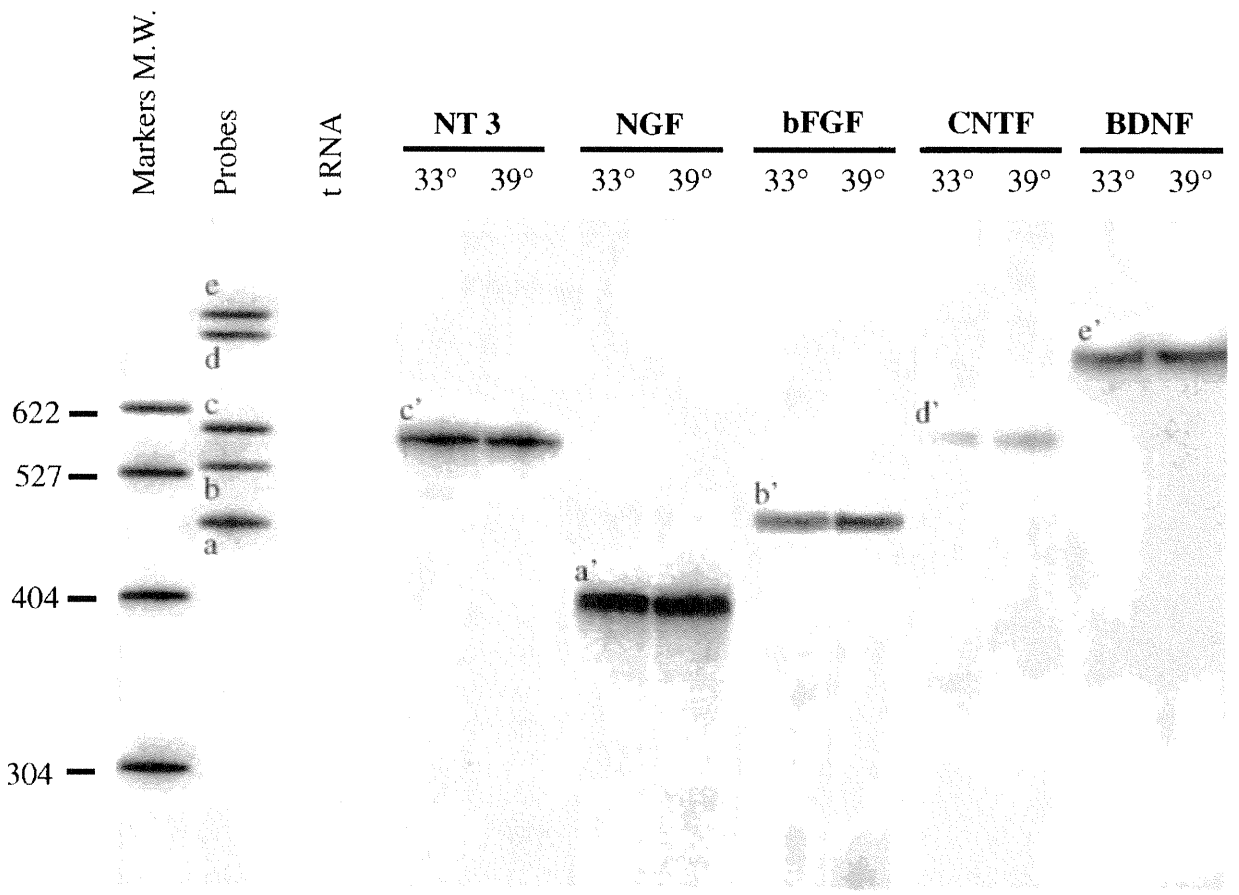

Fig. 4. Expression of growth factors by ST14A cells. Determination of basic fibroblast growth factor (bFGF), ciliary neurotrophic factor (CNTF), nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and neurotrophin 3 (NT-3) mRNA levels in ST14A cells grown at 33°C or at 39°C. The RNase protection assay was conducted as described in Materials and Methods. Twenty μg of total RNA were used for RNA determination. Markers M.W.: ^{32}P-end-labeled MspI-digested pBR322 fragments; probes: aliquotes (4,000 c.p.m. each) of the hybridization solution containing the different cRNA probes were used. Each probe is indicated by a letter: a, NGF antisense probe (447 bases); b, bFGF antisense probe (524 bases); c, NT3 antisense probe (579 bases); d, CNTF antisense probe (670 bases); e, BDNF antisense probe (696 bases). Lettering with an index (a′, etc.) indicates the protected fragment for each probe. The protected fragments for the various probes are: a′, NGF antisense probe (401 bases); b′, bFGF antisense probe (477 bases); c′, NT3 antisense probe (544 bases); d′, CNTF antisense probe (600 bases); e′, BDNF antisense probe (656 bases). The autoradiographic film was exposed for 24 hr at -70°C with an intensifying screen.

DISCUSSION

In this study, neuroepithelial cells isolated from embryonic day 14 rat striatum primordia were infected with a retrovirus containing the temperature-sensitive mutant of the SV40 Large T Antigen (Frederiksen et al., 1988) and the neo gene coding for antibiotic resistance.

The use of this conditional oncogene permits regulation of the state of the cells through alteration of the temperature of growth in culture (Jat and Sharp, 1989). At 33°C, the cells proliferate normally, but at the nonpermissive temperature (39°C), the immortalizing oncoprotein is inactivated and the cells stop dividing. Among the cell lines established in our experiments, the ST14A cells were investigated further. Numerous studies have shown that cell lines obtained after immortalizing rodent CNS primary cells retain some of the properties of the immature progenitors from which they were derived (Frederiksen and McKay, 1988; Redies et al., 1991; Mehler et al., 1993). As for the primary embryonic striatal cells, ST14A cells maintained the expression of Nestin at the permissive temperature and exhibited a response to bFGF stimulation. We found that upon shifting to the nonpermissive temperature, the expression of the Large T Antigen was downregulated, as has also been shown by in vitro experiments conducted in other such derived cell lines (Renfranz et al., 1991; Almazan and McKay, 1992;

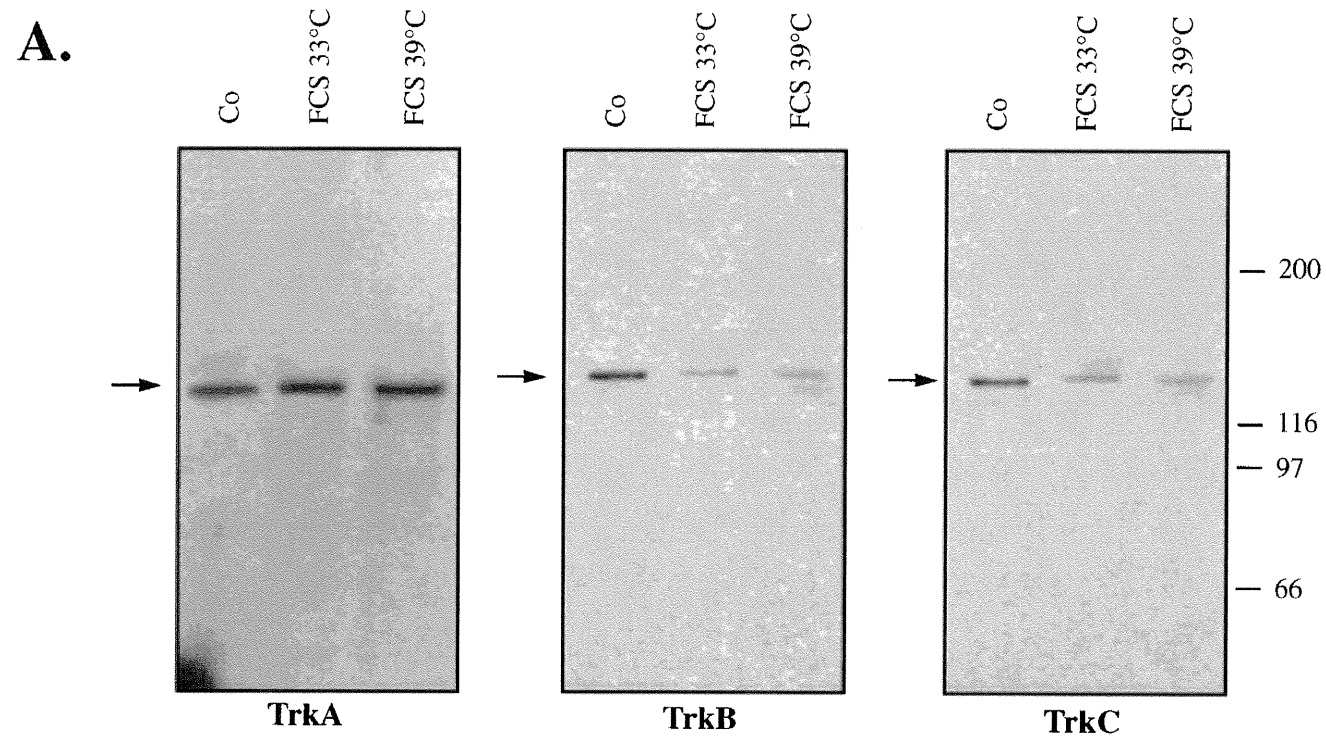

Fig. 5. Neurotrophin receptors expressed by ST14A cells. **A:** Western blot analyses showing TrkA (**left**), TrkB (**center**), and TrkC (**right**) protein levels in lysates from control (adult rat striatum for TrkA detection and adult rat cortex for TrkB and TrkC detections) and from ST14A cells grown at 33°C or at 39°C. Fifty μg of proteic extracts were loaded for each sample (20 μg were loaded for the controls). Immunoreactive bands were detected by ECL. **B:** Reverse transcriptase polymerase chain reaction (RT-PCR) analyses for the expression of the $p75^{NGFR}$ in RNA extracts from ST14A cell grown at 33°C and at 39°C. As a control, total RNA extracted from the E18 rat brain was used.

Whittemore et al., 1994) as well as in vivo transplantation studies on ST14A cells (Cattaneo et al., 1994; Magrassi et al., 1996; Lundberg et al., 1997). Grafting experiments have demonstrated that changes in the expression of Nestin is accompanied by the loss of the expression of the immortalizing oncoprotein and by a restriction of the growth potential of the cells (Cattaneo et al., 1994; Lundberg et al., 1997). These studies also revealed that ST14A cells do not form tumors and are capable of surviving and differentiating in vivo (Cattaneo et al., 1994; Lundberg et al., 1997). Our new data indicate that, upon withdrawal of serum, ST14A cells are able to express MAP2 (Fig. 3B). In a preliminary study we also found expression of DARPP-32 (Ehrlich et al., 1997), an antigen used as a marker for mature striatal medium spiny neuronal phenotype (Campbell et al., 1995).

It is well known that neurotrophic factors regulate the proliferation, survival, and differentiation of specific neuronal populations by interacting with two classes of receptors (Raffioni and Bradshaw, 1993; Cattaneo and

Pelicci, 1998). The first is represented by the Trk family of tyrosine protein kinase receptors. To this family belong the TrkA (gp 140^{TrkA}, the high-affinity NGF receptor; Martin-Zanca et al., 1989), and the two related tyrosine protein kinases TrkB (gp 145^{TrkB}, which binds preferentially to BDNF and NT-4; Klein et al., 1989; Middlemas et al., 1991) and TrkC (gp 145^{TrkC}, which is the receptor for NT-3; Lamballe et al., 1991). To the second class of receptors belongs the low-affinity receptor $p75^{NGFR}$ that binds all members of the NGF family of neurotrophins with similar nanomolar affinities (Chao, 1992). In this study, we have demonstrated that ST14A cells produce several neurotrophic factors and the related receptors, and in particular, as has been observed for striatal neurons (Maisonpierre et al., 1990; Martin-Zanca et al., 1990; Lamballe et al., 1994), we found that ST14A cells express elevated levels of NGF mRNA. ST14A cells also express the TrkA and $p75^{NGFR}$ receptors. Although this property is peculiar to the mature cholinergic striatal interneurons, this receptor system is also expressed in immature striatal neuroblasts as observed by cross-linking experiments conducted with iodinated NGF (Cattaneo, unpublished).

Over the last few years, multipotent CNS progenitor cells have been shown to constitute a particularly interesting source of donor cells for intracerebral transplantation studies, and vehicle for genes of potential therapeutical interest, because of their ability to differentiate into the host brain (Renfranz et al., 1991; McKay, 1992; Martinez-Serrano and Bjorklund, 1997). In particular, ST14A cells have been shown to retain properties of their "in vivo" counterparts such as Nestin expression, and the capacity to differentiate into neurons and glia following transplantation into the embryonic (Cattaneo et al., 1994; Magrassi et al., 1996) or adult environment (Lundberg et al., 1997). Furthermore, ST14A cells have also been used extensively for genetic manipulation experiments, in order to express genes of interest (Corti et al., 1996; Cattaneo et al., 1996). The extremely high transfection efficiency of ST14A cells with the LipoFectamine-based method (50–60% of efficiency, data not shown) indicates that these cells are an in vitro system with great potential for biochemical, molecular, and biological studies, such as the pharmacological modulation of Alzheimer's amyloid metabolism (Salvietti et al., 1996). Taken together, immortalized ST14A progenitor cells may provide an interesting tool for functional analyses of the differentiative and neurodegenerative processes that are typical of neuronal cells.

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