Differentiation of the immortalized adult neuronal progenitor cell line HC2S2 into neurons by regulatable suppression of the *v-myc* oncogene

(retroviral vector/tetracycline/electrophysiology)

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ABSTRACT A regulatable retroviral vector in which the *v-myc* oncogene is driven by a tetracycline-controlled transactivator and a human cytomegalovirus minimal promoter fused to a *tet* operator sequence was used for conditional immortalization of adult rat neuronal progenitor cells. A single clone, HC2S2, was isolated and characterized. Two days after the addition of tetracycline, the HC2S2 cells stopped proliferating, began to extend neurites, and expressed the neuronal markers tau, NeuN, neurofilament 200 kDa, and glutamic acid decarboxylase in accordance with the reduced production of the *v-myc* oncoprotein. Differentiated HC2S2 cells expressed large sodium and calcium currents and could fire regenerative action potentials. These results suggest that the suppression of the *v-myc* oncogene may be sufficient to make proliferating cells exit from cell cycles and induce terminal differentiation. The HC2S2 cells will be valuable for studying the differentiation process of neurons.

Much of our understanding of the molecular mechanisms that control the development and the function of nervous system cells has been derived from studies of cells in culture. Clonal cultures of progenitor cells are useful for studying aspects of the differentiation pathways leading to the formation of mature neurons or glia. Primary cultures of progenitor cells, being heterogeneous in nature, do not offer such an opportunity. As an alternative approach, neural cell lines have been generated by various techniques (1–5). One common approach has been to immortalize cells by transducing neuroepithelial or neural progenitor cells from developing brain with retroviral vectors encoding the simian virus 40 (SV40) large tumor (T) antigen or *myc* oncogenes (1–12). Studies have shown that the immortalization process arrests cells at specific stages of development and prevents their terminal differentiation (9). As a result, cells at specific stages in the development can be immortalized and used to study the nature and potentiality of the cells at this particular stage in the lineage and how they can be further differentiated down that particular pathway.

In neural cell lines developed by constitutively expressing oncogenes such as *myc*, the mitotic activity of the oncogene is always present, and cells proliferate continuously in culture (6, 9, 13). Differentiation of the immortalized progenitor cells into neurons may require sufficient down-regulation of the oncogene, but attempts to induce such terminal differentiation often induce apoptosis (14). However, partial differentiation of *c-myc*-immortalized cells has been achieved by treatment with growth factors (6). To obtain a regulatable expression of the oncogene, a temperature-sensitive mutant of SV40 large T antigen (tsA58) has been used for conditional immortalization (3, 7, 8, 10–12, 15). At a permissive temperature when the large T antigen is expressed, the immortalized cells are undifferen-

tiated and are multipotent in nature (3, 7, 8), as has been seen with *myc*-immortalized cells (6). Although at nonpermissive temperature the expression of T antigen is considerably down-regulated and the cells are not under the mitotic drive of the oncogene, the temperature shift results in only incomplete differentiation of cells into neurons (7, 8, 11, 12, 15). A combination of factors and substrates is needed to further differentiate the cells *in vitro* (12). A simpler system in which the regulatable suppression of oncogene expression in immortalized neuronal progenitor cells could allow their differentiation into neurons without the help of complex factors and substrates would be useful.

To build a system in which the oncogene expression can be regulated by exogenous agents, we have taken advantage of a tetracycline-controlled gene expression system (16–19). In this system, a tetracycline-controlled transactivator (tTA), which is a fusion protein of the repressor (*tetR*) of the Tn10-derived tetracycline-resistance operon of *Escherichia coli* and the acidic domain of VP16 of herpes simplex virus, strongly activates transcription from P_{hCMV}⁺, a minimal promoter from human cytomegalovirus (hCMV) fused to the tetracycline (*tet*) operator sequences in the absence of tetracycline. Low concentrations of tetracycline (0.01–1.0 μ g/ml), at which no toxic effect is evident, almost completely abolishes transcription activation by tTA (16, 17). Using this vector system, we constructed a retroviral vector (LINXv-*myc*) in which the *v-myc* oncogene is transcribed in a tetracycline-regulated fashion. Neural progenitor cells cultured from adult rat hippocampus were transduced with the retroviral vector LINXv-*myc*, and a stably transfected colony was isolated and grown. Here we report that tetracycline suppresses the production of *v-myc* and allows the progenitor cells to terminally differentiate into neurons. Since the progenitor cell line differentiates into neurons only, we may have immortalized a cell that has already been committed to a neuronal lineage. This cell line will be useful for studying the mechanisms of cell differentiation *in vitro*.

EXPERIMENTAL PROCEDURES

Vector Construction. The retroviral vector LINXv-*myc* was constructed as follows: the 1.02-kb *EcoRI*–*Bam*HI fragment of the tTA was excised from pUHD15-1 (16) and inserted into pHENA (20) just upstream of the internal ribosome entry site (IRES) of the encephalomyocarditis virus. The resultant 2.78-kb *EcoRV*–*Bam*HI fragment containing tTA, IRES, and

Abbreviations: FGF-2, basic fibroblast growth factor; tTA, tetracycline-controlled transactivator; IRES, internal ribosomal entry site; GAD, glutamic acid decarboxylase; GFAP, glia fibrillary acidic protein; SV40, simian virus 40; T, tumor; LTR, long terminal repeat; NFH, neurofilament 200 kDa.

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neomycin phosphotransferase was inserted into the polylinker site (*Hap* I and *Bam*HI) of LXSHD (21). Then, the portion of LXSHD spanning the SV40 early promoter and histidinol dehydrogenase cDNA was replaced by a $P_{hCMV^{*1-1}}-v-myc$ (3.32 kb) fragment. $P_{hCMV^{*1-1}}$ was derived from pUHD10-3, and a 2.87-kb *Sac* I-*Sph* I fragment of *v-myc* was derived from pMC38 (American Type Culture Collection).

Cell Culture. All packaging and producer cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. ψ -2 cells were transfected with 10 μ g of LINXv-*myc* plasmid DNA by the calcium phosphate procedure. Virus-containing medium collected from ψ -2 cells 2 days after transfection was used to infect the amphotropic PA317 cells. One day after the infection, the cells were split at a 1:10 ratio, plated, and selected for G418 (400 μ g/ml) resistance. Colonies were picked after selection for 8 days and tested for the proper integration of the vector, production of *v-myc*, and the titer of the retrovirus.

Cells from adult (3-month-old) rat hippocampus were isolated and cultured as described (22) with the following modifications. Cells isolated from tissue after enzymatic dissociation were resuspended in DMEM/Ham's F-12 (1:1, vol/vol) high glucose medium (Irvine Scientific) containing 10% fetal bovine serum and then plated onto uncoated plastic tissue culture flasks (1×10^6 cells per 75 cm² flask). The cells were grown at 37°C in a 5% CO₂ incubator. The next day, the serum-containing medium was replaced with serum-free DMEM/Ham's F-12 medium containing N-2 supplement (insulin at 5 μ g/ml, human transferrin at 50 μ g/ml, 20 nM progesterone, 100 μ M putrescine, 30 nM sodium selenite, 2.5 mM glutamine; GIBCO) and basic fibroblast growth factor (FGF-2; human recombinant) at 20 ng/ml. Confluent cultures of cells were passaged to polyornithine/laminin-coated plates and cultured. Proliferating cultures, maintained for about a year through 19 passages were split at a 1:3 ratio and 1 day later infected for 20 hr with a mixture of one volume of the conditioned medium of the producer cell line and two volumes of DMEM/Ham's F-12 containing N-2 supplements, FGF-2 at 20 ng/ml, and Polybrene at 4 μ g/ml. The infected cells were split at a 1:4 ratio and selected in the presence of G418 (100 μ g/ml).

Northern Blot Analyses. Total RNA was isolated by the CsCl/guanidinium thiocyanate method (23). Fifteen micrograms of total RNA was separated on formaldehyde/agarose (1.5%) gels and transferred onto a Magnagraph nylon membrane and probed with randomly primed *v-myc* or cyclophilin (pB1B15).

Immunofluorescence Staining. Cells were plated in the absence or presence of tetracycline (1 μ g/ml) onto Lab-Tek glass chamber slides (Nunc) coated with polyornithine/laminin and cultured for 1 (without tetracycline) or 5 (with tetracycline) days and then fixed for 10 min with 4% paraformaldehyde. The cells were incubated sequentially with the primary antibody in PBS containing 4% donkey serum and 0.3% Triton X-100 overnight at 4°C followed by fluorescein isothiocyanate-conjugated secondary antibody (Jackson ImmunoResearch Laboratories; used at 1:500) for 4 hr at room temperature. Slides were mounted in 24% glycerol and 9.6% poly(vinyl alcohol) containing 2.5% 1,4-diazobicyclo[2.2.2]octane. The monoclonal antibodies used were to tau (Boehringer Mannheim; used at 1:250), neurofilament 200 kDa (NFH) (clone RT97; Boehringer Mannheim; used at 2 mg/ml), and NeuN (used at 1:5; ref. 24); polyclonal antibodies used were to vimentin (Amersham; used at 1:10), glial fibrillary acidic protein (GFAP) (Chemicon; used at 1:2000), nestin (used at 1:10,000), and *v-myc* (Upstate Biotechnology; used at 1:8000). For immunofluorescent staining for bromodeoxyuridine (Brd-Urd), cells were incubated with BrdUrd (Amersham; used at 1:1000) for 18 hr, fixed with 95% ethanol/5% acetic acid, and then incubated with monoclonal anti-BrdUrd antibody (Am-

ersham; undiluted). Immunoreactivity was detected with anti-mouse biotinylated IgG followed by streptavidin-Texas Red conjugate.

Confocal microscopic images of cells were obtained using a Bio-Rad MRC600 confocal microscope equipped with a krypton/argon laser and coupled to a Zeiss Axiocvert microscope. Images were collected sequentially using the K1/K2 filter blocks matched to the appropriate excitation filter for each channel. Transmitted light images of differential interference contrast optics were captured in registration with the fluorescent signals using the transmitted field detector. Instrument settings to preclude background fluorescent signals were set against a negative control well where the primary antibodies had been omitted; gain and black levels were adjusted to preclude any signal from this well before imaging other wells for positive signal. Collected digital images were composited in ADOBE PHOTOSHOP 3.0 and printed on a Fujix Pictography 3000. Phase-contrast images were photographed on a Nikon Diaphot using T-Max film and the negatives digitized using a Leaf Lumina. These images were printed as described above.

Reverse PCR Analysis. One hundred nanograms of total RNA was reverse transcribed using avian myeloblastosis virus reverse transcriptase (Promega) with random hexamers (10 μ M) as primers in a 20- μ l reaction mixture containing 10 mM Tris Cl (pH 8.4), 50 mM KCl, 3.8 mM MgCl₂, 1 mM each dATP, dTTP, dCTP, and dGTP, and 20 units of RNasin (Promega). After 75 min at 42°C, the reaction was terminated by heat inactivation at 95°C for 5 min. For PCR amplification, several sets of specific oligonucleotide pairs (10 ng/ μ l) were incubated with the above reaction mixture and 5 units of *Taq* polymerase (Perkin-Elmer) in a 100- μ l reaction mixture containing 10 mM Tris Cl (pH 8.4), 50 mM KCl, 1 mM MgCl₂, and 2 μ Ci of [α -³²P]dCTP (1 Ci = 37 GBq). Cycle parameters were 2 min at 94°C, 2 min at 60°C, and 2 min at 72°C for 23 cycles followed by a final 10-min incubation at 72°C. Forty microliters of each reaction was analyzed by electrophoresis on 8% polyacrylamide gels followed by autoradiography. Control experiments using primers for the rat ribosomal protein L27a (internal control) showed that the amount of amplified PCR product was directly proportional to the amount of input RNA after 23 cycles of amplification (data not shown). The sequences of genomic DNA between two primers were selected so that they contained one or two introns for discrimination between products from RNA and contaminating genomic DNA. The following oligonucleotides were used as primers (nucleotide positions are shown in parentheses): rat ribosomal protein L27a (25) 5' primer: 5'-ATCGGTAAGCACCG-CAAGCA-3' (69-88), 3' primer: 5'-GGGAGCAACTCCAT-TCTTGT-3' (302-283); rat GFAP (26) 5' primer: 5'-ACCT-CGGCACCCTGAGGCAG-3' (459-478), 3' primer: 5'-CCA-GCGACTCAACCTTCCTC-3' (599-580); rat glutamic acid decarboxylase (GAD) (27) 5' primer: 5'-AAGGTTTGGAC-TTCCACCAC-3' (589-609), 3' primer: 5'-CATAAGAA-CAAACACGGGTGC-3' (855-835); rat NFH (28) 5' primer: 5'-GAGGAGATAACTGAGTACCG-3' (247-266), 3' primer: 5'-CCAAAGCCAATCCGACACTC-3' (548-529).

Electrophysiology. The whole-cell configuration of the patch clamp technique was used to study voltage-gated currents. Pipettes (3- to 5-M Ω resistance) were pulled from Boralex glass (Rochester Scientific), coated with Sylgard (Dow Corning Corp.) and fire-polished. They were filled with internal solution containing 108 mM cesium methanesulfonate, 4 mM MgCl₂, 9 mM EGTA 9 mM Hepes, 4 mM ATP, 14 mM creatine phosphate (Tris salt), 0.3 mM GTP (Tris salt), and creatine phosphokinase (50 units/ml) at pH 7.4 with CsOH. Whole-cell recordings were initially established in the bath solution [Tyrode's: 150 mM NaCl, 4 mM KCl, 2 mM MgCl₂, 10 mM glucose, 10 mM Hepes (pH 7.4 with NaOH), 2 mM CaCl₂ and, in some cases, 4 mM BaCl₂ added]. Sodium currents were characterized in this bath solution, whereas calcium currents

were assessed with external solution containing 160 mM tetraethylammonium chloride, 10 mM BaCl₂, and 10 mM Hepes (pH 7.4) with tetraethylammonium hydroxide and 1 μ M tetrodotoxin (Sigma) added. External solutions flowed from an array of microcapillary tubes (internal diameter, 140 μ m), driven by gravity; solution exchange was complete in less than 500 msec.

Whole-cell currents were recorded using an Axopatch 200A patch clamp amplifier and the BASIC-FASTLAB interface system (Indec Systems, Santa Cruz, CA). Voltage-dependent currents were filtered at 10 kHz (4-pole Bessel low-pass) and digitized every 25 μ s (sodium current) or 50 μ s (calcium current). Series resistance compensation was employed, typically for 80–95% of the series resistance measured from the uncompensated capacity transient (dividing the decay time constant by cell capacitance) or from the potentiometer used for nulling the capacity transient. Data were accepted for sodium and calcium current only if the remaining voltage error (calculated as the current times the uncompensated series resistance) was <1 mV and if voltage control was adequate as judged by a graded increase in peak current as test depolarizations were increased. Reported potentials have been corrected for a liquid junction potential of –10 mV between the internal solution and the Tyrode's solution in which the pipette current was zeroed

before sealing onto the cell. Sodium and calcium channel currents were corrected for leak and capacitive currents by subtraction of an appropriately scaled current elicited by a hyperpolarization from –80 mV to –90 mV. All experiments were done at 21–25°C. Statistics are given as the mean $\pm$ SEM.

RESULTS

Design of the Vector and Generation of the Producer Cell Line. The control elements of tetracycline-resistance operon encoded in *E. coli* Tn10 have been used to generate a tetracycline-regulatable vector system (16, 17). In this vector the prokaryotic *tet* repressor was converted to a eukaryotic transactivator by fusion of the repressor with the activating domain (C-terminal) of herpes simplex virus VP16 protein. This transactivator strongly activates transcription from the minimal promoter P_{hCMV}*-1 fused to *tet* operator sequences (16). Although this promoter has very low basal activity in HeLa cells or in most tissues of transgenic mice, the synthesis of tTA activates the P_{hCMV}*-1 promoter (16–19). Using this vector system, we constructed a retroviral vector to express the *v-myc* oncogene from the P_{hCMV}*-1 promoter in a tetracycline-regulated fashion (Fig. 1A). The long terminal repeat (LTR) of Moloney murine sarcoma virus transcribed a 7.8-kb mRNA

FIG. 1. Structure of LINXv-myc. (A) The LTR transcribes the 7.8-kb mRNA, which produces two proteins: tTA and neomycin phosphotransferase (neo), with the assistance of the encephalomyocarditis virus IRES. (B) In turn, tTA activates the tTA-dependent promoter (P_{hCMV}*-1) and transcribes the 3.5-kb mRNA, which produces the *v-myc* oncoprotein. (C) Tetracycline inhibits the ability of tTA to transcribe the 3.5-kb mRNA. (D) Immunofluorescence staining of HC2S2 cells for *v-myc* and BrdUrd. The HC2S2 cells were grown in the absence of tetracycline [Tc(–)] for 1 day (a and c) and in the presence of tetracycline [Tc(+)] (1 μ g/ml) for 2 days (d) or 3 days (b). The cells were stained with anti-*v-myc* antibody (a and b) or incubated with BrdUrd for 18 hr and stained with anti-BrdUrd antibody (c and d).

containing both tTA and neomycin phosphotransferase genes by means of an IRES (Fig. 1B). The tTA binds to *tet* operator sequences present in the hybrid promoter in the absence of tetracycline and stimulated transcription from $P_{hCMV^{*}1}$ to yield a 3.5-kb mRNA, which produced the *v-myc* oncoprotein (Fig. 1B). Tetracycline (1 μ g/ml) suppressed the function of the tTA almost completely and inhibited the production of *v-myc* (Fig. 1C). An amphotropic producer cell line producing LINXv-*myc* retrovirus was selected and expanded. The virus had a titer of 10^5 colony-forming units per ml for NIH 3T3 cells and did not contain helper viruses.

Isolation of Immortalized Neuronal Progenitor Cells. Cultured adult rat hippocampal cells were infected with the LINXv-*myc* retrovirus and selected for stable transfectants. One culture consisting of several colonies, HC2, showed some cells with neuronal morphology 3 days after the addition of tetracycline at 1 μ g/ml. HC2 cells were plated at low density (1:1000), and a single colony was isolated (HC2S2).

The HC2S2 cells were polygonal and had very small processes (Fig. 2A). Southern blot analysis showed that these cells had a single retroviral genome integration site and therefore can be considered a single clone (data not shown). The HC2S2 cells grew very rapidly (doubling time of 12 hr) and were not contact inhibited for growth. However, 2 days after the addition of tetracycline at 1.0 μ g/ml, the cells stopped dividing. Most of the cells became phase-bright and started to extend processes, which began to be interconnected by 3 days after the addition of tetracycline (Fig. 2B). BrdUrd incorporation studies showed that, when grown in the absence of tetracycline, essentially all HC2S2 cells incorporated BrdUrd (Fig. 1Da). However, tetracycline treatment for 2 days followed by incubation with BrdUrd resulted in only 1–2% of the cells incorporating the label (Fig. 1Dd).

Northern blot analysis showed the production of large amounts of 3.5-kb *v-myc* transcript in HC2S2 cells grown in the absence of tetracycline. However, amounts of the transcript were reduced after the addition of tetracycline. These results suggest that tetracycline can regulate the transcription of *v-myc* from the hybrid promoter in the HC2S2 cells (Fig. 2C). The transcription from the LTR promoter was also reduced (Fig. 2C). At the protein level, the expression of *v-myc* oncoprotein in the nucleus of the HC2S2 cells was also down-regulated after the addition of tetracycline (Fig. 1Da and Db). Although the

suppression of *v-myc* production by tetracycline was enough to stop proliferation of the HC2S2 cells and induce differentiation, the suppression of the oncogene was not complete.

Suppression of the *v-myc* Production Is Sufficient to Differentiate Immortalized Neuronal Progenitor Cells into Neurons. The expression of the neural precursor cell markers nestin and vimentin and the neuronal markers tau, NeuN, and NFH in HC2S2 cells under proliferative and differentiating conditions was examined. When grown in the absence of tetracycline, cells expressed nestin and vimentin (data not shown). Although a few cells were cytoplasmically stained by the anti-NFH antibody (Fig. 3A), no staining of cells by the anti-tau or anti-NeuN antibodies was observed (Fig. 3C and E). Five days after the addition of tetracycline, almost all cells in culture became positive for NFH, and over half of the cells became positive for tau and NeuN (Fig. 3B, D, and F). Cell bodies as well as processes were stained by the anti-NFH and anti-tau antibodies (Fig. 3B and D), and nuclei were stained by the anti-NeuN antibody (Fig. 3F). However, nestin and vimentin staining was no longer present in the cells (data not shown).

In parallel to the immunological studies, PCR analyses showed the expression of differentiated neural cell markers by HC2S2 cells. Designs for primers predicted PCR products of 234 bp for the ribosomal protein L27a, 267 bp for the adult type of GAD, 353 bp for the embryonic type of GAD, 141 bp for GFAP, and 302 bp for NFH. The primer design for GAD can discriminate between the embryonic type of GAD, which is a truncated inactive enzyme, and the adult type of GAD, which is an active enzyme (29). The PCR study demonstrated a marked increase of both types of GAD mRNA in the differentiated HC2S2 cells 3 days after the addition of tetracycline (Fig. 4). A very weak NFH PCR product was detected in the HC2S2 cells grown without tetracycline and paralleled the finding that only a few cells were immunoreactive for anti-NFH. However, there was an increase of the NFH PCR product after the addition of tetracycline (Fig. 4). GFAP mRNA could not be detected in cells grown with or without tetracycline (Fig. 4).

Immortalized Cells Express Sodium and Calcium Currents After Suppression of *v-myc* Production. Electrophysiological

FIG. 2. The cultures derived from a single clone (HC2S2) were grown in the absence of tetracycline for 2 days (A) or in the presence of tetracycline (1 μ g/ml) for 3 days (B). (C) The Northern blot analysis shows the transcripts from the LTR (7.8-kb mRNA) and the transcripts from $P_{hCMV^{*}1}$ (3.5-kb mRNA) in the cells grown in the absence [Tc(-)] or presence [Tc(+)] of tetracycline. The photograph of the gel stained with ethidium bromide is presented as an internal control.

FIG. 3. Immunofluorescence staining for neuronal markers. The cells were grown in the absence of tetracycline [Tc(-)] for 1 day (A, C, and E) or in the presence of tetracycline [Tc(+)] (1 μ g/ml) for 5 days (B, D, and F). The cells were stained with anti-NFH antibody (A and B), anti-tau antibody (C and D), and anti-NeuN antibody (E and F).

recordings from HC2S2 cells after growth in proliferative and differentiating conditions provided additional evidence that differentiated HC2S2 cells represent neurons (Fig. 5). HC2S2 cells were examined with whole-cell voltage clamp after growth in proliferative (FGF-2, -Tc) and differentiating (FGF-2, +Tc for 6 days) conditions. In proliferative conditions, cells were quiescent, exhibiting virtually no sodium (5 ± 1 pA/pF; 12 cells) or calcium (4 ± 1 pA/pF; 11 cells) current. After growth with tetracycline, substantial sodium and calcium currents were present (Fig. 5A and B; +Tc). The densities of restored sodium and calcium currents (Fig. 5C) were 96 ± 18 pA/pF (16 cells) and 21 ± 3 pA/pF (10 cells), respectively, which is similar to those present in primary, differentiated fetal rat hippocampal cells in culture (D.W.Y.S., unpublished observations). Sodium and calcium currents were activated at potentials more positive than -40 mV, peaked at -10 ± 2 mV (10 cells) and -3 ± 2 mV (4 cells), and reversed at 78 ± 2 mV (8 cells) and 55 ± 9 mV (3 cells), respectively. The restored sodium current in differentiated HC2S2 cells was of the neuronal form, exhibiting a voltage dependence of inactivation with a midpoint of -60 ± 1 mV (14 cells) and rapid kinetics (time-to-peak of 0.8 ± 0.1 msec; 13 cells). In addition, the restored sodium current was blocked by $1 \mu\text{M}$ tetrodotoxin ($95\% \pm 1\%$ inhibition, 6 cells). Concomitant with sodium current induction, HC2S2 cells acquired the ability to fire regenerative action potentials after differentiation with tetracycline (data not shown).

DISCUSSION

Advances in cell and molecular biology are predicated on reliable and versatile *in vitro* systems. The paucity of brain-derived neuronal cell lines that can differentiate into mature neurons *in vitro* is a hindrance to a more complete understanding of the central nervous system at cellular and molecular levels. The temperature-sensitive variant of SV40 large T antigen (tsA58) has been the main choice for the conditional immortalization of the neuronal progenitor cells (7, 8, 10–12, 15). However, most of the studies have failed to demonstrate full differentiation of the immortalized cells by simply switching the temperature from a permissive to a nonpermissive one (7, 8, 11, 12, 15). The presence of several cytokines, or forskolin or growth factors on specific substrates, in addition to shifting the temperature, is needed for the differentiation of immortalized precursor cells (11, 12). Although some of these cell lines express NFH after differentiation, the precursor cell marker nestin is still expressed in them (8, 15). These results may indicate that, although the cells express the marker for differentiated neurons, cells are still in the precursor state and not fully differentiated. A simpler system in which the pro-

duction of oncoprotein can be regulated by exogenous agents to induce differentiation of the progenitor cells will be useful.

Recently, the bacterial tetracycline-resistance operon regulatory system from *E. coli* Tn10 has been used to develop a tetracycline-responsive promoter system for eukaryotic cells (16, 17). The system functions well in mammalian cells (16) and has been used to generate transgenic mice (18, 19). Transgene expression can be temporally regulated in most tissues of the animal by providing tetracycline exogenously (19). The vector has also been used to control SV40 large T antigen expression and conditional transformation of pancreatic β cells in transgenic mice (18). In this experimental system, the expression of T antigen can be inhibited *in vivo* by providing tetracycline to the animals, but the oncoprotein expression can be restored by removal of the drug. We constructed a retroviral vector in which the expression of *v-myc* oncogene is transcribed by the tetracycline-responsive binary system. The transcription of *v-myc* from the hybrid $P_{hCMV}\cdot 1$ promoter in hippocampal neuronal progenitor cells was found to be efficient and strong enough to immortalize these cells. Although the down-regulation of *v-myc* production by tetracycline was enough to stop proliferation of the HC2S2 cells and induce differentiation, the suppression of *v-myc* mRNA and protein was not complete. To date, this vector has only been used to immortalize progenitor cells from adult rat hippocampus. It will be important to immortalize progenitor cells from other brain regions to examine the regional specificity of the differentiation process.

FIG. 4. PCR products of embryonic (E) type of GAD (353 bp), adult (A) type of GAD (267 bp), ribosomal protein L27a (234 bp), GFAP (141 bp), and NFH (302 bp) detected in the cells grown in the absence [Tc(-)] or presence [Tc(+)] of tetracycline. NFL, neurofilament 68 kDa (155 bp).

FIG. 5. Cells were grown with FGF-2, without (-Tc) or with (+Tc) tetracycline for 6 days. (A) Sodium currents elicited by depolarizations from a holding potential of -80 mV to test potentials of -50 , -30 , -10 , 0 , and 30 mV. (B) Calcium channel currents elicited by depolarizations from a holding potential of -80 mV to test potentials of -40 , -20 , 0 , and 20 mV. (C) Average sodium (I_{Na}) and calcium channel (I_{Ba}) current densities after growth with or without tetracycline. Current densities are current amplitudes normalized to total cell capacitance. Each bar represents the mean from 16 (I_{Na} , +Tc), 12 (I_{Na} , -Tc), 10 (I_{Ba} , +Tc), or 11 (I_{Ba} , -Tc) cells; error bars indicate the SEM.

The differentiation of HC2S2 cells to neurons was demonstrated by morphological and immunocytochemical characteristics of cells. Neurons with phase-bright cell bodies and interconnected long and thin processes expressing specific neuronal markers, tau, NFH, and NeuN were observed. In addition, unlike temperature-sensitive tsA58 immortalized cells, the HC2S2 cells stop expressing nestin upon differentiation (data not shown). Electrophysiological studies have provided further evidence for the differentiation of HC2S2 cells into neurons. Cells treated with tetracycline acquired large sodium currents and the ability to fire action potentials. Moreover, the induced sodium current was of the neuronal form, exhibiting rapid kinetics and a midpoint of inactivation of -60 mV (compared to a midpoint at -80 to -85 mV in glial cells; ref. 30). The restored calcium current was of the high-threshold class, based on the voltage dependence of activation and lack of inactivation (31). Thus differentiated HC2S2 cells exhibit functional properties essential to central nervous system neurons: sodium current, which is necessary for initiation and propagation of the action potential, and calcium current, which is required for neurotransmitter release. Taken together, these results suggest that HC2S2 cells were derived from an immortalized neuronal progenitor cell and that they can be differentiated into neurons after suppression of the *v-myc* oncogene. Moreover, since these cells are derived from the adult hippocampus, progenitor cells with the potential for neural differentiation must exist in the adult nervous system and they are amenable to isolation and expansion *in vitro*. It is now possible to study the mechanism of the cell cycle arrest and subsequent differentiation into neurons that is induced by the down-regulation of the overexpressed *v-myc* oncogene.

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