Generation and Initial Characterization of Conditionally Immortalized Chromaffin Cells

Mary J. Eaton,[1,2]* Beata R. Frydel,[1] Tomas L. Lopez,[1] Xing T. Nie,[1] Jian Huang,[1] John McKillop,[1] and Jacqueline Sagen[1,2]

[1]The Miami Project To Cure Paralysis, University of Miami School of Medicine, Miami, Florida 33136
[2]Department of Neurological Surgery, University of Miami School of Medicine, Miami, Florida 33136

Abstract   Adrenal chromaffin cells have been successfully used to attenuate chronic pain when transplanted near the spinal cord, but primary cells are neither homogeneous nor practical for routine use in human therapy. Conditional immortalization with the temperature-sensitive allele of the large T antigen (tsTag) and creation of stable chromaffin cell lines would advance our understanding of both the use and limits of cell lines that contain this immortalization gene for such therapies. Cultures of embryonic day 17 rat adrenal and neonatal bovine adrenal cells were immortalized with the temperature-sensitive allele of SV40 tsTag and chromaffin cell lines established. The rat chromaffin line, RAD5.2, and the bovine chromaffin cell line, BADA.20, both expressed immunoreactivities (ir) for all the catecholamine enzymes: tyrosine hydroxylase (TH), the first enzyme in the synthetic pathway for catecholamines, dopa-β-hydroxylase (DβH), and phenylethanolamine-N-methyltransferase (PNMT). At permissive temperature (33°C), these chromaffin cells are proliferative, have a typical rounded chromaffinlike morphology, and contain detectable TH-, DβH-, and PNMT-ir. At nonpermissive temperature (39°C), these cells stop proliferating, decrease Tag expression, and change the expression of TH-, DβH-, and PNMT-ir in vitro, suggesting increased differentiation at nonpermissive temperature. The chromaffin cell lines also express immunoreactivity for the opioid met-enkephalin (ENK) at permissive and nonpermissive temperatures. The expression of TH-ir in the bovine chromaffin cells is upregulated by the addition of dexamethasone (DEX) or forskolin during differentiation; TH-ir is not affected by the addition of DEX or forskolin in the rat chromaffin cells. The addition of forskolin during differentiation upregulates the expression of DβH-ir in the rat chromaffin cells. PNMT-ir is not affected by differentiation or agents in either cell line. However, catecholamine synthesis was not detectable by high-performance liquid chromatography, suggesting incomplete differentiation under current conditions, or influence by continued low levels of Tag expression. Both cell lines have been carried over many passages in vitro for more than 3 years and were repeatedly frozen and thawed. These data describe an initial step in the conditional immortalization of chromaffin cells that can maintain the phenotype of primary chromaffin cells in vitro over long periods. The use of such chromaffin cell lines that are able to deliver neuroactive molecules offers a novel approach to pain management. J. Cell. Biochem. 79:38–57, 2000.   © 2000 Wiley-Liss, Inc.

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The recovery and improved function of the nervous system after various injuries or disease states has been increasingly possible with the development and use of cell transplant therapies [Zompa et al., 1997]. Primary chromaffin cells derived from the adrenal gland have been used for transplantation and delivery of therapeutic molecules into recipients for a variety of therapeutic indications, including pain, Parkinson's disease, traumatic brain injury, stroke, and depression [Sortwell and Sagen, 1995]. Secretion of neurotransmitters, neurotrophic factors, opioids, neuropeptides, excitatory amino acid antagonists, or other unknown substances from these cells has been demonstrated to effectively halt or reverse various disease processes, such as debilitating chronic pain [Hama and Sagen, 1994]. Chromaffin cells from both human and bovine sources have been used in early-stage clinical

trials for the treatment of Parkinson's disease and chronic pain [Tkaczuk et al., 1997]. However, a serious limitation in the widespread application of this approach for the therapeutic application is the necessity of harvesting fresh cells from donors, because chromaffin cells are mostly postmitotic. This approach is costly, time consuming, and inconvenient. In addition, the resultant cell preparation is obtained from different donors, possibly requiring complete safety screening for each batch of cells. Finally, this primary culture approach could result in different mixtures of cell types that are incompletely characterized and nonhomogeneous.

The generation of chromaffin cell lines would overcome many of the limitations of primary chromaffin cell culture. However, past experience in other laboratories has indicated that, with the exception of tumor cell lines such as pheochromocytomas, chromaffin cell lines are difficult to generate. Pheochromocytoma cell lines are inappropriate for therapeutic use because of the great risk of unrestricted cell division and tumor formation [Hefti et al., 1985]. Moreover, pheochromocytoma cell lines, such as the rat PC12 line, may not behave as normal mature chromaffin cells, in that they appear to maintain or dedifferentiate to a less mature or neuronal phenotype after transplant [Zompa et al., 1993]. For example, PC12 cells synthesize dopamine and occasionally norepinephrine, but only minimal levels of epinephrine, a principal catecholamine in mature chromaffin cells. In support for this, these cells have also been found to lack detectable levels of phenylethanol-amine-*N*-methyltransferase (PNMT), which catalyzes the conversion of norepinephrine to epinephrine [Greene and Tischler, 1976].

Several attempts have been made to generate immortalized chromaffin cell lines. Birren and Anderson [Birren et al., 1990] have reported v-myc immortalized sympathoadrenal progenitor cell lines from embryonic rat adrenal glands. Suri et al. [1993] have generated adrenal cell lines from tyrosine hydroxylase-SV40 T antigen transgenic mice. However, like PC12 cells, in both of these cases, the immortalized cells generated produced dopamine and some norepinephrine, but not epinephrine, and lacked the synthetic enzyme PNMT [Cairns et al., 1997].

An additional limitation using both of these approaches is that the immortalized cells generated are oncogenically transformed and continue to divide, creating a risk of tumorigenesis if transplanted into an immune-privileged site such as the central nervous system (CNS). An exception to this in the v-myc immortalized lines is the differentiation of a small percentage of cells to postmitotic neurons. However, these postmitotic cells become nerve growth factor (NGF)–dependent and rapidly die in its absence [Birren and Anderson, 1990]. Conditional immortalization is a means to generate an immortalized cell line that can later be disimmortalized to stop cell division. Several approaches for conditionally immortalizing cell lines have been described in the literature. One approach involves the use of the temperature-sensitive mutant of the SV40 large T antigen (tsTag) [Giordano et al., 1993] and has been used successfully to conditionally immortalize embryonic CNS neurons [Frederiksen and McKay, 1988; Frederiksen et al., 1988; Selmaj et al., 1991; Whittemore and White, 1993]. Using this approach, transfected cells undergo continual cell division at low temperature conditions (e.g., 32–34°C), but differentiate and become postmitotic when the temperature is raised (37–39°C). The host CNS environment to receive these tsTag-immortalized cell lines has a temperature about 36–39°C, and transplantation allows the cells to cease proliferation and continue differentiation without tumor formation. Thus, conditional immortalization with the tsTag construct incorporates the advantages of cell lines, including the convenience of growing large quantities that can be characterized and safety tested and the ability to genetically engineer in additional therapeutic molecules, while reducing the disadvantages of tumor cell lines.

Here we describe and expand our recent preliminary report [Frydel et al., 1999] for conditional immortalization of chromaffin cells using the tsTag. Data describing the use of these grafted cell lines for the relief of neuropathic pain has been recently reported [Eaton et al., 2000b], suggesting that immortalized chromaffin cells that do not synthesize catecholamines are still able to have a antinociceptive function. This approach for conditional immortalization of chromaffin cells is expected to provide a useful model for the creation of similarly derived human cell lines and their application to a strategy for the alleviation of chronic pain after human peripheral nerve injury and spinal cord injury.

METHODS

Primary Culture and Immortalization of Dissociated Rat and Bovine Adrenals

Methods to culture chromaffin cells from rat embryonic adrenal tissue has been previously described [Frodin et al., 1994]. Briefly, primary cultures of rat chromaffin cells were established from Sprague Dawley rat embryos on fetal day 17 (E17). The whole adrenal gland was dissected out and pooled in Mg^{2+}-Ca^{2+}-free Hanks' balanced salt solution with pen/strep (CMF-HBSS) on ice. The tissue was incubated at 37°C for 15 min in 1 ml DNAse I (1x)/0.125% trypsin/CMF-HBSS. After trituration through increasingly finer bore cotton-plugged glass pipettes, the settled tissue was rinsed one time with heat-inactivated horse serum (HI-HS) to inactivate the trypsin. Tissue was settled, the top layer of cells was diluted to 2 ml with the growth media [Dulbecco's Modified Eagle Medium (DMEM)/RMPI-1640, 1:1, vol/vol]/20% HI-HS/pen-strep/glutamine (2 mM). Cells (0.5–1×10^4), counted by trypan-blue exclusion, were plated on a six-well plate (Costar, Cambridge, MA). After 6–12 h, at 37°C, CO_2 5%, media was changed to remove dead cells and cultures were grown overnight before infection with immortalizing viruses.

Cultures were incubated overnight with the conditioned media from the competent retroviral producer line ψ2-tsTag, containing the sequence for tsTag expression and resistance to the antibiotic G418, in the presence of polybrene 4µg/ ml (Sigma, St. Louis, MO). Cultures were rinsed with media to remove virus and were allowed to rest for 2 days at 33°C, before treating cultures with the addition of 250µg/ml of the selection antibiotic G418 (Genticin; Gibco, Grand Island, NY) in selection medium (DMEM/F12, D/F media; GIBCO) plus 10% fetal bovine serum (FBS)/pen-strep).

Similarly, primary cultures of chromaffin cells were established from young neonate calf [Sortwell et al., 1994]. The whole adrenal glands were obtained from a local slaughterhouse and were immediately perfused with a Ca^{2+} and Mg^+-free Locke's solution. Glands were next perfused with 0.1% collagenase solution and the medullary tissue was dissected out. The medullary portions were minced, filtered, and washed several times before purification of the cell population on a Renografin gradient (Squibb Meyers, New Brunswick, NJ).

The band containing viable chromaffin cells and some fibroblasts was removed, washed, and plated on 100-mm tissue culture (tc) dishes in medium (1:1, D/F media) supplemented with 5% FBS and allowed to stabilize 24 h before replating at 37°C in a CO_2 (5%) incubator. The remaining cells, counted by trypan-blue exclusion, were replated (0.5–1.0×10^4) on a six-well plate (Costar). Again, cultures were allowed to grow overnight before infection. The bovine chromaffin cultures were infected overnight at 37°C with conditioned media from the retroviral producer line containing the tsTag construct and antibiotic resistance in the presence of polybrene, 4 µg/ ml. Cultures were rinsed with media to remove virus and allowed to rest for 2 days at 33°C, before treating cultures with the addition of 250 µg/ml of the selection antibiotic G418 (Genticin; Gibco).

In both rat and bovine cultures, 250 µg/ml G418 was added to the media, and the media changed daily for selection (D/F media/ 10%FBS/pen-strep). When cells were visible after 3–5 days, the FBS was increased to 20% to increase the rate of proliferation, the G418 was decreased to 125 µg/ml for maintenance, and cultures were purified by differential plating, to reduce the fibroblast population, before subcloning in 100-mm TC dishes with subcloning rings to isolate individual colonies of chromaffin cells. Good survival of chromaffin clones required that some fibroblasts were carried through each subcloning, and each final chromaffin line contains a small number of adherent fibroblasts that must be removed by differential plating before use. The rat chromaffin cell line used for characterization is called RAD5.2; the bovine chromaffin line is called BADA20.

For differentiation at 39°C, rat and bovine chromaffin cell lines were grown at nonpermissive temperature (39°C) in B16 base/1% bovine serum albumin (BSA; Boehringer Mannheim)/ TCM/pen-strep ((B16 base medium [Romijn et al., 1984; Brewer and Cotman, 1989] containing 1% (wt/vol) BSA, TCM proprietary serum-free replacement ingredients (Celox Labs), for the number of days indicated in each experiment. Differentiation was usually continued in differentiation media, or with the addition of forskolin (2 µg/ml) or 1 µM dexamethasone (DEX), or both agents (see below).

After expansion with proliferation, both rat and bovine chromaffin cultures were suspended in Cellvation (Celox Labs) freezing medium, according to the manufacturer's directions, for permanent liquid N_2 cell storage. Various passages of both cell lines have been frozen, warmed, reconstituted, and expanded for more than 2 years, although passages of approximately numbers 12–15 are most commonly used for the in vitro characterization. No passage number greater than number 45 has been used for experiments, but no detectable difference in growth or phenotype characteristics has been observed with these later passage numbers.

With regard to the treatment of the rat embryos and killing of the rat dams for removal of embryos, euthanasia was in accordance with the Laboratory Animal Welfare Act, *Guide for the Care and Use of Laboratory Animals* (NIH, DHEW Pub. No. 78-23, Revised, 1978] and guidelines provided by the Animal Care and Use Committee of the University of Miami, Miami, Florida.

Differential Plating of Chromaffin Cells

The method for purification of chromaffin cells is a modification of differential plating procedures described by Unsicker and Muller [1981]. Briefly, on day 1, cells were lifted from the tissue culture dish with 0.5 mM EDTA/Dulbeco's phosphate-buffered saline (DPBS) (sterile, 37°C). The cells were centrifuged 3–5 min at $150 \times g$. The pellet was resuspended in D/F media/10% FBS/pen-strep. Cells were replated in 100 mm non–tissue-culture plates (catalog number 25384-208, VWR; Plainfield, NJ) with 7–10 ml media. Cell were incubated 4 h at 33°C, for cell lines. Sessile cells (the supernatant that contained cells that did not attach to the plate) were removed into tissue culture flasks (T-25 Blue-top, Falcon, Lincoln Park, NJ) and incubated for 2 h at 33°C. The supernatant was then moved into a T-25 Blue-top flask and further incubated overnight at 33°C.

The next morning, on day 2, the supernatant was removed and saved in a 15-ml tube. The flask of attached cells was rinsed briefly with 0.5–1 ml EDTA, 3 ml of media added, and the total solution in the flask used to wash off loose cells was then added to the 15-ml tube. The cells were again replated on non-TC plates for 4 h, to remove any remaining fibroblasts.

Sessile cells in the supernatant were replated to TC flasks (T25 Blue-top, Falcon) for 2 h. Media was added to the flask to expand the cells.

Immunocytochemistry

For characterization of the chromaffin cell lines using immunocytochemistry, cells were seeded onto eight-well chamber slides and proliferated to near confluence at permissive (33°C) or differentiated for 7–21 days at nonpermissive temperature (39°C) in differentiation media described above. Cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4, for 20 min, rinsed with phosphate-buffered saline (PBS), and nonspecific background blocked with the preimmune serum, for a few hours at room temperature. Cells were reacted with primary antibodies for 18–48 h at 4°C, followed by several rinses and incubation in secondary fluorescent antibodies (1–2 h at room temperature). Secondary antibody reporters were: goat anti-mouse IgG-Cy3, Sigma (St. Louis, MO); and goat anti-rabbit IgG oregon-green, Alexa green, and Alexa red from Molecular Probes (Eugene, OR). After reactions were completed, slides were coverslipped using antifade mounting medium. All slides for color photography were scanned using an inverted Olympus fluorescent microscope, with fluorescein isothiocyanate filter for oregon green (absorbance 488 nm, emission 520 nm) and rhodamine filter for Cy3 visualization (absorbance 550 nm, emission 570 nm). Color images were taken with the assistance of a color Optronics DEI-750 camera and Image Pro-plus software for MacIntosh. Black and white images were taken from stained eight-well slides of cultures, with fluorescent antibody reporters, with a Zeiss AxioplanII on TMAX400 professional film and scanned at 200 dpi into TIF format, and images were collected in Adobe Photoshop.

Tag

Double- and single-labeled cultures of cell lines examined for Tag-ir were stained at the permissive temperature of 33°C and at various time points after the beginning of differentiation at 39°C to determine whether the immortalized chromaffin cells continued to express functional Tag protein in vitro, which would reflect the possibility of continued proliferation

under the mitotic control of the large T antigen. Antibody staining for Tag in the cells is a modification of the methods described for in vitro staining for Tag in other conditionally immortalized cells [Whittemore and White, 1993]. The antibody source for the large T antigen was collected from the Tag hybridoma (hybridoma PB101, clone 412), American Type Culture Collection (Rockville, MD). Cells were either grown to near confluence at permissive temperature (33°C), or differentiated in differentiation media from 7 to 21 days at nonpermissive temperature (39°C) before anti-Tag immunohistochemistry. After fixation, chromaffin cells were labeled with monoclonal antibody to T-antigen (Tag supernatant) at the dilution range from 1:1 to 1:5, in PBS/0.4% Triton X-100 (TX)/5% normal goat serum (NGS), followed by Cy3- or oregon green–labeled secondary antibody (goat anti-mouse IgG, Sigma and Molecular Probes, respectively) for color images. Cells stained for the black and white images used Alexa green or Alexa red (Molecular Probes) as the secondary antibody. Cells that were only stained for Tag were double-labeled with the nuclear marker bis benzamide (1 μM) to identify viable cells. Negative controls were done in the absence of primary antibody and positive controls on noninfected cells (primary chromaffin cells).

Tyrosine Hydroxylase

Briefly, before primary antibody incubation, cells were incubated with 5% NGS in PBS/0.4%TX, for a few h at the room temperature. Cells were then incubated for overnight at 4°C with rabbit polyclonal antibody to tyrosine hydroxylase (TH) (Chemicon, Temecula, CA), diluted 1:300 in PBS/0.4% TX/5% NGS, rinsed, followed by anti-rabbit IgG–fluorescein conjugate (Alexa green, Molecular Probes), diluted 1:100 in PBS, and incubated 1 h at room temperature.

Dopa-β-Hydroxylase

Some cultured cells were stained for DβH expression to assess whether the chromaffin cell lines were capable of synthesizing norepinephrine during the proliferation and differentiation conditions. Antibody staining for DβH in the cells is a modification of methods described previously [Aunis et al., 1980). The anti-DβH polyclonal antibody, raised in rabbit (1:300; Incstar, Stillwater, MN) was incubated with TX-permeabilized (0.4%/PBS) cells overnight at 4°C, followed by a anti-rabbit Alexa green secondary reporter.

Phenylethanolamine-*N*-Methyltransferase

Some cultured cells were colabeled for Tag and also stained for PNMT expression to assess whether the chromaffin cell lines were capable of synthesizing epinephrine during the course of proliferation and differentiation conditions. In a separate experiment that used double labeling, the Tag marker was also used in these cultures to examine whether PNMT-positive cells contained Tag-ir. Antibody staining for PNMT in the cells is a modification of methods described previously [Cahill et al., 1996]. The anti-PNMT polyclonal antibody, raised in rabbit (1:300; Incstar; Stillwater, MN) was incubated under the same conditions as described above for polyclonal antibodies.

Met-enkephalin

Some cultured cells were stained for met-enkephalin (ENK) to assess whether the chromaffin cell lines were likely synthesizing enkephalin during the course of proliferation and differentiation conditions. The anti-ENK polyclonal antibody, rabbit (1:300; Incstar, Stillwater, MN) was incubated with TX-permeabilized (0.4%/PBS) cells overnight at 4°C, followed by an anti-rabbit Alexa green secondary reporter.

Quantitative Image Analysis of Antigens

For each chromaffin cell type, after proliferation at 33°C, 2.5×10^5 freshly purified (by differential plating) cells were either plated on eight-well slides (3.0×10^4/well) or on 100-mm TC dishes, and differentiated for 7 days at 39°C in differentiation media , media plus either 2 μg/ml forskolin, or 1 μM dexamethasome (DEX), or forskolin/DEX. Media with these additions were made freshly, and changed every 2 days for the 7 days of the differentiation. The cell contents of each dish was lifted with a nonenzymatic chelator solution (Cellstripper; Mediatech, Inc., Herndon, VA) and distributed evenly over an eight-well slide for fixation and staining, as described above. Cells were fixed for 20 min with buffered 4% paraformaldehyde and stained for: TH, DβH, PNMT, and met-enkephalin as described above, with two wells stained only with the secondary antibody, to

provide negative controls. Randomly chosen fields of stained cells (6–10/experiment/treatment paradigm, after proliferation at 33°C, differentiation at 39°C for 7 days, and differentiation plus agents) were counted at the 40× magnification on an Olympus IX70 inverted microscope with an aid of Optronix DEI-750 digital camera and Image Pro Plus 3.0.1 software. Images of cells that had the primary antibody absent were used to determine nonspecific staining levels. Positive reaction was considered when antibody staining was above the background levels. Images for each treatment paradigm and each primary antibody were examined with the same microscope and camera settings, to ensure that images were comparable. Primary rat and bovine chromaffin cells were used to determine both the specificity of antibody labeling and intensity of staining for each antigen. Exposure setting represents intensity of staining. Exposure 1 is very low intensity of staining , and ¼ and lower fractions represent more bright staining (more antigen/antibody binding). Change after differentiation or treatment from an exposure setting from 1.0 to 1/15 represents a four-step increase in density of that antigen in the cells. Data represent the results of 6–10 randomly chosen fields/well from three independent experiments.

Statistical Analysis

Statistical significance of all quantitative data was determined with a multivariate analysis of variance. Comparisons of differences between individual means were tested using the Tukey honest significant differences method or the unequal N least significant difference test. All of the analyses were performed with a commercially available software package (Statistica, Statsoft, 1990). P values <0.01 were considered statistically significant.

High-Performance Liquid Chromatography for Catecholamines in Chromaffin Cell Lines

Chromaffin cell lines were examined for the secretion and release of catecholamine neurotransmitters, norepinephrine, and epinephrine, under proliferation and differentiation conditions at permissive (33°C) and nonpermissive (39°C) temperatures, respectively. HPLC methods to examine catecholamines from cells were a modification of those reported previously [Cheng et al., 1992, 1993]. For proliferation of rat and bovine chromaffin cells, cell lines were grown at permissive temperature (33°C), in D/F media/10% FBS/125 µg/ml G418 as described previously. For differentiation, rat and bovine chromaffin cell lines were either grown for 10 days at nonpermissive temperature (39°C) in differentiation medium: 1) B16/1% BSA/TCM /pen-strep; or 2) with the addition of 1 µM DEX; or 3) with the addition of 1 µg/ml forskolin for 10 days; or 4) 5 days in differentiation medium, followed by 5 days in differentiation medium, with no BSA or TCM/10% FBS/pen-strep. In all proliferation and differentiation conditions, cell cultures were differentially plated to remove fibroblasts, the chromaffin cells were counted by trypan-blue exclusion, and plated (2×10^6/well) in eight-chamber plastic slides, and the cells settled for 6 h before use for HPLC studies. Cells were rinsed one time, for 15 min, with normal Hanks' buffer (140 mM NaCl/5.9 mM KCl/1.2 mM $MgSO_4 \cdot 7H_2O$/2.5 mM $CaCl_2 \cdot H_2O$/15.0 mM HEPES/11.0 mM glucose, pH 7.4), then 125µl/well of release buffer (Hanks' +75 mM KCl (final) or nicotine (61 µM/normal Hanks') added to the cells for 15 min. A 10-µl aliquot of the last was injected onto the HPLC (BAS-200, Analytical System, Inc.) C18 column (150 × 1 mm) with electrochemical detection. The potential used was +750 mV versus Ag/AgCl, with a classic 3-mm glassy carbon electrode. The ranges of sensitivity for the electrode were 50 nA and 5.0 nA, with a flow rate of 0.10 ml/min. Media from chromaffin cell lines were examined at the 5.0-nA sensitivity. The mobile phase included: 25 mM NaH_2PO_4/50 mM Na citrate/25 µM disodium-EDTA/10 mM diethylamine · HCl/2.2 mM 1-octanesulfonic acid (sodium salt), with the pH adjusted to 3.2 with H_3PO_4. An additional 30 ml of methanol and 22 ml of dimethylacetamide were added to 1 l of this buffer before use. Primary cultures of chromaffin cell were used as positive controls; some samples had stock neurotransmitters added to spike the media and served as internal standards. Ordinarily, the norepinephrine appeared at ~3 min; the epinephrine at ~3.5 min.

Chemicals

Ham's F12 media (D/F, 1:1, vol/vol) and Geneticin (G418) were obtained from Gibco; TCM serum replacement constituent was from Celox Labs, and all other powdered media, attach-

ment factors, and chemicals were purchased from Sigma Chemical Co. BSA (fraction V, protease-free) was obtained from Boerhringer Mannheim (Indianapolis, IN), FBS from Hyclone (Logan, UT). Cellstripper (Mediatech, Inc., Herndon, VA), a proprietary nonenzymatic cell dissociation solution, was used to lift chromaffin cells after differentiation for antibody staining. Vectashield mounting medium 4′,6-Diamino-2-phenylindol (DAPI) was obtained from Vector Laboratories (Burlingame, CA). Nontissue cultures plates for differential plating were obtained from VWR.

RESULTS

Loss of Expression of Tag-ir in Chromaffin Cell Lines After Proliferation at Nonpermissive Temperature (39°C)

Immunohistochemistry for Tag was used to examine the chromaffin cell cultures at various time points to assess whether the cell lines were actually differentiating (with decreased Tag), rather than continuing to proliferate and express high levels of Tag-ir. The reduction of Tag-ir during differentiation in the chromaffin cell cultures is illustrated in Figure 1. Both rat (Fig.1A–D) and bovine chromaffin cells (Fig.1E–H) were stained for Tag-ir at 33°C (A,E) and 1 week (Fig. 1B,F) and 3 weeks (Fig. C,G) after the switch to differentiation conditions at nonpermissive temperature (39°C). At 33°C, during proliferation, 100% of the cells contain Tag-ir. After 7 days of differentiation at nonpermissive temperature (39°C), many of the cells cease to label for Tag. By 3 weeks of differentiation at 39°C, many or most surviving cells cease to express detectable Tag-ir. To ensure that the disappearance of Tag-ir at 3 weeks was not a result of poor cell survival before anti-Tag immunohistochemistry, these cell cultures were counterstained with the nuclear Hoechst dye, which stains viable cell nuclei a fluorescent blue. These counterstained RAD5.2 (Fig. 1D) and BADA.20 (Fig. 1H) cells were both viable and no longer expressed any detectable Tag-ir (arrows) after 3 weeks. Some cells, even after 3 weeks of differentiation, continued to express low levels of Tag-ir, and probably represent "leaky" cells for tsTag-ir, e.g., not a complete loss of Tag expression. Control primary chromaffin cells do not stain for Tag-ir (data not shown).

Regulation of Tyrosine Hydroxylase Expression with Differentiation and After Forskolin and Dexamethasone in Chromaffin Cell Lines In Vitro

Immortalized rat, RAD5.2, and bovine, BADA.20, chromaffin cell lines were stained with an antibody specific for TH to assess whether the cells expressed the chromaffin catecholamine phenotype at permissive (33°C) and nonpermissive (39°C) temperatures in vitro, and with the addition of DEX or forskolin.

The expression of TH-ir is shown in rat RAD5.2 and bovine BADA.20 (Fig.2) chromaffin cell lines in vitro. The immortalized rat chromaffin cells, RAD5.2 cells, develop a typical rounded chromaffin cell–like morphology and contained detectable TH-ir at 33°C (Fig. 2A), with no change in TH- ir after 7 days of differentiation (in serum-free media without additions) at 39°C (Fig. 2B), the addition of forskolin (Fig. 2C), DEX (Fig. 2D), or both agents simultaneously (Fig. 2E) at 39°C. The immortalized bovine chromaffin cells, the BADA.20 cell line, also appear rounded, with barely detectable TH-ir at 33°C (Fig. 2F), which did not change after 7 days of differentiation at 39°C (Fig. 2G). However, unlike the rat chromaffin cells, the BADA.20 had increased TH-ir after the addition of forskolin (Fig. 2H) or DEX (Fig. 2I) during 7 days of differentiation.

Regulation of Dopamine-β-hydroxylase and PNMT-ir with Differentiation and After Forskolin and Dexamethasone in Chromaffin Cells

The other enzymes for catecholamine synthesis, PNMT and DβH, were examined in the chromaffin cell lines under proliferation and differentiation conditions DβH- and PNMT-ir in the cells as shown in Figures 3 and 4, respectively. The immortalized rat chromaffin cells, RAD5.2 cells (Fig. 3), contained detectable DβH-ir at both 33°C (Fig. 3A) and equivalent levels at 39°C (Fig. 3B); the immortalized bovine chromaffin cells, BADA.20 cells, also contained detectable DβH-ir at 33°C (Fig. 3F), but increased DβH-ir at 39°C (Fig. 3G). The addition of forskolin during differentiation increased DβH-ir in rat RAD5.2 (Fig. 3C), but not bovine BADA.20 (Fig. 3H) cells. The addition of DEX during differentiation had no effect on
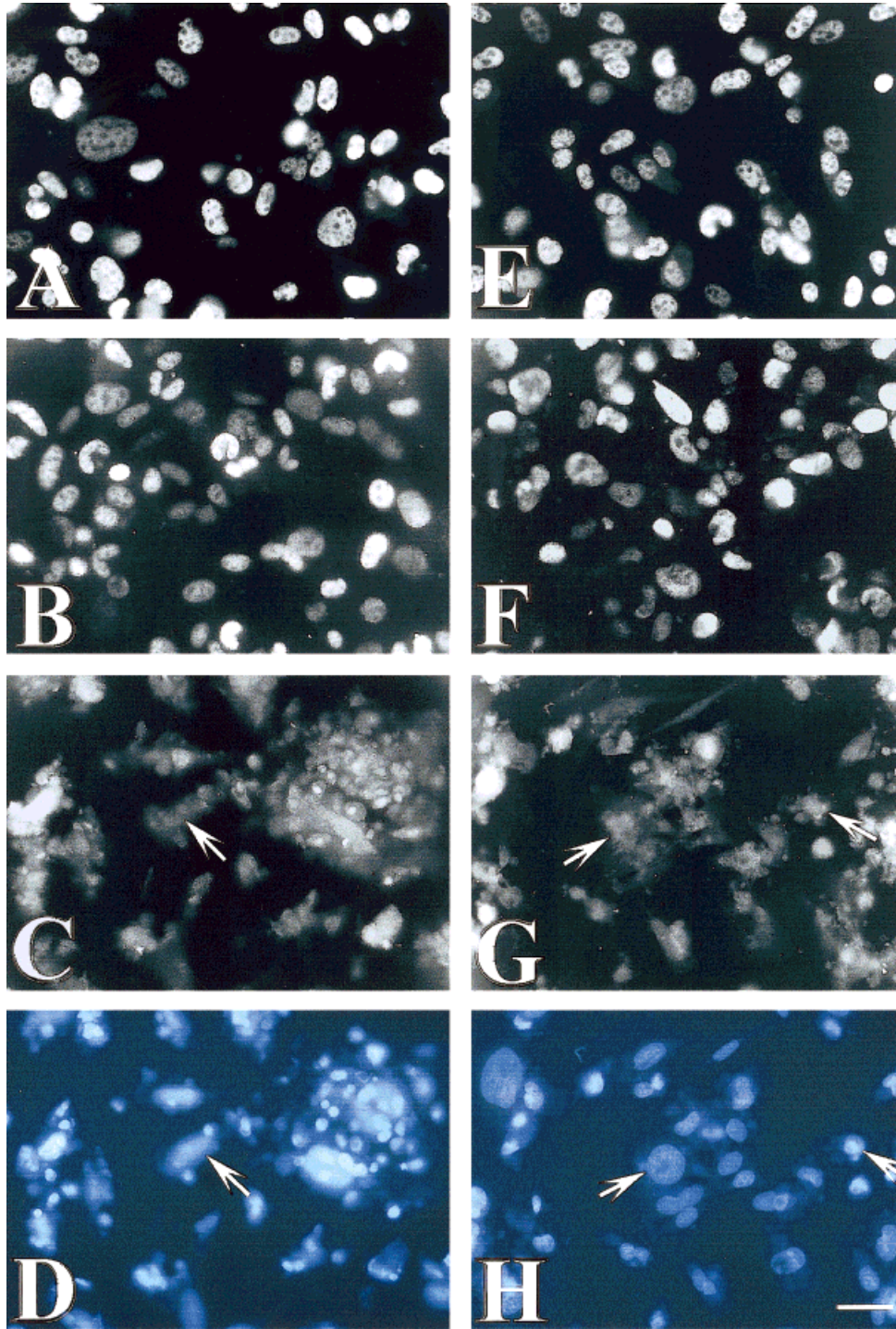
Fig. 1. Reduction of large T antigen immunoreactivity (Tag-ir) with differentiation in vitro. Both rat RAD5.2 (**A–D**) and bovine BADA.20 (**E–-H**) cells were stained with an antibody specific for the Tag protein. Compared to a ubiquitous Tag-ir at 33°C in rat (A) and bovine (E) cells, Tag-ir begins to decline at 7 days after the beginning of differentiation at 39°C (B,F). The near complete absence of Tag-ir is more apparent after 21 days (C,G; arrows) in culture. These 21-day cultures were counterstained with the nuclear Hoechst dye, bis benzamide (D,H) to mark viable surviving chromaffin cells (arrows) with no Tag-ir. Scale bar = 70μm.
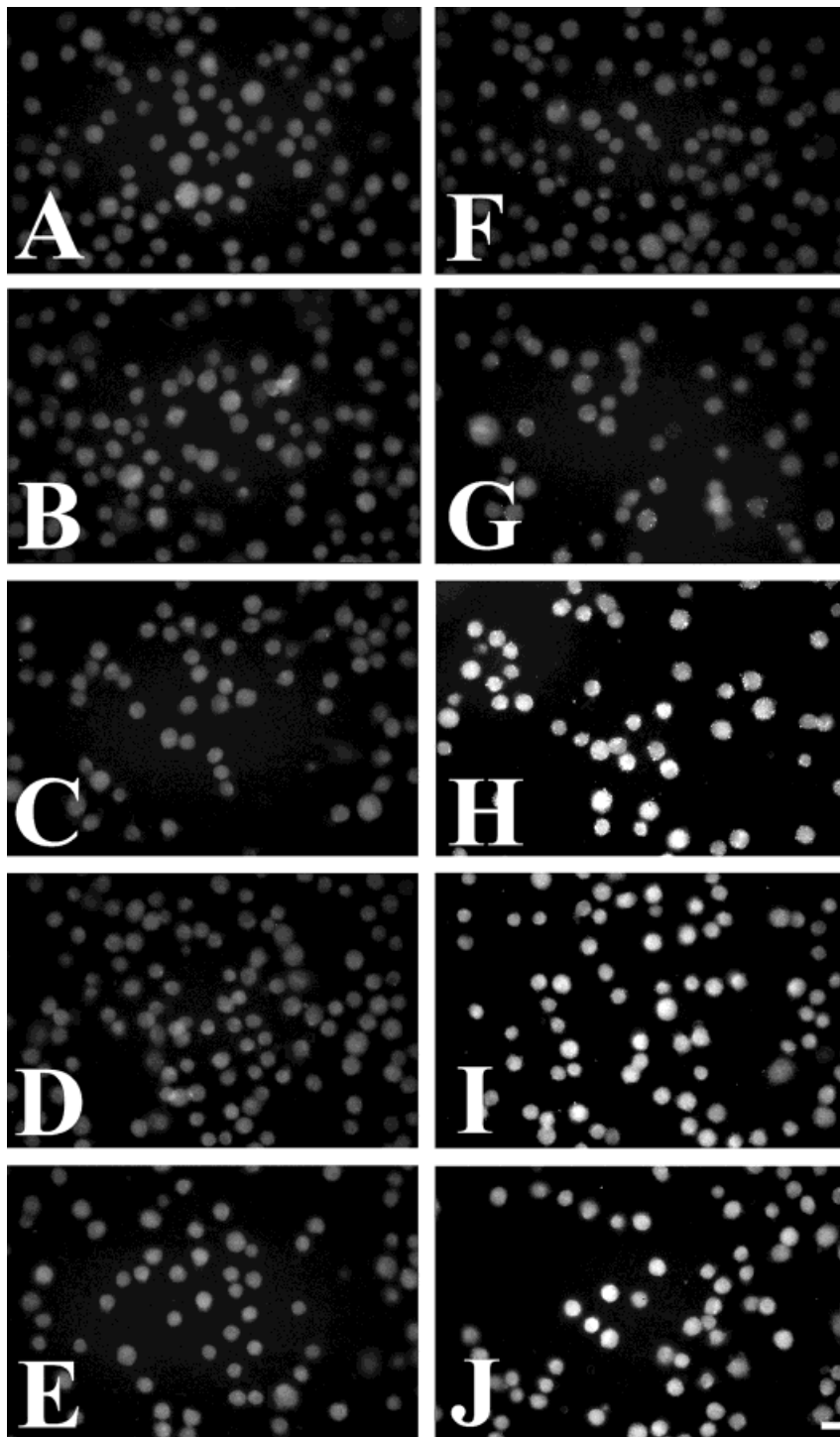
Fig. 2. Regulation of tyrosine hydroxylase immunoreactivity (TH-ir) in rat and bovine chromaffin cells at 33°C and 39°C in vitro. After proliferation at 33°C (**A,F**) and differentiation for 7 days at 39°C in differentiation media alone (**B,G**), or with the addition of 2µg/ml forskolin (**C,H**), or 1 µM dexamethasone (DEX) (**D,I**), or DEX/forskolin (**E, J**), the RAD5.2 rat (A–E) and bovine BADA.20 (F–J) cells were stained with an antibody for TH. During proliferation, levels of TH-ir are barely detectable (A,F), whereas after 7 days of differentiation at 39°C plus for-skolin (H) or DEX (I), only the BADA.20 cells contained in-creased TH-ir. The addition of forskolin or DEX to the differen-tiation media for 7 days did not effect TH-ir in the rat RAD5.2 cell line (C,D,E). In both the rat (D) and bovine (I) chromaffin cell lines, the addition of DEX during differentiation increased chromaffin cell survival, whereas the addition of forskolin for 7 days decreased cell survival, most noticeably in the bovine BADA.20 chromaffin cells (H). Scale bar = 50 µm.
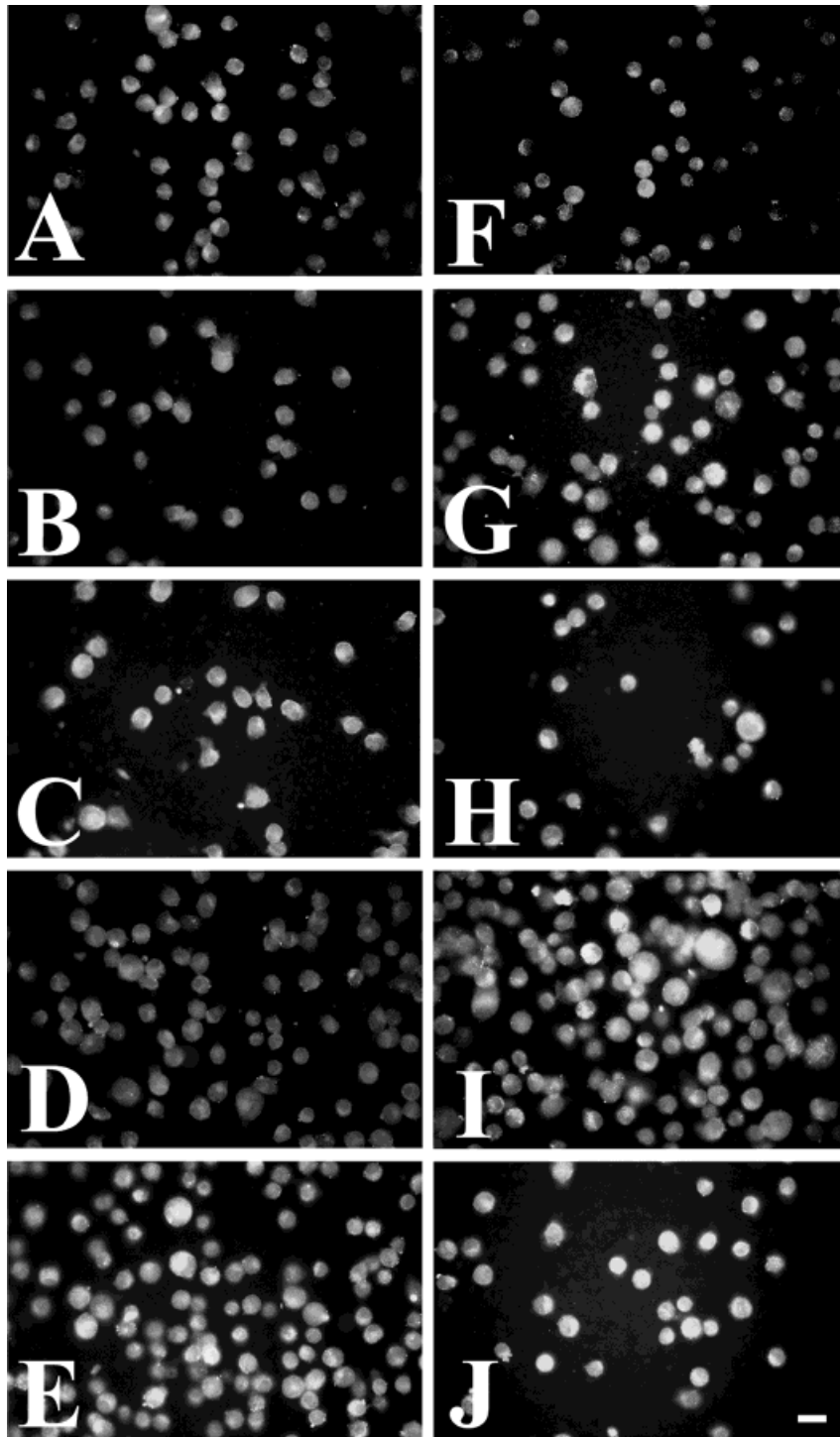
Fig. 3. Regulation of dopa-β-hydroxylase immunoreactivity (DβH-ir) in rat and bovine chromaffin cells at 33°C and 39°C in vitro. After proliferation at 33°C (**A,F**) and differentiation for 7 days at 39°C in differentiation media alone (**B,G**), or with the addition of 2 μg/ml forskolin (**C,H**), or 1 μM dexamethasone (DEX) (**D,I**), or DEX/forskolin (**E,J**), the RAD5.2 rat (A–E) and bovine BADA.20 (F–J) cells were stained with an antibody for DβH. During proliferation at 33°C, levels of DβH-ir are barely detectable, while after seven days of differentiation at 39°C, only the BADA.20 cells contained increased DβH-ir (G). The addition of forskolin for 7 days decreased cell survival and increased DβH-ir (H) in the bovine BADA.20 cells, but only increased DβH-ir in the rat RAD5.2 (C) chromaffin cells, without significantly affecting cell survival. The addition of DEX to the differentiation media for 7 days increased cell survival but did not affect DβH-ir in the rat RAD5.2 cell line (D), whereas the addition of DEX increased both cell survival and DβH-ir in the bovine BADA.20 (D) cells. Scale bar = 50 μm.
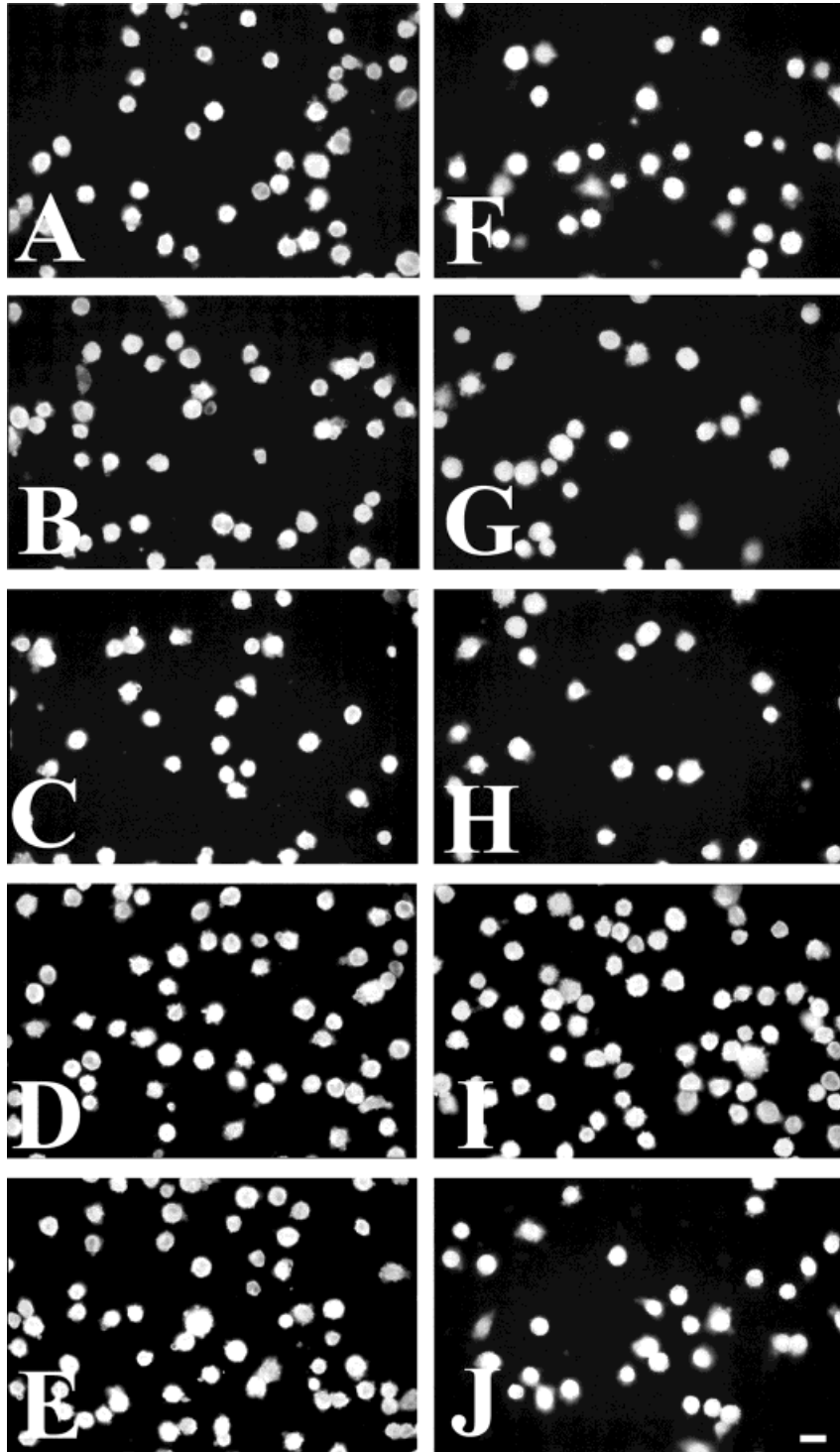
Fig. 4. Regulation of phenylethanolamine-*N*-methyltransferase immunoreactivity (PNMT-ir) in rat and bovine chromaffin cells at 33°C and 39°C in vitro. After proliferation at 33°C (**A,F**) and differentiation for 7 days at 39°C in differentiation media alone (**B,G**), or with the addition of 2 μg/ml forskolin (**C,H**), or 1 μM dexamethasone (DEX) (**D,I**), or DEX/forskolin (**E,J**), the RAD5.2 rat (A–E) and bovine BADA.20 (F–J) cells were stained with an antibody for PNMT. During proliferation at 33°C, levels of PNMT-ir are readily detectable, whereas after 7 days of differentiation at 39°C, both rat RAD5.2 (B) and bovine BADA.20 (G) cells contained equivalent levels of PNMT-ir. The addition of forskolin for 7 days decreased BADA.20 cell survival, without affecting PNMT-ir (H) in both the bovine BADA.20 (H) and rat RAD5.2 (C) chromaffin cells. The addition of DEX to the differentiation media for 7 days increased cell survival but did not affect PNMT-ir in both the rat RAD5.2 (D) and bovine BADA.20 (I) cells. Scale bar = 50 μm.

DβH-ir in either rat RAD5.2 (Fig. 3D) or bovine BADA.20 cells (Fig. 3I).

The RAD5.2 rat chromaffin cell line (Fig.4) contained significant PNMT-ir at both 33°C (Fig. 4A) and 39°C (Fig. 4B). The BADA.20 bovine chromaffin cell line (Fig. 4) also contained easily detectable PNMT-ir at both 33°C (Fig. 4F) and 39°C (Fig. 4G). The addition of forskolin during differentiation had no effect on PNMT-ir in either rat RAD5.2 cells (Fig. 4C) or bovine BADA.20 cells (Fig. 4H). The addition of DEX during differentiation had no effect on the PNMT-ir in either rat RAD5.2 (Fig. 4D) and bovine BADA.20 (Fig. 4I) chromaffin cells.

Regulation of Met-enkephalin Expression After Differentiation and the Addition of Forskolin and Dexamethasone in Chromaffin Cells

DEX and forskolin were used as treatment paradigms to examine the regulation of ENK-ir in rat RAD5.2 and bovine BADA.20 chromaffin cells (Fig. 5). RAD5.2 cells contained barely detectable ENK-ir at 33°C (Fig. 5A), which increased with 7 days of differentiation at 39°C (Fig. 5B). The immortalized bovine chromaffin cells, BADA.20 cells, also contained barely detectable ENK-ir at 33°C (Fig. 5F), which also increased with 7 days of differentiation at 39°C (Fig. 5G). The addition of forskolin (Fig. 5C,H), as well as the separate addition of DEX (D,I) had no further effect on ENK-ir in both cell lines after 7 days of differentiation at 39°C.

Quantitation of Cell Numbers and Antibody Labeling After Differentiation and Addition of Forskolin and Dexamethasone in Chromaffin Cells

The results of quantitative image analysis of rat and bovine chromaffin cells after proliferation and differentiation with the addition of forskolin, DEX, and both agents together is summarized in Table I. A nuclear DAPI stain was used to assess viable cells/field, with counterstaining for TH-, DβH-, PNMT-, and ENK-ir in each case to identify the chromaffin cells/field. In all cases, nearly 100% (82.4–100%) of viable cells/field contained the antigenic markers, but in all experiments the addition of DEX during 7 days of differentiation significantly increased cell survival in both the rat RAD5.2 and bovine BADA.20 chromaffin cells over differentiation media alone. The addition of forskolin tended to decrease cell survival, espe-

cially and significantly in the bovine BADA.20 chromaffin cells. The addition of forskolin and DEX simultaneously only had a moderate effect on increasing cell survival.

A change in exposure number of relative staining intensity (described in Methods) was used to identify the condition(s) that regulated increases in TH-, DβH-, PNMT-, and ENK-ir after differentiation and treatment with forskolin and DEX. Treatment with forskolin and DEX doubled the TH-ir only in the bovine BADA.20 cells. Differentiation alone increased DβH–ir 200% in the BADA.20 cells; forskolin alone increased DβH–ir 100% in the rat RAD5.2 cells. No agent or differentiation affected PNMT-ir in either type of chromaffin cells. Differentiation alone doubled ENK-ir in both types of chromaffin cells in vitro.

HPLC for Catecholamine Neurotransmitters

Because immortalized rat and bovine chromaffin cells express the major synthetic enzymes for these neurotransmitters, both proliferation and differentiation conditions, and differentiation with the addition of serum, forskolin, DEX, and with the addition of the tetrahydrobiopterin cofactor were used to examine the content and release of catecholamines in rat RAD5.2 and bovine BADA.20 cells. Even with the use of standard HPLC methods, including appropriate Ca^{2+} and K^+ ions, to examine catecholamine content in these cells [Cheng et al., 1992, 1993], no detectable neurotransmitter content or release was observed in contrast to preparations of primary chromaffin cultures (data not shown).

DISCUSSION

These studies support the idea that mitotic cells found in adrenal tissue can be conditionally immortalized with the temperature-sensitive Tag oncogene so that the differentiated cell type keeps many of the phenotypic features of primary chromaffin cells. Immortalized rat and bovine chromaffin cells express many of the markers found in primary chromaffin cells, and when differentiated in vitro, as the oncogenic Tag protein is degraded and mitosis ceases, these markers remain and are able to be regulated by continued differentiation, by agents such as DEX and by stimulation of the cAMP pathway with forskolin, mechanisms seen in primary chromaffin cells. Such
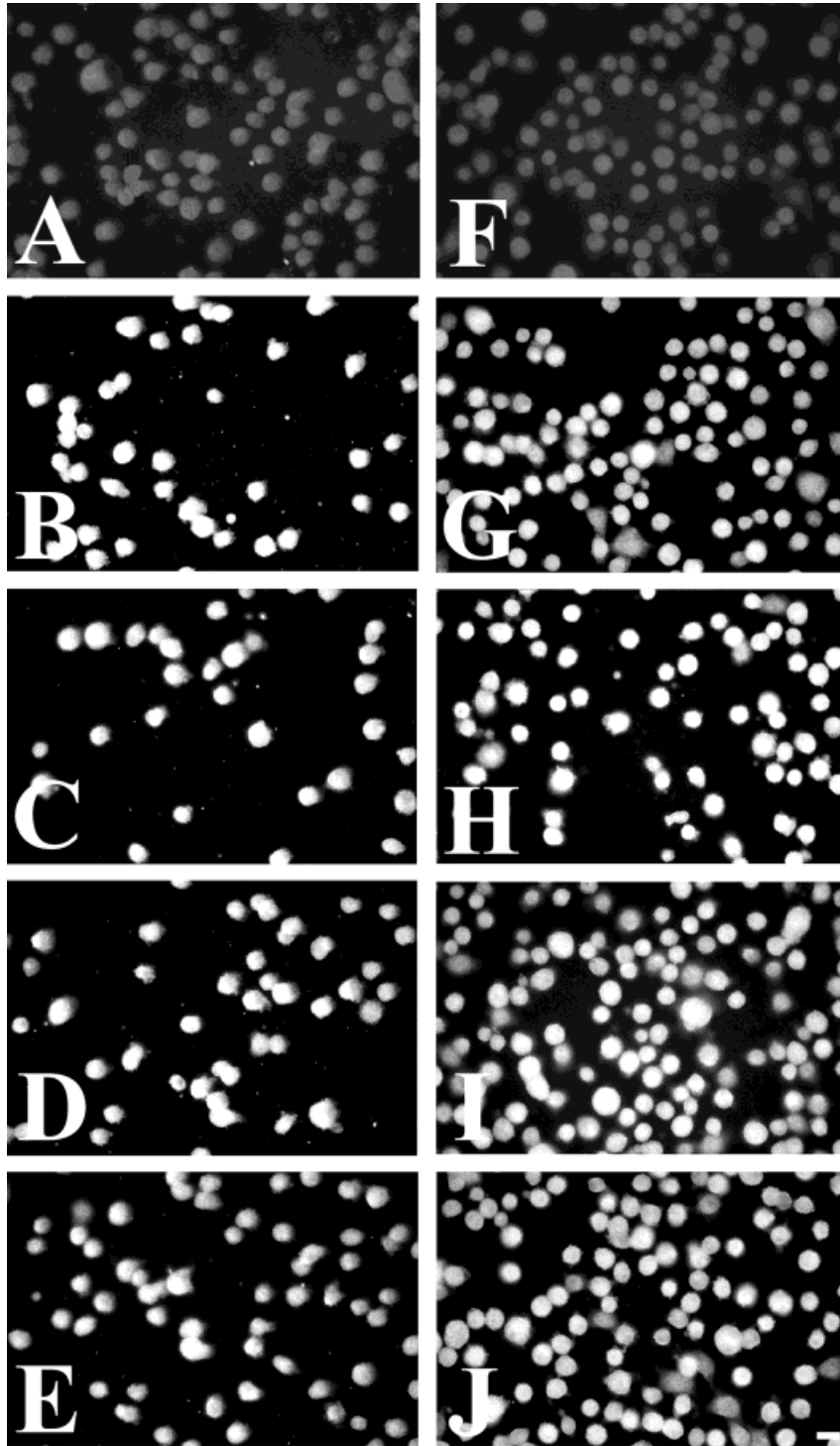
Fig. 5. Regulation of met-enkephalin immunoreactivity (ENK-ir) in rat and bovine chromaffin cells at 33°C and 39°C in vitro. After proliferation at 33°C (**A,F**) and differentiation for 7 days at 39°C in differentiation media alone (**B,G**), or with the addition of 2 μg/ml forskolin (**C,H**), or 1 μM dexamethasone (DEX) (**D,I**), or DEX/forskolin (**E,J**), the RAD5.2 rat (A–E) and bovine BADA.20 (F–J) cells were stained with an antibody for ENK. During proliferation at 33°C, levels of ENK-ir are barely detect-able, whereas after 7 days of differentiation at 39°C, both rat RAD5.2 (B) and bovine BADA.20 (G) cells contained increased levels of ENK-ir. The addition of forskolin for 7 days decreased BADA.20 cell survival, without affecting ENK-ir in both the bovine BADA.20 (H) and rat RAD5.2 (C) chromaffin cells. The addition of DEX to the differentiation media for 7 days increased cell survival but did not affect ENK-ir in both the rat RAD5.2 (D) and bovine BADA.20 (I) cells. Scale bar = 50 μm.

TABLE I. Regulation of Survival, Enzymes, and Met-enkephalin in Immortalized Chromaffin Cells[a]

	TH		DβH		PNMT		ENK	
	Bovine	Rat	Bovine	Rat	Bovine	Rat	Bovine	Rat
33°C								
Cells/field	41.5 ± 0.7	28.8 ± 4.4	33.2 ± 0.7	28.2 ± 3.6	34.9 ± 0.3	30.2 ± 1.6	36.3 ± 3.8	37.1 ± 5.5
Exposure no.	(1)	(1)	(1/4)	(1/8)	(1/60)	(1/60)	(1/8)	(1/8)
39°C, 7 days								
Cells/field	32.4 ± 3.9	12.8 ± 2.9	31.0 ± 3.4	13.2 ± 2.1	30.5 ± 3.6	12.6 ± 4.1	30.6 ± 3.6	12.3 ± 2.3
Exposure no.	(1)	(1)	(1/15)	(1/8)	(1/60)	(1/60)	(1/15)	(1/15)
Forskolin, 2 μg/ml, 7 days								
Cells/field	19.5 ± 2.9	13.4 ± 1.4	20.6 ± 3.8	12.7 ± 2.1	23.6 ± 5.1	11.7 ± 2.2	22.1 ± 3.2*	14.5 ± 1.4
Exposure no.	(1/2)	(1)	(1/15)	(1/15)	(1/60)	(1/60)	(1/15)	(1/15)
Dexamethasone, 1 μM, 7 days								
Cells/field	44.7 ± 4.6*	22.2 ± 4.2*	39.6 ± 1.7*	26.2 ± 2.4*	44.5 ± 2.6*	24.6 ± 3.1*	45.4 ± 4.9*	16.9 ± 1.4*
Exposure no.	(1/2)	(1)	(1/15)	(1/8)	(1/60)	(1/60)	(1/15)	(1/15)
Forskolin/DEX								
Cells/field	31.6 ± 4.7	20.2 ± 4.1*	31.9 ± 6.5	20.9 ± 2.4*	32.6 ± 7.7	22.7 ± 4.3*	32.1 ± 7.2	18.8 ± 2.7*
Exposure no.	(1/2)	(1)	(1/15)	(1/15)	(1/60)	(1/60)	(1/15)	(1/15)

TH, tyrosine hydroxylase; DβH, dopa-β-hydroxylase; PNMT, phenylethanolamine-N-methyltransferase; ENK, met-enkephalin; DEX, dexamethasone.

[a]Immortalized bovine BADA20 and rat RAD5.2 chromaffin cells were either proliferated at permissive temperature (33°C) or differentiated at nonpermissive temperature (39°C) for 7 days in media alone or with the addition of 2 μg/ml forskolin, 1 μM DEX, or forskolin/DEX. Surviving cells were replated to eight-well slides, stained for TH-, DβH-, PNMT-, or ENK-immunoreactivity (ir), mounted in DAPI-containing mounting media, and fields of cells were quantified with image analysis software for survival (DAPI-staining) and antigen immunoreactivity. Data represent the mean ± SEM of six/fields of cells from three independent experiments. Analysis of variance indicated that the data were statistically significant. Asterisks indicate that treatment with DEX increased the number of TH-, DβH-, PNMT-, and ENK-ir surviving cells (both rat and bovine chromaffin cells) compared to differentiation in media alone. $P < 0.01$.

immortalized chromaffin cells are stable and appear to be homogeneous, suggesting that they could be useful for further genetic manipulation and be used as a source for transplant studies in vivo. Conferring immortalization with the tsTag expression has a variety of effects on cells when the wild-type large T protein is present, including binding of large T and inactivation of the growth suppressors pRB, p53, and SEN6 [Scheidtmann, 1989; Jha et al., 1998], a decrease in G1 and increase in G2 and M cell cycle phase duration [Sladek and Jacobberger, 1992], and the ability of large T antigen to block the differentiation process [Cherington et al., 1988]. However, after immortalization with the temperature-sensitive allele tsTag [Frederiksen et al., 1988; Jat and Sharp, 1989], immortalized cells resume the stage of life span and function of an uninfected cell when they are shifted to nonpermissive temperature conditions [Ikram et al., 1994]. These cells at the nonpermissive temperature have lost the ability to drive cell proliferation, because the large T antigen is labile at the higher temperature conditions [Reynisdottir et al., 1990] and the T antigen is not able to drive mitosis in cells immortalized with the construct, and differentiation is favored [Frederiksen et al., 1988; Jat and Sharp, 1989]. In general, tsTag-immortalized cell lines retain the phenotype of the differentiated lineage of the parent. Cell lines generated with the tsTag retain contact inhibition in vitro [Goodman et al., 1993; Frisa et al., 1994], and do not produce tumors or induce immune rejection even when injected into nude mice [Jat and Sharp, 1986] or rats [Bredesen et al., 1990; Renfranz et al., 1991; Whittemore et al., 1991; Onifer et al., 1993; Shihabuddin et al., 1995; Shihabuddin et al., 1996a, 1996b; Prasad et al., 1998]. A number of functional cell lines have been immortalized with Tag and retain their specific catecholaminergic phenotype [Tornatore et al., 1996; Prasad et al., 1998] and efficacy to reverse neurologic deficits after CNS transplant [Tornatore et al., 1996; Prasad et al., 1998; Clarkson et al., 1998]. Cell lines of adrenal medullary origin have been established from SV40 Tag transgenic mice [Suri et al., 1993; Cairns et al., 1997], SV40-infected postnatal mice [Tischler et al., 1993], and the PC12 cell line from rat adrenal pheochromocytoma tumors [Greene and Tischler, 1976]. Even though these cell lines do not synthesize epinephrine in vitro, they exhibit upregulation of the adrenergic phenotype after transplant in vivo [Tischler et al., 1993], suggesting that adrenal medullary cell lines are subject to some forms of environmental regulation after transplant. When the ts allele is used as the transgene, the adrenal medullary cell lines derived from such mice retain the ability to respond to the nonpermissive temperature in vitro, shutting off proliferation, but they quickly die and are dependent on the Tag expression for viability, and hence are not truly "conditionally immortalized" [Cairns et al., 1997].

The cell biology and developmental responsiveness during differentiation of chromaffin cells [Unsicker, 1993] reveals clues to the differentiation program of conditionally immortalized chromaffin cell lines in vitro. The enzyme TH (EC1.14.3.x) catalyzes the rate-limiting step [Livett et al., 1965] in the biosynthesis of catecholamines in chromaffin cells in the adrenal medulla [Pickel et al., 1975; Totzauer et al., 1995] and has been used as one of the antigenic markers for the mature chromaffin phenotype of primary rat and bovine chromaffin cells in vitro [Czech et al., 1996], as well as DβH and PNMT. Both the rat RAD5.2 and bovine BADA.20 chromaffin cell lines express these catecholamine enzyme immunoreactivities at both permissive (low levels) and nonpermissive temperatures, when the cells are proliferating or differentiating, respectively, although levels of the DβH enzyme appear to change with differentiation at nonpermissive temperature (39°C). However, further increased catecholamine enzyme expression in the chromaffin cell lines requires treatment with forskolin and/or DEX during differentiation, because differentiation at 39°C is in serum-free medium. Differentiated primary chromaffin cells from rat [Livett, 1984; Verhofstad, 1993] and bovine [Livett et al., 1983] sources have often been used to study the synthesis and release of the catecholamine neurotransmitters norepinephrine and epinephrine in vitro. However, even with upregulation of enzyme expression, these immortalized chromaffin rat and bovine cells do not synthesize catecholamines. Because chromaffin cell lines probably require an adequate substrate interaction for a completely normalized chromaffin phenotype, the absence of detectable catecholamine synthesis in differentiated RAD5.2 and BADA.20 cells may be due to removal from

their fibroblast environment. We have observed that both RAD5.2 and BADA.20 cells are dependent on a fibroblast feeder-layer for extended survival in vitro at 39°C. Alternatively, a critical cofactor (e.g., tetrahydrobiopterin) may be lacking, although its addition to differentiation media did not significantly affect catecholamine synthesis (data not shown) . Studies are continuing with these immortalized chromaffin cells to examine their requirements for cellular or molecular substrates and cofactors to stimulate and support catecholamine synthesis in vitro, as well as to improve survival.

Another possible, and more likely, explanation for the absence of catecholamine synthesis is a continued low level of Tag expression, even though it is greatly reduced after 3 weeks of differentiation at 39°C. It is possible that even a low level of Tag suppresses some normal cellular functions, such as neurotransmitter synthesis. We are currently using Cre-lox technology [Eaton et al., 2000a) to disimmortalize the chromaffin cells in vitro, and examine whether complete removal of the Tag sequence before differentiation will allow neurotransmitter synthesis and a more normal phenotype. Such a strategy would allow disimmortalization either in vitro or in vivo, because expression of the recombinase protein Cre to excise the Tag sequence can be controlled with various site-directed recombination methods, such as use of RU486 for translocation of Cre to the nucleus of the cells [Herman et al., 1997, 1999].

However, as seen with this first report of immortalized chromaffin cells, the development of the mature adrenal chromaffin phenotype can occur under the influence of specific molecular signals. The sympathoadrenal progenitor cell differentiates into a sympathetic neuron in response to fibroblast growth factor and NGF, or migrates into the adrenal gland, where adrenal corticosteroids stimulate the endocrine chromaffin phenotype and synthesis of catecholamines [Unsicker et al., 1978; Doupe et al., 1985a, 1985b; Anderson and Axel, 1986; Claude et al., 1988; Stemple et al., 1988; Seidl and Unsicker, 1989a, 1989b; Birren and Anderson, 1990]. Older prenatal and postnatal chromaffin cells are able to respond to growth factors and proceed to neuronal differentiation [Tischler et al., 1980; Tischler et al., 1982; Lillien and Claude, 1985a, 1985b], but this neuronal differentiation is blocked by adrenal cor-

ticosteroids, which preserves the chromaffin cell phenotype, even in the presence of transdifferentiation-inducing agents [Unsicker et al., 1978; Doupe et al., 1985b; Anderson and Axel, 1986; Foreman et al., 1992]. Glucocorticosteroids, such as DEX, act early in chromaffin cell development (at E11.5) to inhibit neuronal differentiation, but later at E17 to induce the adrenergic enzyme PNMT [Michelsohn and Anderson, 1992]. DEX treatment regulates catecholamine synthesis by inducing expression of PNMT mRNA and its activity [Kelner and Pollard, 1985], whereas TH and DβH mRNA induction are alternately sensitive to cAMP elevation and phorbol esters in primary chromaffin cells [Hwang and Joh, 1993]. Because the rat chromaffin cell line, RAD5.2, was derived from ~E17 rat adrenals, and the bovine chromaffin cell line, BADA.20, derived from young neonatal calf, the mitotic chromaffin precursor immortalized with both sources with tsTag was likely a more mature, PNMT-containing, chromaffin cell compared to other preparations reported in the literature and used for chromaffin cell immortalization [Birren and Anderson, 1990; Suri et al., 1993]. These immortalized chromaffin cells from rat and bovine sources contain high levels of PNMT. TH seems to be responsive to DEX and cAMP elevation only in the more mature bovine BADA.20 cells, whereas DβH is only upregulated by forskolin in the rat RAD5.2 cells. Again, even low levels of Tag during differentiation may subvert the normal pathways of enzyme regulation, at least in vitro.

Differentiated primary chromaffin cells are known to synthesize opioids such as met-enkephalin [Eiden and Hotchkiss, 1983; Eiden et al., 1984a,b], as do these immortalized rat and bovine chromaffin cells. Interestingly, glucocorticoids are also known to upregulate the enkephalin peptides in chromaffin cells in vivo [Yoburn et al., 1987; Henion and Landis, 1992] and in vitro [Yanase et al., 1984; Henion and Landis, 1992]. Met-enkephalin upregulation is stimulated by other pathways in chromaffin cells as well, including cAMP levels [Eiden and Hotchkiss, 1983; Eiden et al., 1984a; Boarder et al., 1986; Adams and Boarder, 1987; Tezapsidis et al., 1995], membrane depolarization [Siegel et al., 1985], Ca^{2+} influx [Waschek et al., 1987], and nicotinic receptor occupation [Eiden et al., 1984b]. NGF, on the other hand, downregulates met-enkephalin in chromaffin

cells [Bode et al., 1986]. Such upregulation by glucocorticoids suggests that the mature chromaffin phenotype is regulated by and is sensitive to occupation of steroid receptors and voltage-gated Ca^{2+}-influxes. Because both immortalized chomaffin cell types upregulate ENK expression with differentiation alone, enkephalin expression might be a signal of increasing maturity, and independent of Tag-influenced disregulation, in these cells in vitro.

This report represents a promising first attempt at conditional immortalization of chromaffin cells, demonstrating continual cell lines retaining many features of the mature chromaffin cell phenotype. In summary, the availability of conditionally immortalized chromaffin cell lines for a variety of studies, including their use as transplants in various models of neuropathic and neurogenic pain, reflects the growing interest in the development of molecular biologic techniques of cellular therapy for neurodegenerative diseases and CNS trauma. Such a homogeneous source will also allow for the manipulation of the chromaffin cell's genome to investigate the mechanisms of action responsible for cell grafts to repair the injured CNS environment. Similar immortalization of human chromaffin precursors and creation of human chromaffin lines presage the advent of cellular therapy as a therapeutic strategy for a variety of currently difficult clinical problems, including intractable pain after peripheral nervous system and CNS trauma.

ACKNOWLEDGMENTS

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